Available online at www.sciencedirect.com
SCIENCE DIRECT
Enzyme and Microbial Technology 38 (2006) 148–154
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Poly(β-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1

Jorge Quillaguamán [a,b,*], Osvaldo Delgado [a,1], Bo Mattiasson [a], Rajni Hatti-Kaul [a]

[a] *Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*
[b] *Centro de Biotecnología, Facultad de Ciencias y Tecnología, Universidad Mayor de San Simón, Cochabamba, Bolivia*

Received 15 November 2004; received in revised form 13 May 2005; accepted 20 May 2005

Abstract

The moderate halophile *Halomonas boliviensis*, isolated from a Bolivian saline soil sample, was able to accumulate poly(β-hydroxybutyrate) (PHB) when grown under conditions of nutrient limitation and excess carbon source. The concentration of sodium chloride in the medium influenced the cell-growth, -size, and rate of PHB accumulation. Cultivation in shake flasks led to a PHB accumulation of about 54 wt.% with respect to cell dry weight at 4.5% (w/v) NaCl in a medium with butyric acid and sodium acetate as carbon sources. The production of PHB was substantially improved to a maximum value of 88 wt.% during cultivation under controlled conditions of pH and oxygen concentration in a fermentor. The use of glucose and sucrose, respectively, as carbon source could also lead to the production of PHB at an average level of 55 wt.%.

Keywords: *Halomonas boliviensis*; Moderate halophile; Poly(β-hydroxybutyrate) (PHB) accumulation; Carbon source

1. Introduction

Halophiles constitute a very heterogeneous group of extremophiles [1,2]. They have been distinguished in two groups based on their salt requirements: extreme halophiles, which grow optimally in media containing 15–30% (w/v) NaCl [3] and moderate halophiles, able to grow at NaCl concentrations of 3–15% (w/v) [1,3]. Extremely halophilic organisms are mainly represented by members of the domain Archaea, which accumulate inorganic ions (commonly K^+) intracellularly to provide osmotic balance with the high salt concentration present in their environments [4]. On the other hand, moderate halophiles have most representatives in the domain Bacteria [2,4], which preferentially use organic compatible solutes to provide the osmotic balance [4]. Due to their special characteristics halophiles have been suggested to hold potential for a variety of biotechnological applications such as production of enzymes, compatible solutes, polymers, preparation of fermented foods, degradation of toxic compounds, etc. [1].

Among the interesting products reported to be produced by extremely halophilic archaea is poly-β-hydroxybutyrate (PHB) [5]. PHB is a polymer synthesised intracellularly and stored as carbon and energy reservoir by several bacteria, usually when cellular growth is restricted by the lack of nutrients such as O, P, N, S, and in the presence of excess carbon source [6,7]. The interest in PHB has been due to its unique characteristic of being a biodegradable thermopolyester that can be produced from renewable resources, and has properties similar to those of petroleum derived plastics [8,9].

Haloferax mediterranei, which grows optimally with 25% (w/v) salts in the medium, can accumulate PHB of about 60–65 wt.% of its cell dry weight when supplied with glucose or starch as sole carbon source and under phosphorous limitation conditions [10]. Another extreme halophilic archaeon, isolated from Egyptian saline soil, was reported to store up to 53 wt.% PHB [11].

On the other hand, there have been no reports so far on PHB production by moderate halophiles; the reports on

* Corresponding author. Tel.: +46 46 222 4948; fax: +46 46 222 4713.
E-mail address: Jorge.Quillaguaman@biotek.lu.se (J. Quillaguamán).
[1] Present address: PROIMI-CONICET, Av. Belgrano y Pasaje caseros, 4000 Tucumán, Argentina.

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.05.013

polymer production by these bacteria being limited to biosurfactants and exopolysaccharides [1,2,12]. Nevertheless, investigations on the phenotypic characteristics of some type strains belonging to the genus *Halomonas* has revealed a positive PHB accumulation test for several species [13]. The present paper reports a study on the production of PHB by *Halomonas boliviensis*, isolated recently from Bolivian highlands [14].

