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ScienceDirect
Bioresource Technology 99 (2008) 5749–5755
BIORESOURCE TECHNOLOGY

Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24

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Received 15 July 2007; received in revised form 7 October 2007; accepted 9 October 2007
Available online 26 November 2007

Abstract

Methylobacterium sp. ZP24 produced polyhydroxybutyrate (PHB) from disaccharides like lactose and sucrose. As *Methylobacterium* sp. ZP24 showed growth associated PHB production, an intermittent feeding strategy having lactose and ammonium sulfate at varying concentration was used towards reaching higher yield of the polymer. About 1.5-fold increase in PHB production was obtained by this intermittent feeding strategy. Further increase in PHB production by 0.8-fold could be achieved by limiting the dissolved oxygen (DO) levels in the fermenter. The decreased DO is thought to increase flux of acetyl CO-A towards PHB accumulation over TCA cycle. Cheese whey, a dairy waste product and being a rich source of utilizable sugar and other nutrients, when used in the bioreactor as a main substrate replacing the lactose, led to further increase in the PHB production by 2.5-fold. A total of 4.58-fold increase in the PHB production was obtained using limiting DO conditions with processed cheese whey supplemented with ammonium sulfate in fed batch culture of *Methylobacterium* sp. ZP24. The present investigation therefore reflects on the possibility of developing a cheap biological route for production of green thermoplastics.

Keywords: Acetyl CoA; Dissolved oxygen; Cheese whey; Polyhydroxybutyrate; *Methylobacterium* sp. ZP24

1. Introduction

Polyhydroxyalkanoates (PHAs) are a group of polyesters produced and naturally accumulated by a large number of bacteria as intracellular granules in response to unfavorable growth conditions and nutrient imbalance (Anderson and Dawes, 1990; Khanna and Srivastava, 2005; Vázquez et al., 2003). Because of their total biodegradability and the potential to be produced from renewable carbon and nitrogen sources, these thermoplastic polymers have been the subject of great interest since their discovery (Choi and Lee, 1997; Hänggi, 1995; Kim, 2000). Poly(3-hydroxybutyrate) (PHB) is the most widely studied and best characterized PHA. PHAs share many material properties to synthetic polymers and hence are considered to be good substitutes for petroleum-derived synthetic plastics. Another emerging application of PHB is generation of enantiomerically pure 3-hydroxybutyric acid (3-HB) which acts as an intermediate for the synthesis of many chiral drugs (Lee et al., 1999; Tokiwa and Ugwu, in press).

High cost of production is one of the main factors, which has limited the broader use of PHAs as a biodegradable commodity plastic. Improvement in PHA production strategies can lead to cost reduction implying wider use of PHA in daily life (Li et al., 2007). This has generated a world wide interest in the efficient production of PHB at a low cost by novel microorganisms. Among various PHB producing organism, *Methylobacterium* sp. ZP24, is of significance since it efficiently utilized lactose/sucrose as a sole source of carbon for growth as well as PHB production, accumulating up to 59% of its dry weight (Yellore et al., 1999) and could also dep-

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doi:10.1016/j.biortech.2007.10.017

olymerise the accumulated polymer to 3-hydroxybutyric acid under specified growth conditions (Nath et al., 2005). Current production of polyhydroxyalkanoates (PHAs) via microbial fermentation still cannot challenge the other methods of producing commercial plastics and work leading towards economical production of PHAs is therefore of utmost significance.

Cheese whey, a by-product of the dairy industry, is mainly regarded as a pollutant due to its high biological oxygen demand and its disposal is being managed at considerable cost. Since *Methylobacterium* sp. ZP24 is reported to utilize cheese whey towards production of PHB (Yellore and Desai, 1998) and most PHA producing bacteria do not utilize cheese whey, it can be considered as an attractive raw material towards cost effective production of PHB using this organism. However few reports are available of PHB production from lactose and whey but mainly by recombinant *Escherichia coli* (Woo et al., 2000).

In the present investigation, strategy for efficient production of PHB by limiting dissolved oxygen level together with intermittent feeding of lactose and ammonium sulfate at different concentrations in feed was investigated under fed batch model using *Methylobacterium* sp. ZP24. Replacement effect of lactose by processed cheese whey as the main substrate of fermentation as well as feed, on production of PHB was also studied and the process was scaled up to 30 l in a process controlled fermenter.

