Valorisation of confectionary industry wastes for the microbial production of polyxydroxyalkanoates

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ABSTRACT

By-product and waste streams from the food industry could be regarded as ideal renewable feedstocks for the production of value-added products. The utilisation of food processing wastes could lead to the development of a sustainable chemical industry and a food industry that will cause less environmental concerns. This will be achieved via simultaneous substitution of fossil raw materials and reduction or even elimination of severe environmental burdens caused by the current disposal and treatment of food waste streams. This work is focused on the bioconversion of flour-rich waste streams generated from a confectionary industry for the production of the biodegradable polymer poly(3-hydroxybutyrate) (PHB). The flour-rich waste streams used in this study were taken either as waste generated from the processing line or as out-of-date products that have been returned from the market. These flour streams contained mainly starch and protein. They were converted into a fermentation feedstock (a 95 % of starch to glucose conversion was achieved) using enzyme-rich crude extracts from solid state fermentations conducted on wheat bran by the fungal strain Aspergillus awamori. When an initial concentration of 80 g/L flour-rich wastes were used, the two-stage bioprocess led to the production of hydrolysates that contained 70.8 g/L glucose concentration and 237.65 mg/L free amino nitrogen (FAN). The hydrolysate was employed for the production of PHB via microbial bioconversion in shake flasks and a 3 L bioreactor using the bacterial strain Cupriavidus necator DSM 4058. Shake flask fermentations using different glucose and free amino nitrogen concentrations led to the production of up to 5.95 g/L PHB concentration. The PHB structure and thermal properties were analysed by Fourier infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC).

Keywords: food waste valorisation; microbial bioconversion; biopolymers; polyhydroxyalkanoates

INTRODUCTION

The most promising alternative to petroleum-derived plastics is the production of biodegradable and durable polymers that can be produced from renewable resources [1,2]. Polyhydroxyalkanoates (PHA) is one of the most promising families of biodegradable plastics that can be produced as intracellular energy-reserve granules via microbial fermentation of renewable resources. PHAs have numerous potential applications including food packaging materials, biocomposite production, speciality biopolymers for medical applications, agriculture and flushable hygiene products [3]. They can be accumulated intracellularly by more than 300 microorganisms under unbalanced growth conditions. Poly(3-hydroxybutyrate) is the most widely studied member of the PHA family and the first one that has been produced at industrial scale. It is a homopolymer of 3-hydroxybutyric acid units and can be produced by different bacterial species including Cupriavidus necator under the limitation of a nutritional element such as N, P, Mg, K, O, or S in the presence of excess carbon source.

The commercialisation of PHB is currently hindered by the significant cost of the conventional carbon sources (e.g. glucose, sucrose, vegetable oils) used during fermentation that account for up to 50 % of the total production cost. This means that research initiatives should focus on the development of technologies that utilise industrial or agricultural waste streams and by-products of negligible cost for the production of PHAs. Food processing waste streams are regarded as unique renewable feedstocks that could be used in
future bioprocesses for the production of biopolymers. The utilisation of food processing wastes will eliminate severe environmental burdens caused by the current disposal, treatment or recycling of food waste streams and petroleum-derived polymers. Food industry processing waste streams such as whey, potato processing waste, molasses and waste vegetable and plant oils are some examples that have been considered for the production of PHAs [2,4,5].

The main objective of this study was to investigate the microbial production of PHB from confectionary industry waste streams through the development of a three-stage bioprocess. The bioprocessing stages include: 1) solid state fermentation (SSF) of Aspergillus awamori growing on bran-rich wheat milling by-products to produce amylolytic and proteolytic enzymes; 2) enzymatic conversion of waste streams into a nutrient-rich fermentation feedstock; and 3) PHB production via fermentation with Cupriavidus necator DSM 4058. Initially, expired flour-based food for infants returned from the market and waste streams generated from the production line (FBFI) were converted into a nutrient-rich fermentation feedstock. The macromolecules in FBFI (i.e. starch, protein) were hydrolysed using crude enzymes (i.e. amylase, protease) produced via solid state fermentation of Aspergillus awamori. FBFI hydrolysates were tested as fermentation media in shake flask and bioreactor fermentations for the production of PHB. The feasibility of bioplastic production from flour-based waste streams has been demonstrated.