2. Materials and methods

2.1. Bacterial strain and maintenance

H. boliviensis LC1^T (=DSM 15516^T) was maintained at 4 °C on solid HM medium [14], containing (%, w/v): NaCl, 4.45; $MgSO_4 \cdot 7H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 0.009; KCl, 0.05; NaBr, 0.006; peptone, 0.5; yeast extract, 1.0; glucose 0.1; granulated agar, 2.0; adjusted to pH 7.5.

2.2. Medium composition for PHB production

Cultures of *H. boliviensis* were grown in a modified HM medium, HM-2 containing (%, w/v): yeast extract, 0.1; peptone, 0.02; NaCl, 4.5; $MgSO_4 \cdot 7H_2O$, 0.038; $CaCl_2 \cdot 2H_2O$, 0.013; KCl, 0.075; NaBr, 0.02; KH_2PO_4, 0.02. The medium was further supplemented with different carbon sources for various experiments.

2.3. PHB production in flasks

H. boliviensis was grown at 30 °C in 60 ml of HM-2 medium supplemented with 0.8% (v/v) butyric acid and 0.8% (w/v) sodium acetate in 250 ml flasks with rotary shaking at 200 rpm. After about 13 h of growth, 15 ml of the culture broth was inoculated in 1 l Erlenmeyer flasks containing 250 ml of the same medium, supplemented with different NaCl concentrations (%, w/v: 0.5, 4.5, 9.5, 14.5). In all cases, pH was adjusted to 7.5 prior to cultivation. Samples were collected at defined time intervals during cultivation and analysed for cell dry weight (CDW), PHB content and residual cell mass (RCM).

2.4. PHB production in a fermentor

For PHB production in a fermentor, *H. boliviensis* was first grown at 30 °C in 1 l flasks containing 200 ml of HM-2 medium (pH 7.5) and a defined carbon source, with shaking at 200 rpm for 13 h. This culture was used to inoculate a 2 l fermentor vessel (Voyager, Luton, UK) containing 1.4 l of the cultivation medium. Antifoam was added before the inoculation of the medium and pH was maintained at 8.0 by using 0.5 M HCl/NaOH (unless otherwise stated). The air inflow rate and agitation speed were initially adjusted to 0.5 l min^{-1} and 700 rpm, respectively, during the fermentations. When a decrease from the initial dissolved oxygen concentration (i.e. 85%) was detected, the air inflow was increased to avoid oxygen limitation in the cells. Samples (ca. 10 ml) were collected at different time intervals for analysis.

2.5. PHB isolation for NMR-spectroscopic analysis

H. boliviensis LC1 cells, containing the polymer, were harvested from 500 ml of culture broth by centrifugation at 10,000 × *g* for 10 min, washed once with sterile water, and finally resuspended in water and lyophilized. PHB was recovered from lyophilized cells by extraction for 30 h with chloroform in a Soxhlet apparatus, and concentrated by evaporating the solvent under vacuum. The polymer was precipitated from the concentrated solution with 10 vol. of ethanol [11] and the resulting PHB granulates were filtered twice through cellulose extraction thimbles (Whatman, Maidstone, England). The ^{1}H nuclear magnetic resonance (NMR) spectrum was recorded at 500 MHz with a Bruker ARX500 Spectrometer at room temperature using deuterated chloroform as internal reference solvent. The spectrum was evaluated using standard Bruker UXNMR software.

2.6. Quantitative analysis

Cell dry weight (CDW) was determined by centrifuging 3 ml of the culture samples at 2000 × *g* for 15 min, the pellet washed twice with distilled water, and dried at 75 °C until constant weight was obtained.

PHB quantification was based on the Law and Slepecky method [15]. For this, dried cell pellets containing intracellular PHB were hydrolysed using concentrated sulphuric acid for 1 h to obtain crotonic acid, which was quantified by measuring absorbance at 235 nm. Samples were analysed in duplicates from shake flasks, and in triplicates for samples from the fermentor.

