

# Optimizing conditions for poly( $\beta$ -hydroxybutyrate) production by *Halomonas boliviensis* LC1 in batch culture with sucrose as carbon source

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**Abstract** *Halomonas boliviensis* LC1 is able to accumulate poly( $\beta$ -hydroxybutyrate) (PHB) under conditions of excess carbon source and depletion of essential nutrients. This study was aimed at an efficient production of PHB by growing *H. boliviensis* to high cell concentrations in batch cultures. The effect of ammonium, phosphate, and yeast extract concentrations on cell concentration [cell dry weight (CDW)] and PHB content of *H. boliviensis* cultured in shake flasks was assayed using a factorial design. High concentrations of these nutrients led to increments in cell growth but reduced the PHB content to some extent. Cultivations of *H. boliviensis* under controlled conditions in a fermentor using 1.5% (w/v) yeast extract as N source, and intermittent addition of sucrose to provide excess C source, resulted in a polymer accumulation of 44 wt.% and 12 g l<sup>-1</sup> CDW after 24 h of cultivation. Batch cultures in a fermentor with initial concentrations of 2.5% (w/v) sucrose and 1.5% (w/v) yeast extract, and with induced oxygen limitation, resulted in an optimum PHB accumulation, PHB concentration and CDW of 54 wt.%, 7.7 g l<sup>-1</sup> and 14 g l<sup>-1</sup>, respectively, after 19 h of cultivation. The addition of casaminoacids in the medium increased the CDW to 14.4 g l<sup>-1</sup> in 17 h but reduced the PHB content in the cells to 52 wt.%.

**Keywords** *Halomonas boliviensis* · Moderate halophile · Poly( $\beta$ -hydroxybutyrate) (PHB) production

## Introduction

Biopolyesters, the biodegradable polymers produced by several microorganisms from renewable resources, have been considered as good candidates to substitute petroleum-derived plastics (Lee 1996b; Steinbüchel and Fuchtenbush 1998). These biopolymers, also called polyhydroxyalkanoates (PHAs), are stored intracellularly by several microorganisms as carbon and energy reservoir. The most commonly studied PHA, polyhydroxybutyrate (PHB), is known to possess physical properties similar to those of polyethylene, and has potential applications as disposable bulk material in packing films, containers, or paper coatings, amongst others (Lee 1996a; Reddy et al. 2003).

There are only a few bacterial species considered to be good candidates for large-scale production of PHB. These organisms are pseudomonads and *Wautersia eutropha* (formerly called *Ralstonia eutropha*), which produce PHB in the presence of excess carbon source and the depletion of a nutrient element (Lee 1996a); other well-known species are *Alcaligenes latus*, *Azotobacter vinelandii* UWD, and recombinant *Escherichia coli*, which do not require nutrient limitation for PHB synthesis (Lee et al. 1994; Lee 1996a). The former two strains are able to produce the polymer during their active growth phase. Furthermore, both *A. vinelandii* and *E. coli* can be grown in complex nitrogen medium, and yet lead to high cell densities and polymer contents (Page and Cornish 1993; Lee and Chang 1995).

As the efficiency and economics of the manufacturing process of PHB are determined by the carbon source, fermentation process, and downstream processing of the

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polymer, the development of cultivation conditions for microorganisms that allow high PHA content and productivity from cheap and renewable carbon sources is important (Lee 1996a; Choi and Lee 1999). Thus, *A. latus* and *A. vinelandii* UWD can utilize sucrose as carbon source to produce PHB, implying that inexpensive substrates such as raw sugar, beets, or cane molasses can also be utilized. Both strains are able to store 70–83 wt.% PHB of their cell dry weight (CDW), and reach amongst the highest cell densities reported so far (Page and Cornish 1993; Wang and Lee 1997). On the other hand, the extremely halophilic archaeon *Haloferax mediterranei* is able to store large amounts of PHA (67 wt.%) from starch; however, it also produces an extracellular polysaccharide, which might interfere with purification of the polymer, and requires high concentration of salts (25% w/v) for optimum PHA production (Lillo and Rodriguez-Valera 1990; Rodriguez-Valera and Lillo 1992).