MATERIALS & METHODS

Microorganisms

Solid state fermentations were carried out with an industrial strain of Aspergillus awamori 2B. 361 U2/1 (ABM Chemicals Ltd., Woodley, Cheshire, UK). Submerged fermentations for the production of PHB were carried out with Cupriavidus necator DSM 4058.

Raw material

The renewable resource used for the production of fermentation media was out-of-date commercial food for infants and processing waste from the production line of the same product. These streams will be called flour-based food for infants (FBFI) that mainly contains 84.8 % starch, 7.3 % protein and various minerals.

Solid state fermentation

Solid state fermentation of bran-rich wheat milling by-products (BWMB) was carried out in 250 mL shake flasks for the production of various enzymes. BWMB (5 g) were initially sterilised in shake flasks at 121°C for 30 min. The desired A. awamori spore concentration was formed in sterile distilled water for distributing the spores throughout the sterilised wheat bran. The flasks were placed into a 200 rpm shaker and incubated for 3 days at 30°C. The final moisture content was 65 % on a wet basis (wb).

FBFI hydrolysis

At the end of the SSF, the solids were transferred into a 1 L pre-sterilised Duran bottle that contained 200 mL of an aqueous suspension of FBFI. The pH was adjusted to 4.5. The reaction mixture was stirred with a magnetic stirrer in a water bath at 60°C for 24 h. The purpose of this experiment was the enzymatic hydrolysis of the starch and protein content in FBFI. Samples of 1 mL reaction mixture were taken at random intervals during hydrolysis and transferred to 2 mL eppendorf tubes that contained 1 mL of trichloroacetic acid (5 %, w/v) to deactivate the enzymes. After the end of hydrolysis, the solids were separated by centrifugation (3000×g) from the crude hydrolysate that was used as fermentation medium for PHB production in both shake flasks and bioreactor experiments. The pH of the medium was adjusted to 6.6 to 6.8 and subsequently the liquid was filter sterilised using a 0.2 µm filtration unit. The hydrolysate was rich in glucose, sources of nitrogen (e.g. amino acids and peptides) and various other nutrients (e.g. phosphorous). In some cases that a higher Free Amino Nitrogen (FAN) concentration was required at the beginning of PHB fermentations, yeast extract was added into the hydrolysate prior to the filter sterilization stage.

PHB fermentation

FBFI hydrolysates were used in shake flasks and bioreactor fermentations for PHB production. A volume of 50 mL of FBFI hydrolysate was added in 250 mL presterilised shake flasks (121°C, 20 min) under aseptic conditions. A 16 h inoculum was prepared with 10 g/L glucose, 10 g/L yeast extract and 5 g/L

peptone, and a volume of 1 mL was added to each flask under aseptic conditions. Shake flask fermentations were also carried out with commercial nutrient supplements including 10 g/L yeast extract and 5 g/L peptone. The flasks were placed in a shaker at 30°C and agitation of 200 rpm. A whole shake flask was used each time as a sample for the analysis of fermentation kinetics.

Batch fermentations were also carried out in a 3 L bioreactor (1.5 L working volume) using FBFI hydrolysate medium in three fermentations and commercial nutrient supplements in two fermentations. An inoculum of 10% (v/v) was used in all bioreactor fermentations. The pH and temperature were controlled at 6.7 - 6.9 and 30°C throughout fermentation. Aeration rate was kept at 1 vvm. The agitation rate was set between 300 and 700 rpm to maintain dissolved oxygen (DO) above 30% of saturation.

Analytical methods

Glucose was analysed with an enzymatic method (GLUCOSE PAP SL, ELITECK). FAN concentration was analyzed by the ninhydrin colorimetric method promulgated in the European Brewery Convention [6]. Phosphorus content in liquid samples was analysed by the method published by Herbert et al. [7].

