Bioresource Technology 192 (2015) 677–681

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effective production of low crystallinity Poly(3-hydroxybutyrate) by recombinant *E. coli* strain JM109 using crude glycerol as sole carbon source

Mohan Ganesh, Arivazhagan Senthamarai, Santhanam Shanmughapriya [1], Kalimuthusamy Natarajaseenivasan *

Medical Microbiology Laboratory, Department of Microbiology, Centre for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India

HIGHLIGHTS

- Replacement of commercial carbon sources for P(3HB) production.
- Statistical optimization of culture condition for maximal production of P(3HB).
- Model validation was performed by batch fermentation in a 7.5 L bioreactor.
- Analysis of thermophysical properties of the produced P(3HB).
- Produced P(3HB) had high MW (~2.8×10^6) and low crystallinity of ~30%.

ARTICLE INFO

Article history:
Received 3 March 2015
Received in revised form 8 June 2015
Accepted 9 June 2015
Available online 14 June 2015

Keywords:
Glycerol
P(3HB)
Low crystallinity
Crystallinity index

ABSTRACT

Utilization of bio-diesel by-products (glycerol) for microbial polymer production has created a novel biorefinery concept. In the present study, recombinant *Escherichia coli* JM109 was used for the production of P(3HB) from glycerol as carbon source. Batch fermentation in a 7.5 L bioreactor with the statistically optimized culture condition (pre-treated glycerol: 27.5 g/L and casein hydrolysate: 5.25 g/L) scaled up the P3HB production to 65% (~8 g/L). FTIR, ^{1}H and ^{13}C NMR analysis proved the polymer produced to be P(3HB). Gel permeation chromatography, Differential Scanning Calorimetry (DSC) and thermogravimetric analysis (TGA) demonstrated the produced P(3HB) to have high molecular weight (2.84×10^6) and lowered crystallinity (~30%) compared to commercial polymer. Integrating the production efficiency and the thermal characteristics of the polymer produced by recombinant *E. coli*, the viability and sustainability of biofuels and biopolymers for economic human need could be enhanced.

1. Introduction

Our economy and lifestyle demands the use of fossil resources for transportation fuels and material. However, there has been rising concern over their cost, sustained availability, and impact on global warming and pollution (Hansen et al., 2005). In this scenario, biodiesel seems a promising alternative and renewable fuel to meet the need of mankind. The market for biodiesel is increasing with a projection of its production to reach 37 billion gallons by 2016 (Yang et al., 2012). The biodiesel production process involves the transesterification of vegetable oils or animal fats, containing triacyl glycerols, with methanol or ethanol in the presence of an alkali or acid, to form biodiesel and glycerol as co-product (da Silva et al., 2009; Vasudevan and Briggs, 2008). One mole of glycerol is produced with the production of every 3 mol of methyl esters (i.e., biodiesel), resulting in 10% by weight (wt%) of the total crude glycerol produced (Melero et al., 2012). This crude glycerol stream contains major impurities such as methanol, salts, soaps, heavy metals and residual fatty acids (Venkataramanan et al., 2012; Yang et al., 2012). The increased production of biodiesel has resulted in excess of glycerol with significant drop in the cost of crude glycerol along with simultaneous decrease in demand. Although crude glycerol can be refined, it is not an economically feasible solution (Yazdani and Gonzalez, 2007). The fiscal challenge of disposing the raw glycerol has caused many biodiesel companies to shut down their plants (Pagliaro et al., 2007). Therefore, the future production of biodiesel can be supported only if sustainable cost-effective processes are developed to produce value-added products directly from crude glycerol. Currently, the volarization

* Corresponding author. Tel.: +91 431 2407082; fax: +91 431 2407045.
E-mail address: natarajaseenivasan@rediffmail.com (K. Natarajaseenivasan).
[1] Present address: Center for Translational Medicine, School of Medicine, Temple University, Philadelphia, PA 19140, USA.

http://dx.doi.org/10.1016/j.biortech.2015.06.042
0960-8524/

of this low value residue through microbiological or chemicals routes has gained attention and continuous interest (Papanikolaou and Aggelis, 2009). Biological utilization of crude glycerol serves as a feedstock in various fermentation processes for the production of products including 1,3-propanediol (PDO), dihydroxy-acetone (DHA), bioethanol, butanol, propionic acid, succinic acid, and so on (Dobson et al., 2012).

Poly-hydroxyalkanoates (PHAs) are another value-added product. PHAs are biodegradable polymers produced as intracellular energy-reserve granules by ~300 microorganisms. However, the production of PHAs in large scale is limited to substrates cost as the fermentation media is formulated with expensive carbon sources (Andre et al., 2009). Therefore, the use of crude glycerol for this purpose can be an interesting opportunity. Crude glycerol has been tested as fermentation feedstock for the production of Poly(3-hydroxybutyrate) (P3HB) by a wide range of microbes (Cavalheiro et al., 2009; Mothes et al., 2007; Pappalardo et al., 2014; Hermann-Krauss et al., 2013; Rodríguez-Contreras et al., 2015). P(3HB) is the best known PHA, and its accumulation in recombinant *Escherichia coli* using several carbon sources has been studied (Ahn et al., 2000; Nikel et al., 2006). Previous studies have been conducted to study the efficiency of various recombinant *E. coli* strains harboring PHA biosynthesis genes for production of PHAs from pure and crude glycerol (Mahishi et al., 2003; Nikel et al., 2008; De Almeida et al., 2010; Shah et al., 2014; Phithakrotchanakoon et al., 2013). But unfortunately, PHAs obtained from glycerol were reported to have a significantly lower molecular weight than polymer synthesized from other substrates, such as glucose or lactose (Madden et al., 1999). The physical properties of such low molecular weight polymers are poor and do not find its application for commercialization.