Recently, we reported the production of PHB by a moderately halophilic bacterium *Halomonas boliviensis* LC1 from various carbon sources, including volatile fatty acids, mono-/disaccharides (Quillaguamán et al. 2006), and starch hydrolysate containing a mixture of maltooligosaccharides (Quillaguamán et al. 2005). In contrast to the culture requirements of extremely halophilic archaea, sodium chloride concentrations of 0.5 and 4.5% (w/w) provided the highest cell densities and PHB accumulation in the case of *H. boliviensis* (Quillaguamán et al. 2006). The polymer content obtained was in the range of 55 to 88 wt.%; however, the cell concentration was low (up to  $2 \text{ g l}^{-1}$ ), hence resulting in a low volumetric productivity of PHB. Although some improvements in PHB accumulation and cell mass concentration were reached by the addition of some nutrients, major enhancements in the PHB yield were attained when oxygen limitation was induced in a fermentor (Quillaguamán et al. 2005). This paper reports the optimization of culture conditions with an aim to improve the biomass concentration of *H. boliviensis* LC1 during PHB production, utilizing sucrose as the polymer precursor.

## Materials and methods

### Bacterial strain and maintenance

*Halomonas boliviensis* LC1<sup>T</sup> (=DSM 15516<sup>T</sup>) was maintained at 4°C on solid HM medium (Quillaguamán et al. 2004), containing (% w/v) NaCl, 4.45; MgSO<sub>4</sub>×7H<sub>2</sub>O, 0.025; CaCl<sub>2</sub>×2H<sub>2</sub>O, 0.009; KCl, 0.05; NaBr, 0.006; peptone, 0.5; yeast extract, 1.0; glucose, 0.1; and granulated agar, 2.0. The pH of the medium was adjusted to 7.5 using 3 M NaOH.

### Culture composition

*Halomonas boliviensis* was grown in Mineral Salts (MS) medium containing (% w/v) NaCl, 4.5; MgSO<sub>4</sub>×7H<sub>2</sub>O, 0.190; CaCl<sub>2</sub>×2H<sub>2</sub>O, 0.065; KCl, 0.375; and NaBr, 0.02. Sucrose (Merck, Whitehouse Station, NJ, USA) and yeast extract (Merck) were added to the medium in varying amounts for different experiments.

### Factorial design

*Halomonas boliviensis* was grown at 30°C in 60 ml of MS medium supplemented with 0.1% (w/v) yeast extract and 1% (w/v) sucrose in 250-ml flasks with rotary shaking at 200 rpm for 13 h (OD<sub>600</sub> of 1.20±0.05). Subsequently, 15 ml of the resulting culture broth was inoculated in 1-l Erlenmeyer flasks containing 235 ml of MS medium with 1% (w/v) sucrose, and combination of nutrients according to Table 1. The pH was initially adjusted to 7.5 using 3 M NaOH. Cultures were incubated at 35°C with shaking at 200 rpm and samples withdrawn at defined time intervals for determination of CDW and PHB content.

### PHB production in a fermentor using stepwise addition of sucrose

For PHB production in a fermentor, *H. boliviensis* was first grown in 1-l flasks containing 100 ml of MS medium supplemented with 0.5% (w/v) yeast extract and 1% (w/v) sucrose, at 30°C with shaking at 200 rpm for 13 h (CDW of 2.3±0.1 g l<sup>-1</sup>, OD<sub>600</sub>≈1.5). This culture was used to inoculate a 2-l fermentor vessel (Voyager, Luton, UK) with 1.4 l of MS medium containing 1% (w/v) sucrose. A solution containing MS medium supplemented with 100% (w/v) sucrose was intermittently added to the fermentor to provide excess carbon source to the cells. Antifoam was added when needed. The pH of the medium was initially adjusted to 7.7 with concentrated NH<sub>4</sub>OH, and was maintained at this value