Samples (2 – 10 mL) from bacterial fermentations were taken at random intervals to measure total dry weight, PHB, glucose, FAN and phosphorus. Each sample was initially centrifuged at 3,000 × g for 10 min and the sediment was subsequently washed with distilled water and centrifuged. The supernatant was used for the analysis of glucose, FAN and phosphorous. The solids were re-suspended in acetone and transferred into universal bottles. Dry weight measurements were carried out by drying the solids at 50°C and cooling in a desiccator to constant weight. PHB was measured following the protocol proposed by Riis and Mai [8], employing gas chromatography (Fisons 8060) equipped with a Chrompack column (60 m × 0.25 mm).

PHB characterisation

PHB extraction was carried out by the methodology proposed by Hahn et al. [9]. Fourier transform infrared spectroscopy (FTIR) spectra were recorded with a NICOLET FTIR 520 spectrometer. Thermal properties were determined by differential scanning calorimetry (DSC) with a PERKIN-ELMER PYRIS 1 calorimeter, calibrated with indium and n-heptane standards.

RESULTS & DISCUSSION

Production of fermentation medium

Efficient starch hydrolysis can be achieved only in the case that the starch granules are gelatinized prior to enzymatic treatment. The waste streams used in this study have been processed at a temperature of more than 85°C and therefore the starch granules have been gelatinized. Therefore, further gelatinization of starch prior to hydrolysis was not necessary. This is an additional processing advantage as a heat treatment stage can be avoided. For this reason, the temperature used during hydrolysis was 60°C which is the optimum for glucoamylase produced by A. awamori. Each hydrolysis reaction was completed in 24 h.

Figure 1 shows the glucose and FAN production during enzymatic hydrolysis for each byproduct concentration employed (40, 60, 80 g/L FBFI). The maximum glucose concentrations were 35.5 g/L, 53.2 g/L and 70.8 g/L, respectively. The starch to glucose conversion yield achieved was around 95% for all byproduct concentrations employed. In the case of FAN, the maximum concentrations achieved were 166.5 mg/L, 212 mg/L and 237.65 mg/L, respectively. The FAN content was considered low for microbial growth and therefore supplementation of the medium with commercial nutrient supplements was necessary.

Shake flask fermentations

After the enzymatic hydrolysis of the confectionary industry waste streams, the crude hydrolysate produced was utilized as fermentation medium in shake flask fermentations with C. necator. It should be stressed that the crude hydrolysate contains various nutrients that could be used for microbial growth. These nutrients have been released either from FBFI or solids that remained after the solid state fermentation process. The utilization of this nutrient-rich hydrolysate could lead to the reduction of commercial nutrient supplements that represent a significant cost to PHB production economics.

Table 1 presents the results from four shake flask fermentations with C. necator in FBFI hydrolysates that contained various initial glucose and FAN concentrations. Fermentation H1 was carried out without
yeast extract supplementation, whereas various quantities of yeast extract were added in fermentations H2 – H4. Fermentation H1 showed that the hydrolysate could lead to the production of PHB. However, in bioreactor applications the FAN concentration should be increased in order to enhance microbial growth prior to PHB accumulation. The highest PHB concentration (5.95 g/L) was achieved in fermentation H4. Fermentation H3 shows that high initial FAN concentrations may lead to low PHB accumulation.

Figure 1. Glucose and FAN concentration as a function of enzymatic hydrolysis time. Symbols: (●) 40 g/L FBFI, (■) 60 g/L FBFI, and (▲) 80 g/L FBFI.

Table 1. Shake flask fermentations with FBFI hydrolysates.