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) according to the definition by Lee et al. [16].

2.7. Microscopic analysis

H. boliviensis cells, on attaining maximum PHB content, were separated by centrifugation at 4000 × *g* for 7 min and fixed for 4 h at room temperature in a solution of 4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.1, and 0.1% (w/v) Brij 35, followed by an overnight treatment in the same solution without Brij 35. The cells were then rinsed with 0.1 M sodium cacodylate, pH 7.1, transferred to 2% osmium tetroxide for 8 h at room temperature and subsequently to 2% uranyl acetate in 10% ethanol for 40 min [17]. The cells were dehydrated through a graded series of ethanol–water solutions with a final treatment in propylene oxide, and embedded in epon/araldite resin that was then cut with a diamond knife.

The fine sections of 50 nm were placed on Formvar-coated copper grids, contrasted with a 2% aqueous solution of uranyl acetate and examined under a JEM-123 (HC) transmission electron microscope (JEOL, Massachusetts, USA).

3. Results

3.1. H. boliviensis as a PHB producer

Preliminary studies on the growth of *H. boliviensis* in a medium containing excess carbon (≥1.0%, w/v) and limited nutrient content provided by the low amount of yeast extract in HM-2 medium, showed the presence of intracellular granules. The granules were processed for purification of the components for analysis. The proton displacements and chemical shifts observed in the ^{1}H NMR spectrum confirmed the chemical structure of PHB (Fig. 1) [18].

3.2. Effect of varying salt concentrations during cultivation

Fig. 2A and B depicts the production of PHB and cell growth of *H. boliviensis* at different salt concentrations in a medium containing 0.8% (v/v) butyric acid and 0.8% (w/v) sodium acetate. Sodium chloride concentration of 0.5–4.5% (w/v) provided the highest cell densities and also the PHB accumulation of about 54 wt.%. The pH of the medium was increased during cultivation from 7.5 to about 9.7 ± 0.2. Electron microscopic observation of the cells showed the presence of high amounts of the polymer in most cells; in some cases almost all the cytoplasmic space was filled (Fig. 3A and B). With increase in NaCl concentration in the medium, a considerable decrease in the growth rate of the organism was observed, and accumulation of PHB was only evident after 42 h of cultivation although the final yield of the polymer was about the same as that at lower salt concentrations (ca. 50 wt.%). Increase in the medium pH to 8.3 ± 0.05 was noted. The cells were found to be reduced in size and tended to keep a more coccoid shape but short rods could also be observed under the microscope (Fig. 3C and D). In live cultures, elongated rods were occasionally seen, although a reduction in the thickness of the cells was evident with respect to those grown at low salt concentrations. Irrespective of the salt concentration in the medium, *H. boliviensis* cells contained intracellularly one or two PHB inclusions, with sizes varying between 0.20 and 0.64 μm.

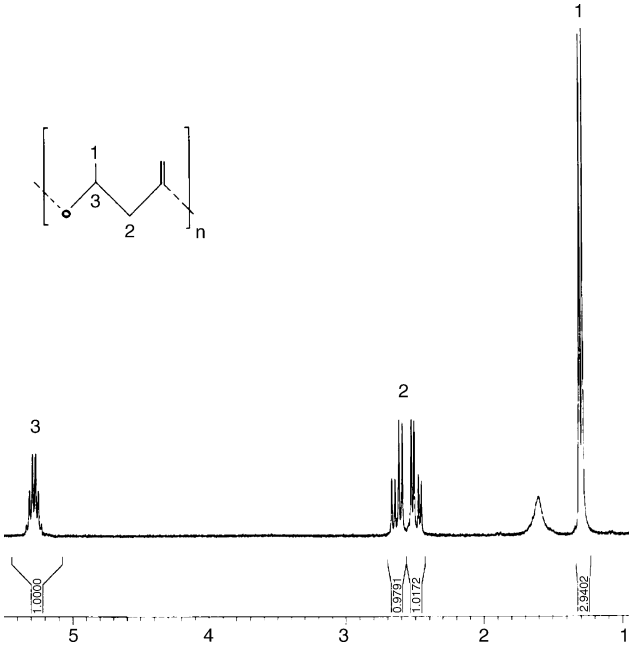

Fig. 1. ^{1}H NMR spectrum showing the carbon composition of the monomers belonging to the polymer extracted and purified from *H. boliviensis* cells. Deuterated chloroform was used as internal reference solvent.