**Table 1** Factorial design 2<sup>3</sup> to study the effect of phosphate and organic and inorganic nitrogen on PHB accumulation and RCM in *H. boliviensis* LC1

Assay no.	Variable changed % (w/v)		
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	KH <sub>2</sub> PO <sub>4</sub>	Yeast extract
1	0.00	0.00	0.05
2	0.00	0.20	0.05
3	0.15	0.00	0.05
4	0.15	0.20	0.05
5	0.00	0.00	0.15
6	0.00	0.20	0.15
7	0.15	0.00	0.15
8	0.15	0.20	0.15

by using 5 M HCl/NaOH. The air inflow rate and agitation speed were initially set to  $1.0 \text{ l min}^{-1}$  and 700 rpm for all fermentations, and were increased up to  $4.0 \text{ l min}^{-1}$  and 1,100 rpm, respectively, when a decrease from the initial dissolved oxygen concentration (i.e., 85%) was detected. The effect of various nutrients such as sucrose,  $\text{KH}_2\text{PO}_4$ , and  $\text{NH}_4\text{OH}$  were studied under these conditions.

#### Batch cultivations in a fermentor

*Halomonas boliviensis* was first grown under the conditions described above in 1-l flasks containing 150 ml of MS medium supplemented with 0.5% (w/v) yeast extract and 2.5% (w/v) sucrose. After 13 h of growth (CDW of  $1.5 \pm 0.17 \text{ g l}^{-1}$ ), the culture was used to inoculate a 2-l fermentor vessel (Voyager) containing 1.35 l of MS medium with 2.5% (w/v) sucrose and 1.5% (w/v) yeast extract. The pH of the medium was maintained at 7.7 by using 5 M HCl/NaOH. The air inflow rate and agitation speed were initially set as described above. Subsequently, air inflow was increased to  $4.0 \text{ l min}^{-1}$ , while agitation speed was modified to 800, 900, and 1,000 rpm, respectively, for different experiments to investigate the effect of oxygen limitation on cell concentration and PHB accumulation by *H. boliviensis*.

The effect of supplementing the medium with 0.2% (w/v) of casaminoacids and whey was also investigated, while maintaining an agitation speed of 900 rpm.

#### Quantitative analysis

CDW was determined by centrifuging 3 ml of the culture samples at  $2,000 \times g$  for 15 min, followed by washing the pellet with distilled water and drying it at  $75^\circ\text{C}$  until constant weight was obtained. PHB quantification was performed by the method of Law and Slepecky (1961), according to which the dried pellets containing intracellular PHB were hydrolyzed using concentrated sulfuric acid for 1 h to obtain crotonic acid, which was quantified by measuring absorbance at 235 nm. Analysis was performed in triplicates in all cases.

Residual cell mass (RCM) concentration was calculated as the difference between cell concentration (CDW) and PHB concentration, while PHB content (wt.%) was obtained as the percentage of the ratio of PHB concentration to cell concentration (CDW) as defined by Lee et al. (2000).

## Results

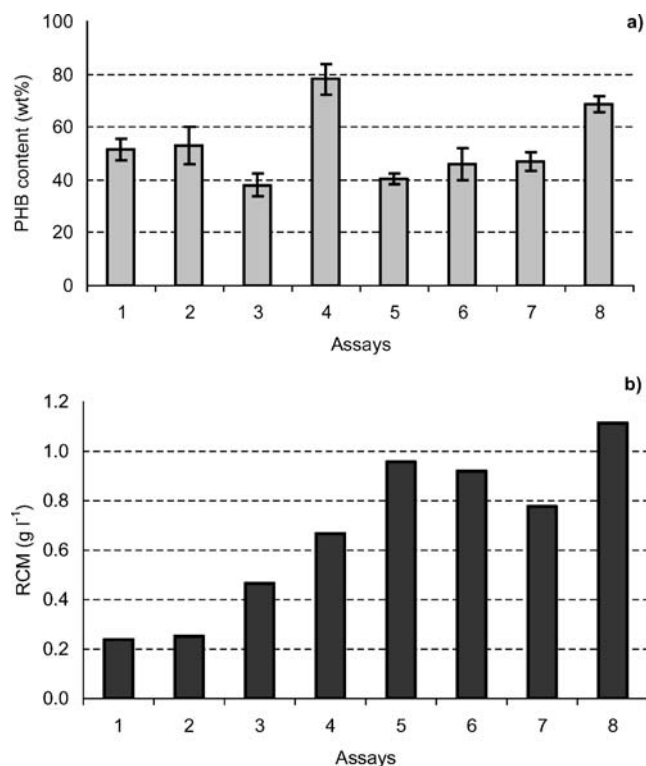
#### Factorial design studies on PHB production by *H. boliviensis*