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Glucose (g/L)</th>
<th>FAN (mg/L)</th>
<th>Time (h)</th>
<th>TDW (g/L)</th>
<th>PHB (g/L)</th>
<th>PHB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>34.4</td>
<td>171</td>
<td>50</td>
<td>10.05</td>
<td>3.86</td>
<td>38.4</td>
</tr>
<tr>
<td>H2</td>
<td>36.3</td>
<td>272.7</td>
<td>28</td>
<td>11.2</td>
<td>4.7</td>
<td>41.8</td>
</tr>
<tr>
<td>H3</td>
<td>33.3</td>
<td>656.4</td>
<td>15</td>
<td>19.6</td>
<td>1.1</td>
<td>5.6</td>
</tr>
<tr>
<td>H4</td>
<td>61.2</td>
<td>492.3</td>
<td>44</td>
<td>14.95</td>
<td>5.95</td>
<td>39.8</td>
</tr>
</tbody>
</table>

Shake flask fermentations were also carried out with commercial nutrient supplements (Table 2). The glucose concentration in C1 and C2 were closer to H1 and H2 respectively. Although FAN concentration was significantly lower in H1 and H2 as compared to C1 and C2 respectively, the total dry weight (TDW) and PHB concentration were at a similar level. This probably occurred because FBFI hydrolysates were richer fermentation feedstocks as compared to the chosen commercial nutrient supplements. This comparison indicates that FBFI hydrolysates could be employed for PHB production.

Table 2. Shake flask fermentations with commercial nutrient supplements.

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Glucose (g/L)</th>
<th>FAN (mg/L)</th>
<th>Time (h)</th>
<th>TDW (g/L)</th>
<th>PHB (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>30</td>
<td>500</td>
<td>30</td>
<td>10.25</td>
<td>3.95</td>
</tr>
<tr>
<td>C2</td>
<td>40</td>
<td>500</td>
<td>50</td>
<td>10</td>
<td>4.64</td>
</tr>
</tbody>
</table>

Bioreactor fermentations

Three batch fermentations were conducted using the FBFI hydrolysate medium. These fermentations were supplemented with different quantities of yeast extract in order to enhance the FAN content in the fermentation broth. Batch fermentations were also carried out using commercial glucose (20-30 g/L) and commercial nutrient supplements i.e. yeast extract (10 g/L) and peptone (5 g/L). The main target of these experiments was to compare commercial media with FBFI-based media for the production of PHB.

Table 3 presents the initial concentrations of glucose, FAN and phosphorus at the beginning of four fermentations carried out in the bioreactor. It also presents fermentation duration, total dry cell weight, final PHB concentration and PHB content in microbial cells. Fermentations B1-B3 were carried out with FBFI hydrolysate under optimal operating conditions. In these fermentations, significant glucose and phosphorus concentrations were still present at the end of the fermentation, whereas FAN was almost completely consumed. This means that fermentations B1-B3 stopped due to nitrogen or other micronutrient limitations. It
can be also deduced that higher FAN concentration led to higher total dry weight, PHB concentration, PHB content and residual biomass. However, fermentations B1-B3 stopped prematurely due most probably to insufficient nutrient content. Fermentations were also carried out on commercial nutrient supplements (i.e. yeast extract, peptone) using optimal operating conditions as in the case of fermentations B1-B3, where PHB accumulation and microbial growth was significantly lower than fermentation B1. This is another indication that FBF1 hydrolysates provide a richer feedstock for PHB production. Fermentation B4 was carried out under oxygen limitation and supplementation of commercial sources of nutrients.

Table 3. Fermentations in bioreactor.

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Glucose (g/L)</th>
<th>FAN (mg/L)</th>
<th>Phosphorus (mg/L)</th>
<th>Time (h)</th>
<th>TDW (g/L)</th>
<th>PHB (g/L)</th>
<th>PHB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>28.4</td>
<td>457.9</td>
<td>267.4</td>
<td>8</td>
<td>13.8</td>
<td>1.55</td>
<td>11.2</td>
</tr>
<tr>
<td>B2</td>
<td>27.3</td>
<td>541.7</td>
<td>325.9</td>
<td>8</td>
<td>15.7</td>
<td>2.5</td>
<td>15.9</td>
</tr>
<tr>
<td>B3</td>
<td>31.4</td>
<td>656.2</td>
<td>328.2</td>
<td>8</td>
<td>18.7</td>
<td>3.2</td>
<td>16.9</td>
</tr>
<tr>
<td>B4</td>
<td>21.8</td>
<td>615.3</td>
<td>-</td>
<td>14</td>
<td>12.7</td>
<td>3.6</td>
<td>28.3</td>
</tr>
</tbody>
</table>