In this present study, the potential utilization of crude glycerol as the carbon source for P(3HB) production by recombinant *E. coli* strain JM109 harbouring the plasmid pGETS109- *phaBCA* was investigated. The gene order of the *phaCAB* operon (*phaABC*, *phaACB*, *phaBAC*, *phaBCA*, *phaCAB*, and *phaCBA*) determines the accumulation level of P(3HB), and the expression plasmid pGETS109- *phaBCA* was known for its most balanced production of Ultra-high Molecular Weight Polyhydroxy-3-butyrate (UHMW-P(3HB) (Hiroe et al., 2012). The recombinant *E. coli* strain JM109 has been shown to produce 41% UHMW-P(3HB) (wt%) in the presence of 20 g/L glucose as carbon source. In this regard, we aimed at studying whether the plasmid that is capable of prominent production of UHMW-P(3HB) can utilize the cheaper carbon source to find its applicability in commercial polymer production. Further we characterized the produced polymer for its thermal stability properties.

2. Methods

2.1. Strains and plasmids

The recombinant *E. coli* strain JM109 harbouring pGETS109-*phaBCA* was used for production of P(3HB). The plasmid pGETS109- *phaBCA* was a kind gift from Dr. Ayaka Hiroe, Tokyo Institute of Technology, Japan. The *E. coli* strain JM109 was maintained in Luria Bertani broth or agar supplemented with ampicillin (100 μg/ml).

2.2. Media and growth conditions

MYAG medium was used for P(3HB) production. The media was composed of Na_2HPO_4 (6.0 g/L), KH_2PO_4 (3.0 g/L), $(NH_4)_2SO_4$ (1.4 g/L), NaCl (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L) and casein hydrolysate (Acid Hydrolyzed; LOBA chemie Pvt. Ltd., India) (5 g/L). Starter cultures for P(3HB) production were grown in MYAG medium supplemented with ampicillin. The growth was monitored at OD_{600nm} and the cultures were used as inoculums as they reach the mid-exponential phase of growth.

2.3. Preparation of carbon sources

Crude glycerol was kindly provided by Vinayaga lubricants (Karur, Tamilnadu, India). A simple method was adopted to separate potential inhibitors from crude glycerol. The original crude glycerol was left overnight in separation funnels to produce pre-treated glycerol (Kachrimanidou et al., 2014). The compositions of crude and pre-treated glycerol are given in Table 1. Fermentation experiments were performed with crude glycerol (20 g/L) or pre-treated glycerol (20 g/L) as carbon source with glucose serving as positive control as the recombinant *E. coli* JM109 is known for its higher P(3HB) yield in the presence of glucose (20 g/L) (Hiroe et al., 2012). The purity of crude or pre-treated glycerol used in each experiment has been taken into consideration and the appropriate calculations were made.

2.4. P(3HB) production

Bacterial fermentations were carried out using MYAG media in 250 mL Erlenmeyer shake flasks (50 mL broth volume). Inoculums of 1 mL mid-exponential phase culture were used for each flask. Each fermentation experiments were repeated three times and at 30 °C. Various concentrations of pre-treated glycerol and casein hydrolysates were used (extended protocols) as carbon and nitrogen sources respectively. The fermentation period was determined preliminarily to be 96 h and kept constant throughout the experiment. After fermentation the samples were centrifuged at 4000*g* for 5 min. The pellet was washed twice with distilled water and P(3HB) was extracted from dried cells.

2.5. Batch fermentation in bioreactor

Large scale batch fermentation experiments were carried out in a 7.5 L Benchtop bioreactor (BioFlo/Celligen 115, New Brunswick, USA). The MYAG medium supplemented with pre-treated glycerol as primary carbon source (27.5 g/L) and casein hydrolysate (5.25 g/L) (as determined by statistical optimization of P(3HB) production; Extended protocol) was used for all fermentation experiments. After inoculation of the starter culture (mid-exponential phase culture; 10% (v/v)) the working volume was set at 3 L. Temperature and pH were maintained constantly at 30 °C and 7, respectively. During fermentation, the pH was adjusted using 2 N HCl or 0.5 N NaOH and the dissolved oxygen content (DOC) was controlled above 20% of saturation, using an aeration rate of 3.6 L_{air}/min and increasing the stirring speed from 200 rpm to 1500 rpm.

2.6. P(3HB) extraction and quantification

P(3HB) extraction was carried out by the dispersion-extraction method (Hahn et al., 1994). Cells were harvested by centrifugation

Table 1
Composition of crude and pre-treated glycerol processed by decanting.

Component	Crude glycerol	Pre-treated glycerol
Glycerol (% w/w)	70 ± 0.9	85 ± 0.4
Sodium and potassium salts (% w/w)	5 ± 0.4	4 ± 0.015
Moisture (%)	10 ± 0.2	5 ± 0.07
Other organics (%)	5 ± 0.6	2–3 ± 0.028
Methanol (%)	10 ± 0.1	<1