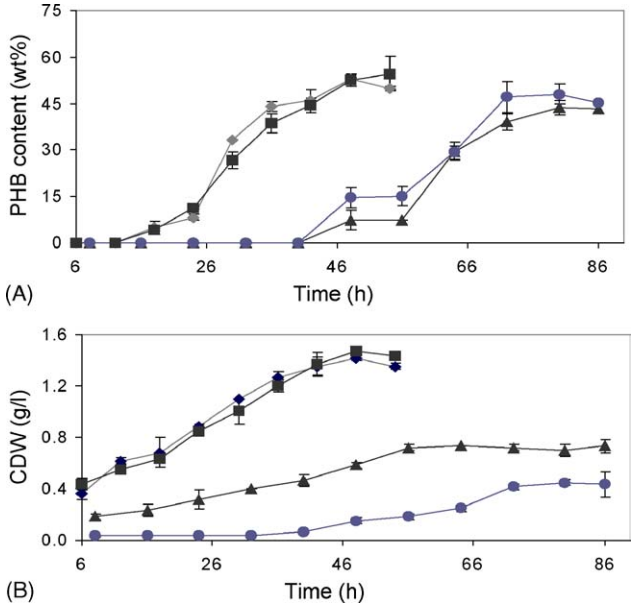

Fig. 2. Effect of sodium chloride concentration on: (A) PHB accumulation and (B) cell concentration by *H. boliviensis* LC1 when cultivated in shake flasks containing HM-2 medium supplemented with 0.8% (v/v) butyric acid and 0.8% (w/v) sodium acetate at 30 °C and 200 rpm. Symbols refer to NaCl concentrations of: (♦) 0.5% (w/v); (■) 4.5% (w/v); (▲) 9.5% (w/v); (●) 14.5% (w/v).

Fig. 4 shows an enhanced micrograph of *H. boliviensis* containing PHB granules. This augmentation reveals that a homogeneous and ordered membrane surrounds the PHB inclusions. This membrane was observed in the cells cultured at all salt concentrations (Fig. 3A and D).

3.3. PHB production from different carbon sources by H. boliviensis

In order to test cell growth and PHB accumulation using different carbon sources and to avoid the potential influence of oxygen limitation and changes in pH, *H. boliviensis* was