To find optimum conditions for cell growth and PHB accumulation by *H. boliviensis*, the influence of various

nutrients and all their possible combinations in the MS culture medium were investigated by a factorial design as indicated in Table 1. Sucrose was used as the carbon source because of its ready availability from renewable agricultural feedstocks. Yeast extract and ammonium sulfate were used as organic and inorganic nitrogen sources, respectively. The results of the factorial design experiments are depicted in Fig. 1a,b. It was observed that increasing the yeast extract concentration from 0.05 to 0.15% (w/v) led to an increase in cell concentration, but the levels of PHB showed a negative trend. Increasing the phosphate concentration at a fixed yeast extract concentration showed an increase in both cell mass and PHB content only in cases when the medium was supplemented with ammonium sulfate. The highest cell mass of  $3.57 \text{ g l}^{-1}$  was achieved in the medium with 0.2% (w/v) phosphate, 0.15% (w/v) yeast extract, and 0.15% (w/v) ammonium sulfate, which provided PHB accumulation of 69 wt.% (Fig. 1a,b). Reducing the yeast extract concentration in the medium to 0.05% (w/v) increased the PHB content to 78 wt.%, but the cell mass was decreased to  $3 \text{ g l}^{-1}$ .

#### Effect of yeast extract and phosphate on PHB production by *H. boliviensis*

Additional investigations on the increments of yeast extract and phosphate concentrations were performed in a fermentor



**Fig. 1** Influence of the amount of phosphate and organic and inorganic nitrogen, assayed as indicated in Table 1, on **a** PHB accumulation and **b** RCM by *H. boliviensis* LC1

tor in which pH and dissolved oxygen were controlled. In all fermentations, aliquots of 100% (w/v) sucrose in MS medium were intermittently added to keep excess carbon source in the medium. The maximum cell mass and PHB accumulation were obtained after 24 h of cultivation. Table 2 shows that while maintaining the yeast extract concentration at 1% (w/v), increase in phosphate levels from 0.15 g resulted first in a slight increase and then a decrease in both cell mass and PHB content. On the other hand, increasing the yeast extract amount while maintaining low phosphate concentration provided the highest cell mass of  $12 \text{ g l}^{-1}$  and PHB content of 44 wt.%. In all cases, inorganic nitrogen ( $\text{NH}_4\text{OH}$ ) was added initially to adjust the pH.

#### Influence of oxygen limitation and complex nitrogen-source supplementation on PHB production

Our earlier studies have shown that the yield of PHB in *H. boliviensis* is improved under conditions of oxygen limitation (Quillaguamán et al. 2005). Oxygen limitation was thus induced in the fermentor by setting different agitation speeds (Fig. 2). The medium was supplemented with  $25 \text{ g l}^{-1}$  sucrose in accordance with other reports using carbohydrate concentration in the range of  $20\text{--}30 \text{ g l}^{-1}$  for optimum cell growth and PHB accumulation in various microorganisms (Page 1992; Lee et al. 1994; Wang and Lee 1997). At agitation speeds of 800, 900, and 1,000 rpm, respectively, the profiles of cell mass and PHB content with time were nearly similar, reaching a maximum at 17–19 h of cultivation, followed by a slight decrease (Fig. 2a,b). The maximum PHB yield of 54 wt.% was obtained in fermentations with agitation at 900 rpm (Fig. 2a). At the lower agitation speed,

