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Research Paper

Biorefinery production of poly-3-hydroxybutyrate using waste office paper hydrolysate as feedstock for microbial fermentation

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ABSTRACT

Waste paper, a major fraction of municipal solid waste, has a potential to serve as renewable feedstock for the biorefineries of fuels, chemicals and materials due to rich in cellulose and abundant at low cost. This study evaluates the possibility of waste office paper (WOP) to serve as a potential feedstock for the biorefinery production of poly (3-hydroxybutyrate). In this study, the WOP was pretreated, enzymatically saccharified and the hydrolysate was used for PHB production. The hydrolysate mainly consists of glucose (22.70 g/L) and xylose (1.78 g/L) and the corresponding sugar yield was about 816 mg/g. Ammonium sulphate and C/N ratio 20 were identified as most favorable for high yield of PHB. The batch fermentation of *Cupriavidus necator* using the pretreated WOP hydrolysate resulted in cell biomass, PHB production and PHB content of 7.74 g/L, 4.45 g/L and 57.52%, respectively. The volumetric productivity and yield achieved were 0.061 g/L/h and 0.210 g/g sugar, respectively. The results suggested that WOP could be a potential alternative feedstock for the biorefinery production of bioplastics.

1. Introduction

Polyhydroxyalkanoates (PHAs) are synthesized under nutrient limiting conditions such as nitrogen, phosphorous, oxygen, sulphur or magnesium, in the presence of excess carbon source (Cesario et al., 2014; Kachrimanidou et al., 2014). Poly (3-hydroxybutyrate) (PHB) is a biodegradable and thermoplastic polymer accumulated intracellularly as carbon and energy storage granules that have the potential to replace the petrochemical based plastics (Kulpreecha et al., 2009). PHB production and commercialization has been intensified in recent years due to its potential applications in packaging, biomedicine, agriculture and many other fields (Rehm, 2006). The global production of microbial bioplastics was counted as 54 kt in 2014 where it is expected to be a 5 – fold increase by 2020 (Ferreira and Schlottbom, 2016). However, the production cost is the major problem that hinders the process since 45% of the total production cost drives for the carbon source (Kulpreecha et al., 2009; Kachrimanidou et al., 2014; Annamalai and Sivakumar, 2016). Several researchers have been intensively working to find a sustainable, renewable and cheaper alternative carbon source which serves as a bioplastic contender for petrochemical plastics market (Zhang et al., 2013; Saratale and Oh, 2015; Cerrone et al., 2015; Annamalai and Sivakumar, 2016).

Lignocellulosic biomasses and other industrial wastes are being considered as a promising renewable and sustainable feedstocks that is becoming rapidly developed and commercialized for production of polyhydroxyalkanoates as a substitute of fossil fuel derived plastics (Obruca et al., 2014, 2015). Waste paper is one among the major components of municipal and industrial solid wastes which accounts more than 35% of total lignocellulosic wastes (Dubey et al., 2012). Annually, more than 400 million tons of waste paper is generated and only about 50–65% is being recycled due to the constraints in recycling of paper fibers which turned into low quality paper products and also the difficult in the process when the paper mixed with other wastes (Wang et al., 2012). However, waste paper has a potential to be used as an excellent alternative feedstock for fermentable sugars production due to its high cellulose content (50–60%), relative abundance and low cost (average $52/ton) (Kadar et al., 2004). Utilization of waste paper as feedstock for production of other value added products is considered as a much valuable and an alternative route for waste management (Wang et al., 2013). There are several attempts have been made recently on utilization of waste paper and paper-derived materials for byproduct production for instance biofuel (Dubey et al., 2012; Wang et al., 2012; Wang et al., 2013; Brummer et al., 2014), however, there was no specific attempt has been made on the production of bioplastics. This study was aimed to explore the possibility of waste office paper to use as a feedstock for poly-3-hydroxybutyrate (PHB) production. The

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paper focuses about the pretreatment and enzymatic hydrolysis of waste office paper and subsequent production of PHB by *Cupriavidus necator* using WOP hydrolysate.

2. Materials and methods

2.1. Microorganisms and chemicals

The PHB producer strain, *C. necator* (Formerly known as *Ralstonia eutropha*) NCIMB 11599, a glucose-utilizing mutant (*nagE_G265RΔnagR*) of wild type strain *C. necator* H16 was procured from the National collection of industrial food and marine bacteria (NCIMB) and was maintained in yeast peptone meat extract (YPM) agar slants [yeast extract −10; peptone − 10; meat extract −5; $(NH_4)_2SO_4$ −5; agar −15 (g/L)] at 4 °C.

The chemicals and materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or as indicated.

The enzymes used in this study such as cellulase from *Trichoderma reesei* ATCC 26921 (C2730) and β– glucosidase from *Aspergillus niger* (49291) were purchased from Sigma-Aldrich (MO, USA). The activity of cellulase and β – glucosidase were estimated as 185 FPU/mL and 500 CBU/mL respectively. The cellulase activity was determined by standard filter paper assay (Ghose, 1987). One unit of enzyme activity (FPU) is defined as the amount of enzyme required to liberate 1 mmol of glucose from filter paper in 60 min at 50°C and pH 4.8. The β – glucosidase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl – β – D-glucopyranoside (*p*NPG) (Parry et al., 2001). One unit of enzyme activity (CBU) is defined as the amount of enzyme required to produce 1 mmol of *p*-nitrophenol from *p* −nitrophenyl-b-D-glucopyranoside (*p*NPG) per minute at 50 °C and pH 5.0.