Figure 2 shows the consumption profiles of glucose, FAN and phosphorus as well as the production profiles of total dry cell weight, PHB and residual biomass in fermentation B3. The glucose consumption stopped at around 8 h and the total concentration consumed was 21.3 g/L. As in the case of fermentations B1 and B2, glucose consumption, microbial growth and PHB accumulation stopped at the same time as the FAN concentration was depleted from the fermentation broth. The final phosphorus concentration was around 123 mg/L. Thus, phosphorus depletion was not the reason that triggered the end of microbial growth and PHB accumulation. Consequently, this fermentation probably stopped as the fermentation broth run out of either nitrogen or other nutrients. The higher amount of FAN resulted in increased TDW, PHB and residual biomass concentration as compared to fermentations B1 and B2.

Fermentation B4 was carried out with commercial sources of nutrients. The major difference with all previous fermentations was the fact that it was carried out under oxygen limiting conditions after 6 h fermentation. This was achieved by controlling the DO concentration at 8-20% of saturation. Figure 3 presents the consumption profiles of glucose and FAN as well as the production profiles of total dry cell weight, PHB and residual biomass. The initial glucose concentration was completely consumed at 14 h fermentation. However, there was still more than 250 mg/L of FAN concentration at the end of the fermentation. After the DO concentration dropped below 20%, oxygen limiting conditions were imposed to microbial cells and this led to the initiation of PHB accumulation. Figure 3 shows that the FAN consumption rate was reduced. However, FAN was never the limiting nutrient during fermentation. The results obtained in fermentation B4 indicate that the presence of nutrients during PHB accumulation is necessary. In all previous fermentations, PHB production stopped because there was not enough amount of nutrients to sustain the maintenance of microbial cells.

Figure 2. Profiles of glucose, FAN, phosphorus, TDW, residual biomass and PHB concentration during fermentation B3 with initial glucose and FAN concentrations of 31.4 g/L and 656.2 mg/L respectively.

Fermentation B4 was carried out with commercial sources of nutrients. The major difference with all previous fermentations was the fact that it was carried out under oxygen limiting conditions after 6 h fermentation. This was achieved by controlling the DO concentration at 8-20% of saturation. Figure 3 presents the consumption profiles of glucose and FAN as well as the production profiles of total dry cell weight, PHB and residual biomass. The initial glucose concentration was completely consumed at 14 h fermentation. However, there was still more than 250 mg/L of FAN concentration at the end of the fermentation. After the DO concentration dropped below 20%, oxygen limiting conditions were imposed to microbial cells and this led to the initiation of PHB accumulation. Figure 3 shows that the FAN consumption rate was reduced. However, FAN was never the limiting nutrient during fermentation. The results obtained in fermentation B4 indicate that the presence of nutrients during PHB accumulation is necessary. In all previous fermentations, PHB production stopped because there was not enough amount of nutrients to sustain the maintenance of microbial cells.
The experiments showed that FBFI hydrolysates could be used for PHB production but further work is required in order to optimize media composition. FTIR and DSC techniques were applied so as to confirm both chemical structure and thermal properties of the biopolymer produced. FTIR spectrum confirmed PHB structure because all characteristic bands present in the PHB molecule were identified (results not reported). Thermal analysis showed that FBFI hydrolysates promote the biosynthesis of a PHB biopolymer with a melting point, fusion enthalpy and crystallinity index of 169.1°C, 88 J/g and 58.9 %, respectively.

![Graphs](image)

**Figure 3.** Profiles of glucose, FAN, TDW, residual biomass and PHB concentration during fermentation B4 with initial glucose and FAN concentrations of 21.8 g/L and 615.3 mg/L respectively.

**CONCLUSIONS**

FBFI hydrolysates could be employed as fermentation media for PHB production. FBFI hydrolysates did not contain enough nitrogen. Shake flask experiments showed that the utilization of FBFI hydrolysate could yield higher PHB concentrations compared to the use of commercial nutrient supplements. Bioreactor experiments showed that further optimisation of media composition and operating conditions is necessary.

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**REFERENCES**


