Journal of Industrial Microbiology & Biotechnology (2000) 25, 276–279

www.nature.com/jim

Effect of total nutrient feed on production of poly-3-hydroxybutyrate by *Methylobacterium* sp. ZP24 grown on sugars

VS Yellore[1], MS Ghatnekar[2], JS Pai[2] and AJ Desai[1]

[1]*Department of Microbiology and Biotechnology Centre, Faculty of Science, Maharaja Sayajirao University of Baroda, Baroda 390002, India;* [2]*Division of Foods and Fermentation Technology, University Department of Chemical Technology, Matunga, Mumbai 400019, India*

Methylobacterium **sp. ZP24 is able to produce poly-3-hydroxybutyrate (PHB) from sucrose and lactose. As the production of PHB is growth-associated, a strategy of intermittent feeding of sugars and other nutrients was assessed for obtaining high yields of the polymer. Higher PHB synthesis was obtained at increased sugar feed rates. Cellular PHB contents of 63% and 71%, with productivities of up to 0.354 and 0.645 g PHB/l h were obtained from sucrose and lactose, respectively. A short-duration semicontinuous production level of up to 2.4 g PHB/l h was achieved in the lactose fermentation.** *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 276–279.

Keywords: polyhydroxybutyrate; fed-batch; sugars

Introduction

Polyhydroxyalkanoates (PHAs) are microbial polyesters synthesized by a variety of bacteria as a storage material of energy and carbon [3,13,16]. Of these biopolymers, poly-3-hydroxybutyrate (PHB), is of much interest as it is a biodegradable and biocompatible alternative to several bulk petroplastics [3]. The cost of PHA production, however, is high due to substrate and recovery costs and this is a major limitation to their widespread use. The substrate cost for PHB production can be as high as 38% of the total operating cost, as is the case for recombinant *Escherichia coli* [6]. This cost can be brought down considerably by the use of cheaper crude substrates, like whey and molasses [7].

The synthesis and accumulation of PHB can be either dependent or independent of nutrition limitation [7]. Nutrition-limitation-dependent synthesis of PHAs in turn is either growth-limited as in *Alcaligenes eutrophus* [8], *Pseudomonas oleovorans* [14] and *Methylobacterium organophilum* [9] or growth-associated as in *Methylobacterium rhodesianum* grown on fructose [1]. Nutrition-limitation-independent PHA production is growth-associated as in *Alcaligenes latus* [18] and *Azotobacter vinelandii* UWD [4]. However, in *A. latus*, nutrition limitation increases PHB production [17]. Growth-associated PHB production is of advantage as the use of crude substrates is easier and a wider range of materials may be used in cultivation.

Methylobacterium sp. ZP24 produces PHB from lactose and sucrose [19,20]. This organism utilizes disaccharides efficiently, and without prior hydrolysis of the substrate as is required by recombinant *E. coli* [20]. Moreover, as a member of a group of bacteria known to achieve high cell densities in culture, it is of interest for PHA production. However, to determine the potential of this strain to produce polymers from cheap raw materials, higher volumetric yields (g/l) and productivity (g/l h) would be required. As the production of PHB is growth-associated, the effect of different fermentation strategies on PHB yield and productivity by *Methylobacterium* sp. ZP24 was tested.

Correspondence: Dr AJ Desai, Department of Microbiology and Biotechnology Centre, Faculty of Science, Maharaja Sayajirao University of Baroda, Baroda 390002, India
Received 6 June 2000; accepted 31 August 2000

Materials and methods

Bacterial strain and culture conditions

Methylobacterium sp. ZP24 was grown in MM-1 medium containing sucrose or lactose, normally at 12 g/l and 2.35 g/l of $(NH_4)_2SO_4$ [19]. Incubations were carried out in a bioreactor or shake flask at 32±2°C and pH 7±0.5. Inocula were prepared by growing the organisms in 200 ml medium per 500-ml flask for 24 h at 200 rpm on a rotary shaker. All components of the medium and feeds used were of reagent grade, with the exception of sucrose, which was obtained from a domestic market.