2.2. Preparation of waste office paper

Waste office paper (WOP) collected from the local market were shredded into small pieces (2 × 5 mm) using a mechanical shredder (Atlas, China). The shredded WOP was soaked with distilled water (5%, w/v), wet milled, filtered using cloth sheath and dried at 60 °C for 24 h. Further, the dried pulp was then milled again and used for further study.

2.3. Pretreatment of waste office paper

The pretreatment was carried out in a screw cap bottle with 0.5% hydrogen peroxide and waste office paper (5% w/v) and then autoclaved at 121 °C for 30 min. The solid residues were collected by filtration using cloth sheath and washed several times with distilled water until neutral pH. The pulp dried at 60 °C for 24 h was milled again and used as a substrate for enzymatic hydrolysis.

2.4. Enzymatic hydrolysis of pretreated waste office paper

Enzymatic hydrolysis of pretreated WOP was carried out in 500 mL hydrolysis flask containing 100 mL of 50 mM citrate buffer (pH 4.8) with 3 % (w/v) solids loadings. The enzyme mixture such as cellulase (37 FPU/g solid) and β-glucosidase (25 CBU/g solid) was used for hydrolysis. After addition of solids and enzymes, the flasks were incubated at 50 °C for 120 h at 160 rpm. Aliquots were withdrawn at regular intervals (24 h), centrifuged at 10,000 ×*g* for 10 min and the supernatant was subjected to sugar analysis. The hydrolysate obtained from the enzymatic hydrolysis (10,000 ×*g*, 10 min) was used for PHB production. The percentage of hydrolysis (%) was calculated based on the amount of sugars in the initial substrate and the sugars released from hydrolysis using the following formula:

$$\text{Percentage of hydrolysis (\%)} = \frac{\text{Sugar released (g/mL)} \times \text{Total volume (mL)} \times 0.9}{\text{Sugar in the initial subtract (mg/mL)}} \times 100$$

2.5. Effect of various nitrogen sources and C/N ratio on cell biomass production and PHB accumulation

The effect of various nitrogen sources on cell biomass and PHB accumulation was studied by growing *C. necator* in mineral salt medium (MSM) [2.4 − KH_2PO_4; 2.5 − Na_2HPO_4; 0.5 − $MgSO_4$ and 0.05- ferric ammonium citrate (g/L)] (pH − 6.8) prepared with WOP hydrolysate (20 g/L) was supplemented with various nitrogen sources (2 g/L) to be investigated. The effect of C/N ratio [10,20, 40, 60, 80 and ∞ (N-free)] was investigated by cultivating in WOP hydrolysate with different concentrations of ammonium sulphate. No ammonium sulphate was added to the medium for N-free condition. The flasks were seeded with 1% inoculum (OD – 1.0 at 600 nm), incubated at 30 °C for 72 h at 200 rpm and the cell biomass and PHB content was estimated. The cell biomass was estimated gravimetrically by centrifuging the culture broth (5 mL) at 5000 ×*g* for 10 min at 4 °C, washed with deionized water and dried at 50 °C for 24 h and expressed in g/L.

2.6. PHB production using WOP hydrolysate

C. necator was precultured in YPM broth at 30 °C for 24 h and the cells with low PHB content were harvested by centrifugation at 5000 ×*g* for 10 min, suspended in sterile distilled water and used as an inoculum (OD – 1.0 at 600 nm). For PHB production, batch fermentation was carried out in a 500 mL Erlenmeyer flasks containing 100 mL of MSM [2.4 − KH_2PO_4; 2.5 − Na_2HPO_4; 0.5 − $MgSO_4$; 0.05- Ferric ammonium citrate and 2.2 − NH_2SO_4 (g/L)] prepared with WOP hydrolysate (24.48 g/L total sugar/22.7 g/L glucose) was filtered through pre-sterilized membrane filter (0.2 mm). The flasks were seeded with 1% (v/v) inoculum and incubated at 30 °C for 96 h with 200 rpm. The dry cell weight (DCW) (g/L), PHB content (%) and residual sugars (g/L) were determined from the aliquots withdrawn at regular interval (24 h).

2.7. Analytical methods

2.7.1. Compositional analysis

The moisture and ash content of waste office paper were determined using the method of NREL/TP – 510 – 42621 (Sluiter et al., 2008a) and NREL/TP – 510 – 42622 methods (Sluiter et al., 2008b), respectively. The structural carbohydrates (cellulose and hemicellulose), acid soluble (ASL) and insoluble lignin of waste office paper were determined by the method of NREL/TP – 510 – 42618 (Sluiter et al., 2012).

2.7.2. Sugars and inhibitors analysis

The glucose, xylose, hydroxymethylfurfural (HMF) and furfural were analyzed using HPLC (Shimadzu; LC10AD) equipped with Aminex HPX − 87H (300 mm × 7.8 mm; Bio-Rad, USA) column at 65 °C using 5 mM sulfuric acid as mobile phase at 1 mL/min flow rate with refractive index detector (Shimadzu; RID10A). The sugars were quantified by external calibration with standards. Total phenolics were determined by Folin – Ciocalteu method (Singleton et al., 1999).

2.7.3. Poly(3-hydroxybutyrate) quantification

The PHB content of dry cell was estimated by gas chromatography (GC) analysis as described earlier (Annamalai and Sivakumar, 2016). Briefly, 50 mg of dried cells were added with 2 mL chloroform and 2 mL acidic methanol (2.8 M H_2SO_4 in methanol) containing octanoic acid (10 mL/L) as an internal standard and heated at 100 °C for 4 h. After cooling, 1 mL of distilled water was added to the mixture, vortex vigorously for 3 min and then centrifuged at 2000 ×*g* for 1 min. After that,