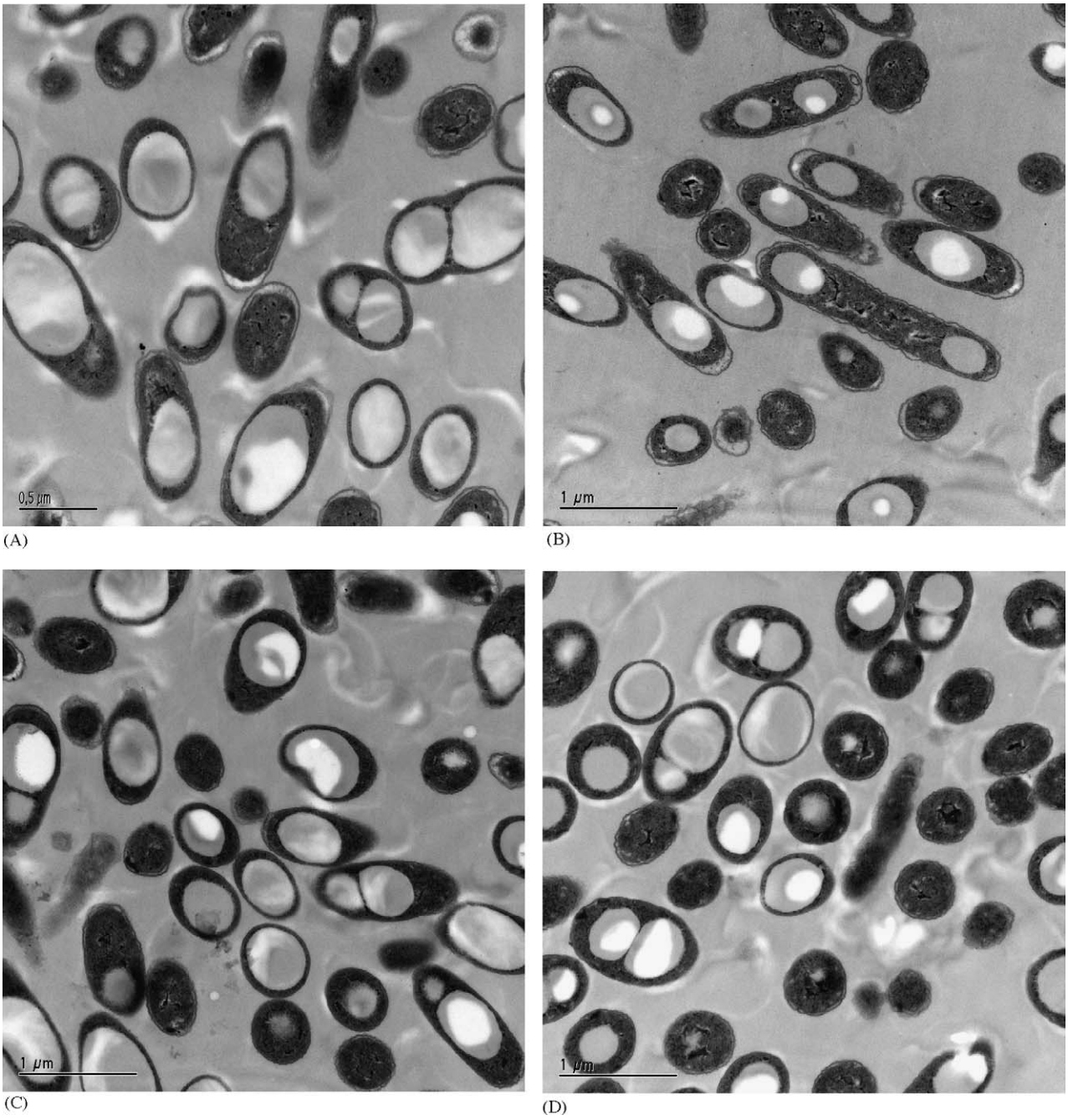

Fig. 3. Transmission electron microscope pictures of *H. boliviensis* grown in HM-2 medium supplemented with 0.8% (v/v) butyric acid, 0.8% (w/w) sodium acetate (w/v), and with NaCl concentration of: (A) 0.5% (w/v), (B) 4.5% (w/v), (C) 9.5% (w/v), and (D) 14.5% (w/v), respectively, in shake flasks at 30 °C and 200 rpm. Cells were harvested on reaching maximum PHB accumulation.

grown in a fermentor under a controlled environment. In all experiments, NaCl concentration was 4.5% (w/v) and the pH of the culture medium was maintained at 8 by adding NaOH/HCl. Under these conditions, the PHB accumulation attained from the combination of butyric acid and sodium acetate was drastically improved, reaching a maximum value of 88 wt.%, however, the cell mass was increased slightly to 2 g l^{-1} as compared to the shake flask cultivations (1.4 g l^{-1}) (Fig. 5). For all cultivations in the fermentor, the residual cell mass of *H. boliviensis* was noted to be approximately constant during the PHB accumulation phase (e.g. Fig. 5).