Fermentation studies

Fermentation studies were carried out in an Electrolab series III bench-top lab fermentor (UK), in a 7-l vessel with an on-line dO_2 probe. Experiments were initiated with 5% inoculum (v/v) in a medium volume of 4 l. Aeration at 1–1.5 vvm and an impeller speed of 200–250 rpm was provided to maintain the dO_2 level above 40% of saturation. High dO_2 levels were possible due to the use of two flat six-blade turbine type impellers with a diametric ratio of 0.5.

For the fed-batch fermentation studies, sterilized feed solution was fed, using a programmed peristaltic pump, at specific dosing intervals. The feed solution was formulated by mixing two separately sterilized solutions, one consisting of the sugar, $(NH_4)_2SO_4$ and yeast extract, and the other containing phosphate salts and the remaining components. The concentrations of the major components were calculated on the basis of their utilization rate during maximum growth phase in batch cultivation. The feed dosings were started after 14–15 h of growth and were made at

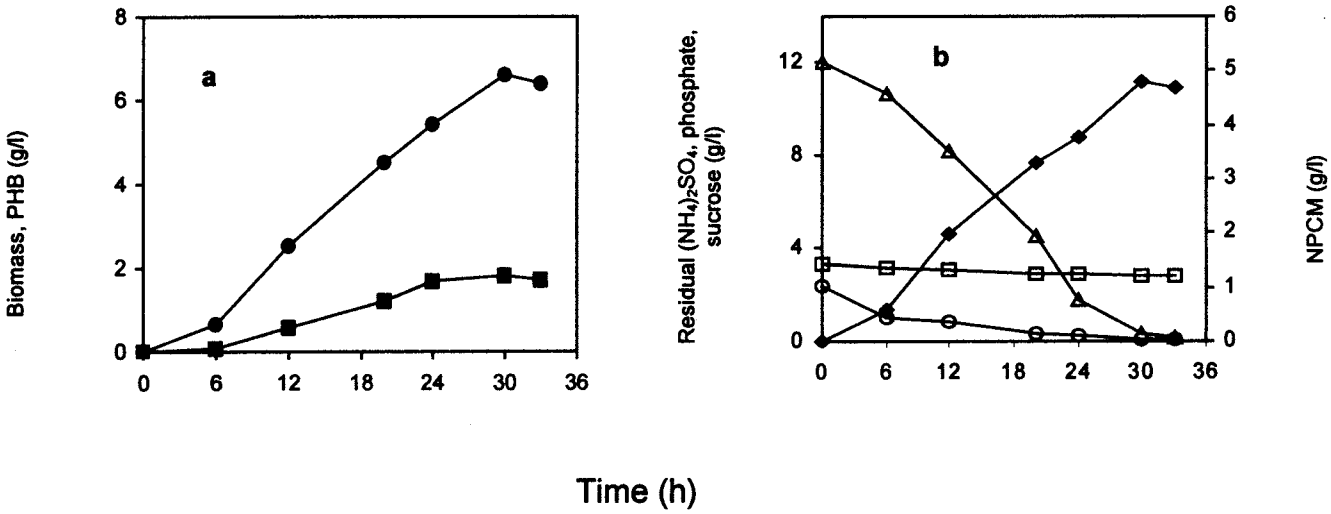

Figure 1 Batch cultivation of *Methylobacterium* sp. ZP24 in liters MM-1 and sucrose. (a) Production of biomass (●) and PHB (■). (b) Residual ammonium sulphate (○), phosphate (□), sucrose (△) and nonpolymer cellular material (NPCM, ◆).

intervals of 3 h. The dosing rates are referred to in terms of the sugar component, though all other components were also provided.

Analyses

Biomass was measured as the cell dry weight, ammonium by the sodium phenolate reaction, and sugars by the phenol–sulphuric acid method as described earlier [19]. Inorganic phosphate was determined by the ascorbate–molybdate method [5], and PHB by conversion to crotonic acid as described by Song *et al.* [15].

Results and discussion

In a 4-l batch cultivation using MM-1 plus 12 g/l sucrose (Batch) biomass accumulation occurred rapidly between 6 and 24 h of cultivation (Figure 1a). Growth was maximal between 6 and 20 h as evident from the rate of formation of nonpolymeric cellular material (NPCM) (Figure 1b). PHB accumulation was initiated from the start of the cultivation and was maximal between 12 and 24 h of growth. Polymer synthesis was observed to be growth-associated as in the case of *M. rhodesianum* MB126 growing on fructose [1].