**Table 2** Effect of phosphate and yeast extract on PHB production and cell concentration (CDW) of *H. boliviensis* LC1 under controlled conditions in a fermentor after 24 h of cultivation at  $35^\circ\text{C}$

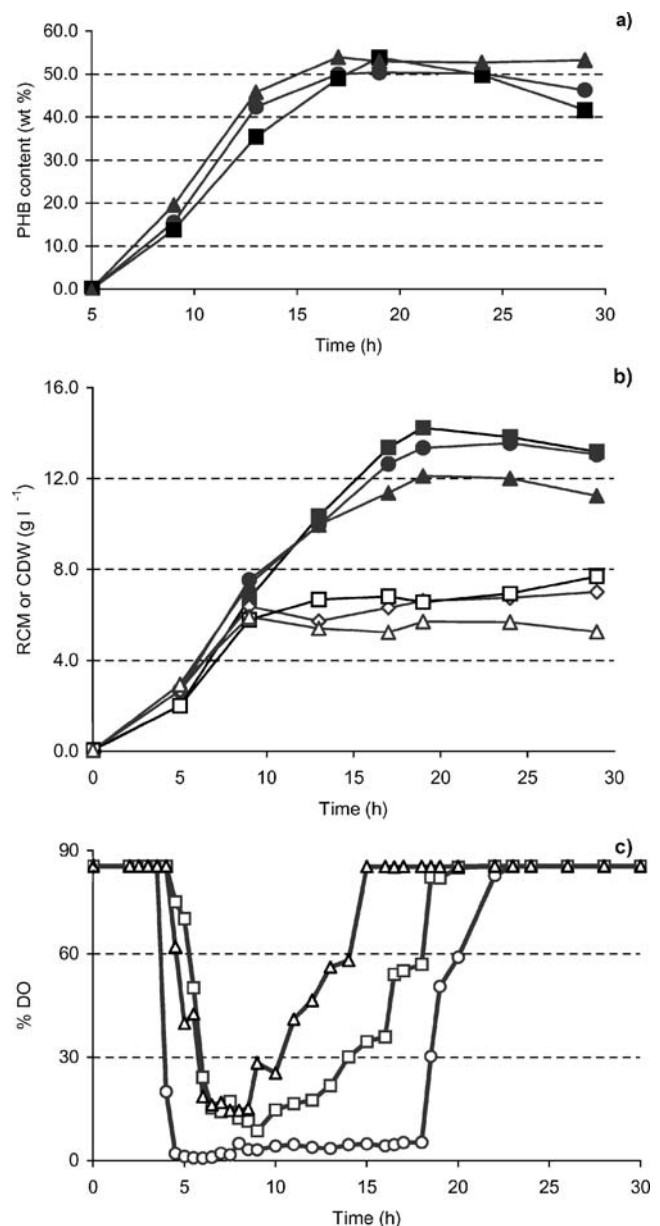
$\text{PO}_4^{3-}$ (g)	Yeast extract % (w/v)	PHB content (wt.%)	CDW ( $\text{g l}^{-1}$ )
0.150 <sup>a</sup>	1.0	37 ( $\pm 1.0$ )	9.3 ( $\pm 0.18$ )
0.525 <sup>b</sup>	1.0	39 ( $\pm 1.5$ )	9.5 ( $\pm 0.28$ )
1.050 <sup>c</sup>	1.0	35 ( $\pm 1.1$ )	6.8 ( $\pm 0.07$ )
0.150 <sup>a</sup>	1.5	44 ( $\pm 2.1$ )	12.0 ( $\pm 0.15$ )