Use of sugars such as glucose and sucrose, respectively, as carbon source, could also induce the formation of PHB in *H. boliviensis*. The maximum PHB accumulation of about 55 wt.% was reached between 23 and 33 h of cultivation (Fig. 6A). Supplementing the medium with sodium acetate slightly increased the rate of PHB formation in the cells but

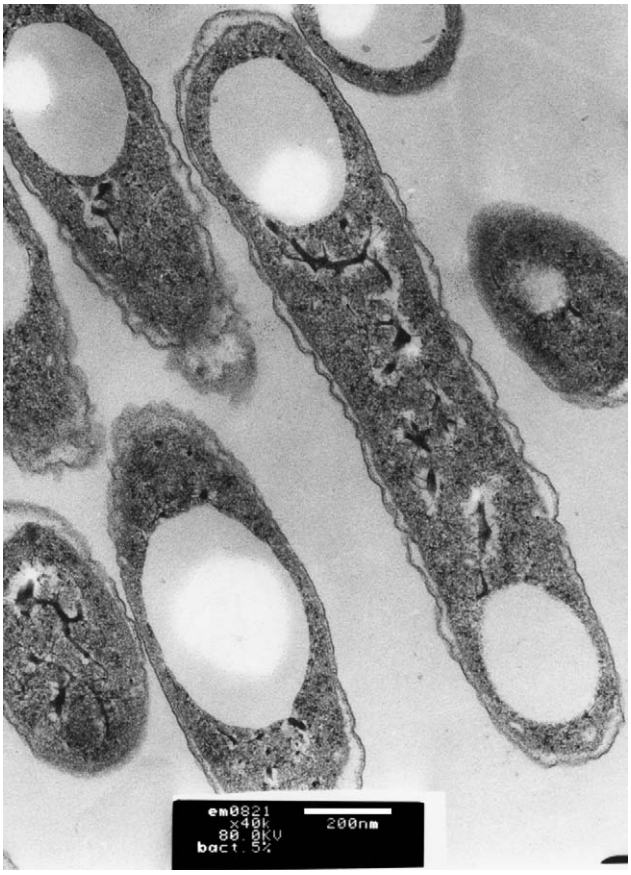

Fig. 4. Enhancement of Fig. 3B showing PHB granules in *H. boliviensis* surrounded by a homogeneous membrane. Magnification 40,000×.

the final PHB accumulation and CDW were about the same as that attained with individual sugars (Fig. 6B). Only restricted growth and polymer production by *H. bolivensis* was detected when starch was added to HM-2 medium (results not shown).

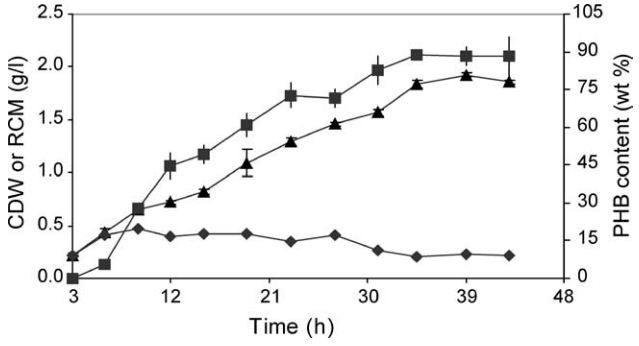

Fig. 5. Profiles of PHB accumulation and cell growth during cultivation of *H. boliviensis* in a fermentor. The HM-2 medium was supplemented with 0.8% (v/v) butyric acid, 0.8% (w/v) sodium acetate, and 4.5% (w/v) NaCl. The pH of the medium was controlled at a constant value of 8.0 by using 1.5 M NaOH/HCl. Agitation speed was kept constant at 700 rpm and air inflow was increased from 1 l min^{-1} when a decrease in the dissolved oxygen of the medium was detected during the cultivation. Symbols: (▲) CDW; (■) PHB content wt.%; (♦) RCM.