Higher yields of PHB were reached by achieving higher growth rates. For this, fed-batch cultivations were carried out using MM-1 medium. As the sucrose utilization rate during maximum growth period in the batch culture was 0.66 g/l h, sucrose was fed at this rate in the first fed-batch fermentation. A fed-batch using sucrose at 0.66 g/l h (FB-1) resulted in the formation of 12.2g/l biomass (Figure 2a). The biomass and PHB were produced concomitantly and the polymer content reached 67% in the cells, which was 2.4-fold higher than that seen in batch cultured cells. This method of feed therefore seemed suitable for further work.

To increase the growth and productivity, a medium feed representing a threefold higher level of sucrose as provided in FB-1 was provided. In this study (FB-2) using sucrose at 2 g/l h, about 1.8-fold higher biomass and PHB yields than FB-1 were obtained (Figure 2b). The higher sugar feed also stimulated a higher production rate than in FB-1 (Table 1). However, the intracellular polymer content did not improve and was about 63% by weight. The 14.1 g PHB/l yield from sucrose and productivity

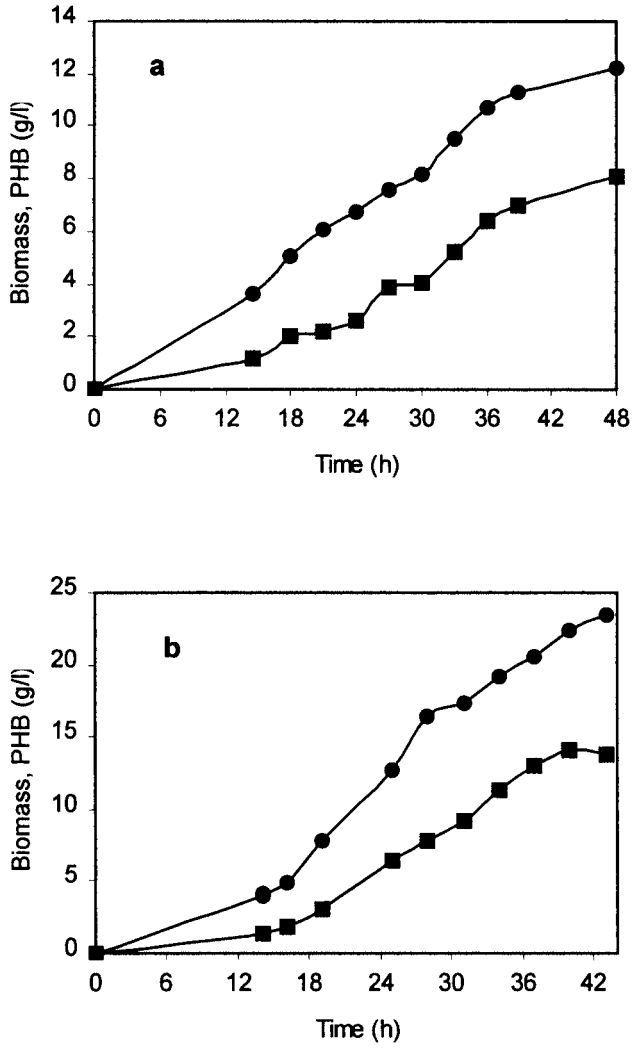

Figure 2 Production of biomass (●) and PHB (■) when *Methylobacterium* sp. ZP24 is fed with (a) MM-1 + 0.66 g sucrose/l h (FB-1) and (b) MM-1 + 2 g sucrose/l h (FB-2).

(0.354 g/l h) was two- to threefold lower than the yields obtained from *A. vinelandii* [4] and recombinant *E. coli* [12].

Methylobacterium sp. ZP24 can produce PHB from lactose, and with higher efficiency than from sucrose [19]. To determine if the approach used to increase productivity of PHB from sucrose could also be used with lactose, a similar feed using this sugar was set up with a slight modification (Lac). After a period of 48 h, 500 ml of culture was removed from the vessel every 8 h, so as to allow further build-up of the culture because the maximum operating volume of the vessel (5 l) was reached. As seen in Figure 3, total nutrient feed with lactose+MM-1 was effective in obtaining higher biomass, PHB and polymer content. The lactose-fed culture also had a higher PHB productivity (0.649 g/l h) than from sucrose. This agrees with a similar trend seen in earlier batch studies (data not shown here).