MS medium supplemented with 100% (w/v) sucrose was added to the fermentor as 7.5 ml after 5 h and 15 ml after 7 and 9 h of cultivation to provide excess carbon source in the culture medium

<sup>a</sup> 1 ml of phosphate buffer [15% (w/v)  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ ], pH 7.7, was added to the fermentor prior to inoculation

<sup>b</sup> 0.5 ml of phosphate buffer [15% (w/v)  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ ], pH 7.7, was added to the fermentor before inoculation and every hour during the first 6 h of cultivation

<sup>c</sup> 1 ml of phosphate buffer [15% (w/v)  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ ], pH 7.7, was added to the fermentor before inoculation and every hour during the first 6 h of cultivation



**Fig. 2** PHB accumulation (a); RCM (open symbols) and CDW (filled symbols) (b), and dissolved oxygen (c) during cultivations of *H. boliviensis* LC1 in MS medium supplemented with 2.5% (w/v) sucrose and 1.5% (w/v) yeast extract in a fermentor. Oxygen limitation was induced for different experiments by changing the agitation speed from 700 rpm to 800 (circles), 900 (squares), and 1,000 rpm (triangles). In all cases,  $0.15 \text{ g PO}_4^{3-}$  was added as indicated in Table 2

the dissolved oxygen in the medium was drastically reduced to about 4% with respect to air saturation, resulting in a reduction in the amount of the polymer (50 wt.%). PHB synthesis rate was increased at 1,000 rpm (reaching 54 wt.% in 17 h) but with a decrease in cell concentration, as seen in the decline of the RCM (Fig. 2b).

While keeping an agitation speed of 900 rpm, the effect of additional supplementation of casaminoacids and whey as nitrogen sources was examined. Addition of 0.2% (w/v) of



casaminoacids resulted in an increase of CDW to  $14.4 \text{ g l}^{-1}$  in 17 h, but the polymer content in the cells was reduced to 52 wt.%. On the other hand, the addition of milk whey hindered both CDW and PHB accumulation, to a maximum of  $12 \text{ g l}^{-1}$  and 42 wt.%, respectively, in 19 h. Comparison of yeast extract and casaminoacids as nitrogen sources for cultivation of *H. boliviensis* in shake flasks showed that, when used individually at low concentrations (0.2% w/v), both allowed accumulation of comparable amounts of PHB, i.e., 25–30 wt.%, whereas, at a higher casaminoacids concentration (0.5% w/v), cell metabolism was directed mainly to cell growth, resulting in only 11 wt.% PHB in comparison to 47 wt.% with the same concentration of yeast extract.

## Discussion

Investigations on PHB production by moderate halophiles have been recently initiated with studies on *H. boliviensis*, which is able to accumulate significant amounts of the polymer (50–88 wt.%) when grown on different carbon sources (Quillaguamán et al. 2005, 2006). The polymer productivity is, however, limited by the low cell densities of the organism during the cultivations.

In this work, the effects of different nutrients and cultivation conditions were examined with an aim to improve the production of PHB by *H. boliviensis*. A high concentration of yeast extract was necessary for high cell growth, but combination with a low phosphate concentration seemed to be necessary for increasing the formation of PHB (Fig. 1, Table 2). Indeed, yeast extract was able to provide enough nitrogen supply for the cells. It is usually expected that the consumption of  $\text{NH}_3$  by the cells would lead to a reduction in the pH of the medium, which is thus regulated by adding  $\text{NH}_4\text{OH}$  (Lageveen et

al. 1988; Kim et al. 1994). In our experiments, however, a reduction in pH was only detected after 5 h of cultivation, during the initial stages of the polymer accumulation by the organism, as has been reported earlier (Quillaguamán et al. 2005).