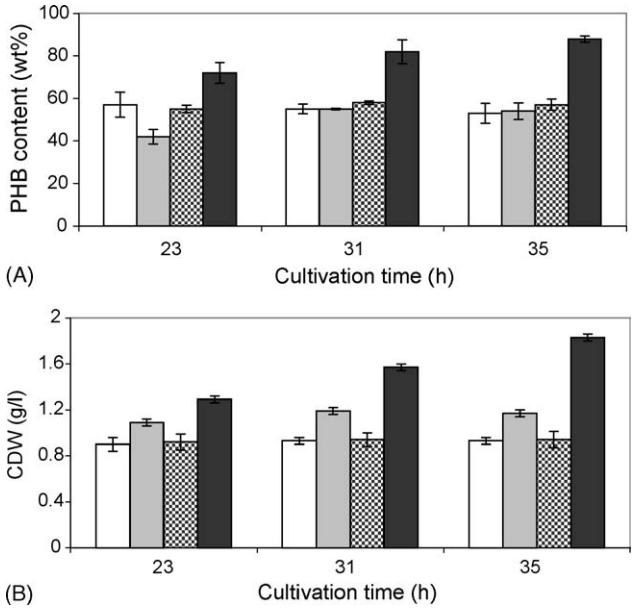

Fig. 6. (A) PHB accumulation expressed as percentage of cell dry weight and (B) CDW, under the same culture conditions indicated for Fig. 5 but with different carbon sources: (□) 1% (w/w) glucose; (▒) 1% (w/w) sucrose; (▩) 0.4% (w/w) sucrose and 0.6% (w/w) sodium acetate; and (■) 0.8% (v/v) butyric acid and sodium acetate 0.8% (w/v).

4. Discussion

The results shown above demonstrate that the moderate halophile, *H. boliviensis* is able to produce high amounts of PHB in the presence of excess carbon source and limited amount of yeast extract in the medium. In contrast to the extremely halophilic archaea, low salts concentrations provide the best environment for PHB production by *H. boliviensis*. This is mainly due to the reduced cell growth at high NaCl concentrations (Fig. 2B). However, like the halophilic archaea [11], the final PHB content in *H. boliviensis* does not seem to be influenced by the NaCl concentration (Fig. 2A).

Physiologically, moderate halophiles differ from extremely halophilic archaea in that they synthesize and accumulate compatible solutes, a process known to be energetically expensive for the cells [2]. It is estimated that the amount of ATP required for the generation of compatible solutes by halophilic heterotrophs is comparable to that needed for biosynthesis of the cell components [4]. Hence, a decrease in cell concentration (CDW) of moderate halophiles with increasing salt concentrations as seen in Fig. 2B, should be expected [4]. Moreover, the reduction in size of *H. boliviensis* cells at high salt concentrations (Fig. 3C and D) could also indicate a restriction in the development of the cell structure. A similar tendency to form more coccoid shapes with reduced-sizes and -cell thickness with increase in NaCl concentration was also previously noted in *Halomonas elongata* and *Holomonas variabilis* [19,20]. Under the culture conditions used for inducing PHB

accumulation by *H. boliviensis*, it seems evident that the biological energy generated by the cells is directed to growth and to adjust their metabolism to the osmotic stress before starting the synthesis of PHB (Fig. 2A and B).

On the other hand, the number and size of PHB granules found in cells is dependent upon the bacterial species; *Wautersia eutropha* (formerly called *Ralstonia eutropha*) cells store between 8 and 12 PHB granules with varying diameters in the range of 0.24–0.50 μm [6] whereas *Azotobacter vinelandii* can accumulate >40 granules per cell, with sizes of 0.5–1.4 μm [21]. Yet a large distribution in the number and sizes of PHB granules can be noted in *Halomonas* spp. when supplied with excess carbon source for the production of exopolysaccharides [22–24]. In contrast, *H. boliviensis* commonly synthesizes one or two granules (0.20–0.64 μm) per cell (Fig. 3A and D), albeit occasionally formation of up to 5 granules in elongated cells can be seen, irrespective of the C-source utilized. The formation of large and uniform PHB granules, as seen in the case of *H. boliviensis*, is suggested to be advantageous for the purification and quality of the polymer [25].