The strategy employed here was to feed the sugar plus other medium components at regular intervals, starting from the stage at which the growth rates were maximum (14–15 h in Batch). The intermittent feeding allowed for maintaining a residual sugar concentration that resulted in a better efficiency of substrate conversion (Table 1). The biomass yield from sucrose fed at low rate in FB-1 was higher than in FB-2 or the Lac experiments. However, PHB productivity from sucrose at a higher concentration was higher in FB-2, which indicated that higher yield and productivity could be achieved by increasing the feed rates. Though the PHB yield from lactose is high, the productivity was much lower than reported for recombinant *E. coli* [12] if the overall process is considered. However, the PHB productivity from sucrose at higher concentration in FB-2 was higher, which indicated that higher yield and productivity could be achieved by increasing the feed rates. Intermittent harvesting carried out during the experiment allowed for a semicontinuous production of PHB at a rate as high as 2.4 g PHB/l h between 48 and 51 h (point productivity), indicating that it would be possible to achieve higher overall production with more efficiency after further fine-tuning of the feed rates. This PHB productivity from lactose is high during 48–51 h, though if the entire process over 54 h is considered, it is much lower than that reported for recombinant *E. coli* [10].

The levels and rates of PHB shown here are lower than those reported for other organisms. However, we have shown the potential of nutrient feed and a cyclic fed-batch approach to increase the productivity of PHB from sugars by a growth-associated PHB-producing organism. This approach allows for higher polymer content to be accumulated and also leads to higher substrate conversions. The raw materials cost (RMC) is a major contributor to the cost of PHB as shown for *A. latus* and *E. coli* [7]. For these, the carbon source (sucrose) contribution is as high as 61%. The carbon source cost of PHB production using

Table 1 Yield of biomass and polymer produced by *Methylobacterium* sp. ZP24 during total nutrient-feed cultivation with sucrose and lactose

Batch	Cultivation time (h)	$Y_{Biomass}$[a] (g/g)	Y_{PHB} (g/g)	$Q_{Biomass}$[b] (g/l h)	Q_{PHB} (g/l h)
Batch	30	0.550	0.151	0.220	0.060
FB-1	48	0.644	0.428	0.254	0.169
FB-2	40	0.354	0.223	0.560	0.354
Lac	54	0.620	0.422	0.952	0.649

[a]Yield of biomass or PHB produced per gram sugar consumed.
[b]Productivity of biomass or PHB produced per liter per hour.

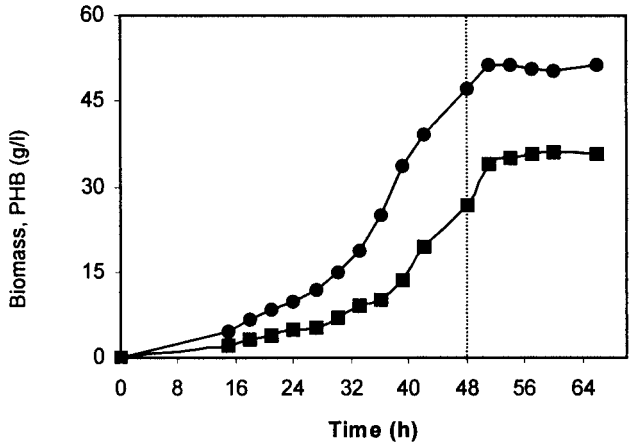

Figure 3 Production of biomass (●) and PHB (■) by *Methylobacterium* sp. ZP24 when fed with MM-1 + 2 g lactose/l h (Lac). Dotted line indicates start of cycling mode.

Methylobacterium sp. ZP24 can be estimated based on the cost of whey and sucrose as reported earlier [7]. The substrate cost due to utilization of sucrose by *A. latus* at a $Y_{P/S}$ of 0.40 (g/g) is $76,412,000, for a production scale of 100,000 tons/annum. Based on these estimates, the cost of substrate for an equivalent production of PHB by *Methylobacterium* sp. ZP24 is 23% of the cost when using whey and 93% of the cost when using sucrose. As described by Lee [10,11], the low polymer content of methylobacteria is a major limitation to their application. An approach as shown in this work shows that *Methylobacterium* sp. ZP24 can accumulate a PHB content as high as 71%, which is sufficiently high to justify its use.