2. Methods

2.1. Microorganism and culture conditions

The bacterial strain used in the present study was isolated from a zoological pond of The Maharaja Sayajirao University of Baroda, Vadodara and identified at IMI Surrey [United Kingdom] as *Methylobacterium* sp. *Methylobacterium* sp. ZP24 was grown in MM1 containing 12 g l^{-1} lactose as carbon source and 2.36 g l^{-1} ammonium sulfate as nitrogen source. The medium (400 ml media/1000 ml Erlenmeyer flask) was inoculated with a 5% of inoculum that had been pre grown overnight in the pre described media and was incubated at 30° ± 2 °C for 24 h under shaking at 200 rpm. Feed (40 ml) was introduced at 30 h in 1000 ml flask containing 400 ml of medium where as no addition was done in case of batch fermentation.

2.2. Chemical analysis of the cell and culture supernatant

Cell growth was monitored by measuring the optical density at 600 nm. PHB concentration was determined by gas chromatography (Shimadzu 14-B) with methyl benzoate as an internal standard (Pal and Paul, 2002) as well as spectrophotometric analysis described by Law and Slepecky (1966). Lactose and ammonical nitrogen was measured from culture supernatant (after spinning @ 7227*g* for 10 min) by DNSA and Berthelot reaction, respectively, as described by Yellore and Desai (1998).

2.3. Preparation of starter inoculum

The starter inoculum of *Methylobacterium* sp. ZP24 was prepared in MM1 containing lactose media, where 1 loop full of colony was inoculated in the same media and allowed to grow for 24 h at 30° C under shaking condition at 200 rpm. *Methylobacterium* sp. was brought to a uniform density by aseptical dilution of the culture with 0.85% saline until they matched freshly prepared McFarland Standard 0.5 as mentioned below. The accuracy was verified by a spectrophotometer, with an optical density reading at 625 nm of 0.137 and at 600 nm of 0.144. McFarland Standard ($BaSO_4$ turbidity standard) was prepared by taking 0.5-ml aliquot of 0.048 mol/l $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) into 99.5 ml of 0.18 mol/l H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. $BaSO_4$ turbidity standard was then stored in dark at room temperature.

2.4. Bioreactor studies

Batch culture of *Methylobacterium* sp. ZP24 was conducted in a process controlled semiautomatic jar fermenter (Biostat B, Sartorius-BBI) with SCADA (MFCS D/A), containing 1 L of MM1 media having following composition. (g l^{-1}): lactose:12; ammonium sulfate: 2.36; Na_2HPO_4: 2.5; K_2HPO_4: 1.0; Yeast extract: 0.5; $CaCl_2 \cdot 2H_2O$: 0.1; $FeCl_3 \cdot 6H_2O$: 0.02; and trace element solution 9 ml, contained (μg l^{-1}): H_3BO_3: 300; $CoCl_2$: 30; $ZnSO_4$: 100; $MnCl_2 \cdot 4H_2O$: 3; Na_2MoO_4: 20; $CuSO_4 \cdot 5H_2O$: 10. The pH was controlled at 7.0 with 2 N Na_2CO_3 or 2 N NaOH. Temperature and dissolved oxygen (DO) were controlled at 30 °C and 60% (or 30%), respectively. Fed batch fermentation was carried out in the same fermenter containing 900 ml MM1 media (pH 7.0) containing 12 g l^{-1} lactose and 2.36 g l^{-1} ammonium sulfate. Feed containing defined lactose and ammonium sulfate concentration (200 ml) was introduced into fermenter at about 30 h of growth. Seed culture (100 ml) was prepared in the MM1 medium as described in Sections 2.1 and 2.3. Dissolved oxygen was maintained at 60% or 30% of air saturation by automatic control of agitation speed (up to 600 rpm) and the air flow rate (up to 3 LPM).

Processed cheese whey along with ammonium sulfate was used to determine if the conditions used for PHB production in the small scale were adequate for use at higher-scale. The organism was cultivated in a 30 l (20 l–25 l working volume) process controlled automatic fermenter (Sartorius-India, Allen Bradley automation), with same percentage of inoculum load and other parameters as described earlier. The initial substrate volume was 15 l. Feeding was done at around 30 h and 48 h, with defined cheese whey and ammonium sulfate concentration.