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Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation

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ABSTRACT

The increasing global demand for sustainable resources necessitates the complete utilization of feedstock. Wheat bran consists of significant amount of cellulose and hemicellulose which can be used as a renewable resource for production of fermentable sugars. In this study, alkaline pretreated wheat bran was enzymatically hydrolyzed using cellulase of *Trichoderma reesei* (37 FPU/g) and β - glucosidase of *Aspergillus niger* (50 CBU/g). Among the nitrogen sources tested, ammonium sulphate was identified as best nitrogen source for the production of polyhydroxybutyrate (PHB). The overall sugar concentration was about 62.91 g/L with the corresponding sugar yield of 629.1 mg/g wheat bran and the sugars released were mainly composed of glucose (48.35 g/L) and xylose (14.56 g/L). The PHB producing mutant strain, *Ralstonia eutropha* NCIMB 11599 grown in wheat bran hydrolysate produced cell density, PHB and yield of 24.5 g/L, 62.5%, and 0.319 g/g sugar respectively, with a productivity of 0. 0.255 g/L/h. Thus, the results suggested that the wheat bran could be a potential alternative feedstock as it does not require any detoxification due to less inhibitory compounds for production of high cell density with significant amount of polyhydroxybutyrate.

1. Introduction

Polyhydroxybutyrate (PHB) is an organic polymer with commercial potential as a biodegradable thermoplastic and a biomaterial (Ramsay et al., 1990). PHB is well known as a carbon and energy reserve produced by a variety of microorganisms and its synthesis is favored by environmental stresses such as nitrogen, phosphate or oxygen limitations (Steinbuchel, 1991; Lee, 1996). PHBs are synthesized and deposited intracellularly in the form of granules and might amount up to 90% of the cellular dry weight (Schlegel et al., 1961). In recent days, the investigations on production of PHB have received much attention due to its potential applications in packaging, medical, agricultural and fisheries (Rehm, 2006). The major limiting factor for its commercial success is the cost of sugars used for production and it was estimated that 3 tons of glucose are required for the production of a ton of PHB (Collins, 1987). Moreover, about 45% of the total production cost is attributed to the raw materials where the carbon source could account for 70–80% of the total expense (Choi and Sang, 1997; Du et al., 2012). Therefore, several efforts have been made continuously in search of suitable renewable and inexpensive carbon sources to reduce the cost of production (Khanna and Srivastava, 2005; Annamalai et al., 2014).

Cellulosic biomass such as agricultural residues are potentially inexpensive renewable feedstock for the biorefineries of alternative fuels, chemicals and materials (Wyman, 2001). Cellulose from lignocellulosic biomass could be hydrolyzed into sugars, such as glucose, which are potential carbon resources for PHB production (Rahman et al., 2007; Chen et al., 2007). Bioconversion of cellulose into fermentable sugars is a bio-refining area that has invested enormous research efforts, as it is a prerequisite for the subsequent production of bioenergy (Kumar et al., 2008). However, the efficient hydrolysis of cellulose from cellulosic biomass to glucose is a major bottleneck due to its recalcitrant composite structure of lignin, hemicellulose and cellulose in plant cell walls which blocks the hydrolysis of polysaccharides into fermentative sugars (Mosier et al., 2005; Hall et al., 2010). The enzymatic saccharification is considered as a promising technology compared to other methods; but its efficiency and effectiveness are affected by many factors, including feedstock characteristics, pretreatment methods and hydrolysis conditions (Mansfield et al., 1999).

Wheat bran is an important by-product of the cereal industry produced in vast quantities worldwide (Martel et al., 2010). It con-

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sists of non-starch polysaccharides [cellulose, arabinoxylans and β - (1,3)/β - (1, 4) - glucan (38%)], starch (19%), protein (18%) and lignin (6%) (Bergmans et al., 1996). Hence, it is assumed that the industrial wheat bran has the potential to serve as a low-cost feedstock for the production of renewable energy or chemicals and its utilization would greatly facilitate potential applications in a biorefinery concept. However, enzymatic hydrolysis of wheat bran is not adequate to liberate sufficient fermentable sugars; therefore, a range of physical or chemical pretreatments are required (Yang and Wyman, 2008). Though several attempts have been made on enzymatic hydrolysis of lignocellulosic biomass for production of biofuels and other chemicals, no attempts were made on its utilization for production of bioplastics. Hence, the present study was made on pretreatment, enzymatic hydrolysis of wheat bran into fermentable sugars and production of PHB through microbial fermentation using glucose utilizing mutant strain *R. eutropha* NCIMB 11599 in order to develop a feasible process for sustainable utilization of this lignocellulosic biomass into bioplastics.

2. Materials and methods

2.1. Materials and microorganism

Wheat bran (WB) purchased from local market was destarched by the method of Zhou et al. (2010). 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 was estimated as 185 FPU/mL and 518 CBU/mL respectively. The cellulase activity was determined by the standard filter paper assay (Ghose, 1987). One unit of enzyme activity (FPU) is defined as the amount of enzyme required to liberate 1 μmol 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). One unit of enzyme activity (CBU) is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol from *p* -nitrophenyl- β-D-glucopyranoside (*p*NPG) per minute at 50 °C and pH 5. Other chemicals and materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or as indicated.