As previously shown, formate and other organic acids were able to enhance the growth of *Methylobacterium* sp. ZP24 growing on sucrose [20]. A fed-batch approach as described here, in combination with an organic acid feed, will enable the further enhancement of PHB levels, and also further increase the PHB content as seen in preliminary observations. Such an approach would ultimately lead to a continuous process, which is ideal for economic cultivation [2].

Acknowledgements

The authors thank Prof P.R. Kulkarni, University Department of Chemical Technology, for use of the instrumental facilities. V.Y., a University Grants Commission Research Fellow, thanks the UGC for the fellowship received, and Dr A.K. Shah for comments.

References

1 Ackermann JU and W Babel. 1997. Growth-associated synthesis of poly (hydroxybutyric acid) in *Methylobacterium rhodesianum* as an expression of an internal bottleneck. *Appl Microbiol Biotechnol* 47: 144–149.
2 Ackermann JU and W Babel. 1998. Approaches to increase the economy of the PHB production. *Polym Degrad Stab* 59: 183–186.
3 Anderson AJ and EA Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* 54: 450–472.
4 Chen GQ and WJ Page. 1997. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. *Biotechnol Tech* 11: 347–350.

5 Chen PS, TY Toribara and H Warner. 1956. Microdetermination of phosphorous. *Anal Chem* 28: 1756–1758.
6 Choi JI and SY Lee. 1999. Efficient and economical recovery of poly (3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnol Bioeng* 62: 546–553.
7 Choi JI and SY Lee. 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Appl Microbiol Biotechnol* 51: 13–21.
8 Kim BS, SC Lee, SY Lee, HN Chang, YK Chang and SI Woo. 1994. Production of poly (3-hydroxybutyric acid) fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. *Biotechnol Bioeng* 43: 892–898.
9 Kim SW, P Kim, HS Lee and JH Kim. 1996. High production of poly-β-hydroxybutyrate (PHB) from *Methylobacterium organophilum* under potassium limitation. *Biotechnol Lett* 18: 25–30.
10 Lee SY. 1996. Bacterial polyhydroxyalkanoates. *Biotechnol Bioeng* 49: 1–14.
11 Lee SY. 1996. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends Biotechnol* 14: 431–438.
12 Liu F, W Li, D Ridgway, T Gu and Z Shen. 1998. Production of poly-β-hydroxybutyrate on molasses by recombinant *Escherichia coli*. *Biotechnol Lett* 20: 345–348.
13 Madison LL and GW Huisman. 1999. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. *Microbiol Mol Biol Rev* 63: 21–53.
14 Preusting H, J Kingma, G Huisman, A Steinbuchel and B Witholt. 1993. Formation of polyester blends by a recombinant strain of *Pseudomonas oleovorans*: different poly (3-hydroxyalkanoates) are stored in separate granules. *J Environ Polym Degrad* 1: 11–21.
15 Song JJ, YC Shin and SC Yoon. 1993. P(3HB) accumulation in *Alcaligenes eutrophus* H16 (ATCC 17699) under nutrient-rich condition and its induced production from saccharides and their derivatives. *J Microbiol Biotechnol* 3: 115–122.
16 Steinbuchel A and B Fuchtenbusch. 1988. Bacterial and other biological systems for polyester production. *Trends Biotechnol* 16: 419–427.
17 Wang F and SY Lee. 1997. Poly (3-hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of *Alcaligenes latus* under nitrogen limitation. *Appl Environ Microbiol* 63: 3703–3706.
18 Yamane T, M Fukunaka and YW Lee. 1996. Increased PHB productivity by high-cell-density fed-batch culture of *Alcaligenes latus*, a growth-associated PHB producer. *Biotechnol Bioeng* 50: 197–202.
19 Yellore V and AJ Desai. 1998. Production of polyhydroxybutyrate from lactose and whey by *Methylobacterium* sp. ZP24. *Lett Appl Microbiol* 26: 391–394.
20 Yellore V, N Thakur and AJ Desai. 1999. Enhancement of growth and poly-3-hydroxybutyrate production from *Methylobacterium* sp. ZP24 by formate and other organic acids. *Lett Appl Microbiol* 29: 171–175.