As was previously noted, the RCM of *H. boliviensis* was approximately constant during polymer accumulation, indicating that PHB is synthesized during the stationary phase of cell growth (Fig. 2b; Quillaguamán et al. 2006). It is thus likely that the depletion of nutrient(s) in yeast extract limits the cell growth and induces PHB accumulation in the cells. Improvements in cell density and inhibitory effect on PHB accumulation obtained on supplementation of the medium with casaminoacids suggest that the depleted components from the yeast extract are restored and could involve one or more amino acids. Amino acids and their derivatives—glutamate, ectoine, and glycine betaine—are essential to maintain cell osmolality and turgor in moderate halophiles to cope with water stress caused by high salt concentrations in their environment (Oren 1999; Kunte 2005). Such compounds are to be synthesized by moderate halophiles at high-energy costs for the cells unless they are provided in the medium (Oren 1999). Yeast extract contains amino acids and glycine betaine (Oren 1999), and could therefore have a positive effect on the growth of *H. boliviensis* (Table 2). It is not common to utilize high amounts of complex nitrogen sources, as they hinder the process of PHB production. Two exceptions are *A. vinelandii* UWD and recombinant *E. coli*. The former produces PHB coupled to the cell growth, and the addition of peptone and yeast extract increases the polymer content but not the final cell mass (Page and Cornish 1993). Yet, both organisms are considered as good candidates for industrial PHA production. On the other hand, the use of large amounts of yeast extract or other complex N sources in the

**Table 3** Comparison of PHA yields achieved in batch systems (shake flasks and fermentors) by *H. boliviensis* with other microorganisms reported in literature

Organism	Carbon source	PHA content (wt.%)	CDW ( $\text{g l}^{-1}$ )	PHA concentration ( $\text{g l}^{-1}$ )	Volumetric productivity ( $\text{g l}^{-1} \text{ h}^{-1}$ )	Reference
<i>Halomonas boliviensis</i>	Sucrose	54.0	14.0	7.7	0.40	This work
<i>Alcaligenes latus</i>	Sucrose	83.0	14.0	11.5	0.39	Wang and Lee 1997
<i>Azotobacter vinelandii</i> <sup>a</sup>	Glucose	74.0	10.0	7.5	0.30	Page 1992
<i>Escherichia coli</i> <sup>a</sup>	Glucose	80.8	8.9	7.2	0.15	Lee et al. 1994
<i>Wautersia eutropha</i>	$\text{H}_2/\text{CO}_2$	76.0	17.0	13.0	0.18	Heinzle and Lafferty 1980
<i>Wautersia eutropha</i> <sup>a</sup>	Glucose	54.0	9.4	5.1	0.11	Doi et al. 1988
<i>Pseudomonas oleovorans</i>	Octane	25.0	2.0	0.5	0.02	Lageveen et al. 1988
<i>Haloferax mediterranei</i>	Starch	67.0	9.7	6.5	NR	Lillo and Rodriguez-Valera 1990; Rodriguez-Valera and Lillo 1992

NR data not reported

<sup>a</sup> Cultivation of the microorganism was performed in shake flasks

medium leads to increased production costs. Hence, identifying the component(s) coupled to the PHB synthesis in *H. boliviensis* may allow the use of a suitable, cheaper nitrogen source at an appropriate concentration.

In the case of *H. boliviensis*, adequate carbon and complex nitrogen sources and oxygen depletion during the process are important for optimal cell growth and polymer production (Fig. 2). As compared with previous studies (Quillaguamán et al. 2005, 2006), the cell mass was increased about sevenfold, hence making it a competitive source of PHB. Table 3 shows that the polymer and cell concentrations attained by *H. boliviensis* were in the range of the best polymer producers reported so far in batch systems.

It may also be noted that the highest PHB production levels have been attained in fed-batch fermentations (Lee 1996b), e. g., a volumetric productivity of up to  $5.13 \text{ g l}^{-1} \text{ h}^{-1}$  was obtained when *A. latus* was cultured with sucrose as the polymer precursor (Wang and Lee 1997). Hence, studies are ongoing to investigate other cultivation strategies and alternative media compositions for further improvements in cell mass and PHB content by *H. boliviensis*, and also effective and environmentally benign downstream processing methods for obtaining the pure polymer in high yields.

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