2.2. Pretreatment of wheat bran

For pretreatment, destarched wheat bran (WB) was added with 1% NaOH at the ratio of 1:10 (w/v) in a 500 mL screw cap bottle and then autoclaved at 121 °C for 30 min. The residue collected by centrifugation at 5000 *xg* for 10 min was washed several times with distilled water till obtain pH between 7 and 8, dried at 50 °C and used for further studies. The moisture and ash contents were determined using NREL/TP – 510 - 42621 (Sluiter et al., 2008a) and NREL/TP – 510 - 42622 (Sluiter et al., 2008b) methods, respectively. The structural carbohydrates and acid soluble and insoluble lignin of both untreated and pretreated wheat bran were determined by NREL/TP – 510 - 42618 method (Sluiter et al., 2012). The cellulose and hemicellulose content was analyzed by HPLC (Shimadzu; LC10AD) equipped with aminex HPX - 87H (Bio-Rad) column at 65 °C using 5 mM sulfuric acid as mobile phase (0.6 mL/min) with refractive index detector (Shimadzu; RID10A) and the sugars (glucose and xylose) were quantified by external calibration with standards.

2.3. Enzymatic hydrolysis of pretreated wheat bran

The enzymatic hydrolysis was carried out in 250 mL conical flask containing 10% (w/v) of wheat bran in 50 mM citrate buffer (pH 4.8), incubated at 50 °C and 150 rpm for 96 h and the enzyme loading was 37 FPU/g and 50 CBU/g of substrate. Samples were withdrawn at regular interval of 12 h, centrifuged at 10,000 *xg* for 10 min and the supernatant was subjected to sugar analysis. The percentage of hydrolysis was calculated based on the amount of glucan (cellulose) and xylan (xylose) in the initial substrates and sugars released from hydrolysis.

2.4. Effect of nitrogen source and C/N ratio for cell growth and PHB production

To study the effect of nitrogen source, the glucose utilizing mutant strain *R. eutropha* NCIMB 11599 was grown in mineral salt medium (MSM) [2.4 - KH_2PO_4; 2.5 - Na_2HPO_4; 0.5 - $MgSO_4$ and 0.1 - Ferric ammonium citrate (g/L) (pH - 6.8) containing glucose (10 g/L) was supplemented with both inorganic and organic nitrogen sources to be investigated such as NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, peptone and tryptone (2 g/L) and incubated (150 rpm) at 30 °C for 72 h. The effect of C/N ratio on growth as well as PHB production was investigated by cultivating *R. eutropha* NCIMB 11599 in MSM (pH - 6.8) with various C/N ratio (20:1, 20:2, 20:3, 20:4 and 20:5) with glucose (10 g/L).

2.5. Production of polyhydroxybutyrate

The strain, *R. eutropha* NCIMB 11599 was precultured in a nutrient medium containing 10 g/L of yeast extract, 10 g/L of peptone, 5 g/L of meat extract, and 5 g/L of $(NH_4)_2SO_4$ at 30 °C for 48 h. The cells with low PHB content were harvested by centrifugation at 5000 *xg* for 10 min, resuspended in sterile distilled water and used as inoculum for PHB production. After enzymatic hydrolysis, the hydrolysate was collected by centrifugation at 10,000 *xg* for 10 min, adjusted to pH - 6.8 and used directly for PHB production without detoxification. Further, 2.4 - KH_2PO_4; 2.5 - Na_2HPO_4; 0.5 - $MgSO_4$; 0.1- Ferric ammonium citrate and 4.8 - NH_2SO_4 (g/L) were added to the hydrolysate (C/N - 20) and filtered through pre-sterilized membrane filter (0.2 μm), seeded with 1% (v/v) inoculum and incubated in a rotary shaker at 150 rpm for 72 h at 30 °C. Cell growth was monitored by measuring optical density at 600 nm. The cell dry weight (DCW) was estimated by centrifuging the culture broth (5000 *xg* for 10 min at 4 °C) and washed twice with deionized water and dried at 70 °C (g/L).

2.6. Extraction and quantification of PHB

PHB from the harvested cells was extracted by adding 6% sodium hypochlorite and chloroform (1:1) at 37 °C at 300 rpm for 2 h. The chloroform solutions were centrifuged (5000 *xg*, 10 min) to remove cell debris and concentrated by rotary evaporation. The PHB polymer was precipitated using chilled methanol (1:9), separated by centrifugation at 3000 *xg* and the methanol-chloroform mixture was decanted. The precipitated polymer was once again dissolved in chloroform and precipitated with methanol to obtain highly purified polymer. Finally, the pellets were vacuum dried and weight of the pellet was measured.

PHB content was estimated by gas chromatography (GC) analysis through methanolysis of the polymers in sulfuric acid and methanol as described in previous studies (Wei et al., 2011; Zhang et al., 2013). Briefly, 30 mg of freeze dried cells were added with 2 mL chloroform and 2 mL acidic methanol (2.8 M H_2SO_4 in methanol) and heated at 100 °C for 4 h in a water bath. Octanoic acid (10 mL/L) was dissolved in acidic methanol and used as an internal standard. After cooling, 1 mL of distilled water was added to the mixture, shaken vigorously for 3 min till separation of clear phase and the organic phase was then filtered and subjected to GC analysis (Varian 450). The gas chromatographic separation was performed using an Agilent J&W HP-5 capillary column