The membrane covering the PHB granules is known to be composed of enzymes, non-enzymatic proteins (called 'phasins'), and/or phospholipids, with arrangement depending on the bacterial species [25,26]. The ordered structure of the membrane sequestering the PHB inclusions resembles those found in pseudomonads, *A. vinelandii* and *Rhodococcus* spp. (formerly called *Nocardia corallina*) but differs from *W. eutropha* and recombinant *E. coli* strains [26].

As seen in Fig. 5, using butyric acid and sodium acetate as carbon feed, the amount of accumulated PHB was significantly improved by performing cultivations of *H. boliviensis* under controlled conditions in a fermentor. The constant residual cell mass observed during cultivations clearly implies that PHB is produced during the stationary phase of growth, as commonly found in other microorganisms [8]. The organism is also able to accumulate high amounts of PHB from various renewable carbon sources – sugars (Fig. 6A), which have been suggested to be more suitable substrates from an economical point of view [8,27]. The maximum PHB accumulation reached by *H. boliviensis* with different carbon sources are comparable to the highest reported so far by non-halophilic organisms, e. g. *Alcaligenes latus, Wautersia eutropha, Azotobacter vinelandii*, pseudomonads, and recombinant *Escherichia coli* strains [5,9,16].

Further work involving different cultivation strategies to improve the cell growth and polymer productivity by *H. boliviensis* grown on sugars is ongoing.

Acknowledgements

We would like to thank the Swedish International Development Cooperation Agency (Sida) and its research branch, SAREC, for supporting this work.

References

[1] Ventosa A, Nieto J, Oren A. Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 1998;62:504–44.
[2] Oren A. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. J Ind Microbiol Biotechnol 2002;28:56–63.
[3] Kushner DJ. Life in high salt and solute concentrations: halophilic bacteria. In: Kushner DJ, editor. Microbial life in extreme environments. Academic Press: London; 1978. p. 318–58.
[4] Oren A. Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 1999;63:334–48.
[5] Rodriguez-Valera F, Lillo JAG. Halobacteria as producers of polyhydroxyalkanoates. FEMS Microbiol Rev 1992;103:181–6.
[6] Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 1990;54:450–72.
[7] Lee SY. Bacterial polyhydroxyalkanoates. Biotechnol Bioeng 1996;49:1–14.
[8] Lee SY. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol 1996;14:431–8.
[9] Steinbüchel A, Füchtenbush B. Bacterial and other biological systems for polyester production. Trends Biotechnol 1998;16:419–27.
[10] Lillo JG, Rodriguez-Valera F. Effects of culture conditions on poly(β-hydroxybutyric) acid production by *Haloferax mediterranei*. Appl Environ Microbiol 1990;56:2517–21.
[11] Hezayen FF, Rehm BHA, Eberhardt R, Steinbüchel A. Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. Appl Microbiol Biotechnol 2000;54:319–25.
[12] Margesin R, Schinner F. Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 2001;5:73–83.
[13] Mata JA, Martínez-Cánovas J, Quesada E, Béjar V. A detailed phenotypic characterisation of the type strains of *Halomonas* species. Syst Appl Microbiol 2002;25:360–75.
[14] Quillaguamán J, Hatti-Kaul R, Mattiasson B, Alvarez MT, Delgado O. *Halomonas boliviensis* sp. nov., an alkalitolerant, moderate halophile bacterium isolated from soil around a Bolivian hypersaline lake. Int J Syst Evol Microbiol 2004;54:721–5.
[15] Law JH, Slepecky RA. Assay of polyβ-hydroxybutyric acid. J Bacteriol 1961;82:33–6.
[16] Lee SY, Wong HH, Choi J, Lee SH, Lee SC, Han CS. Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of *Pseudomonas putida* under phosphorus limitation. Biotechnol Bioeng 2000;68:466–70.
[17] Poirier Y, Erard N, Macdonald-Comber Petétot J. Synthesis of polyhydroxyalkanoate in the peroxisome of *Saccharomyces cerevisiae* by using intermediates of fatty acid β-oxidation. Appl Environ Microbiol 2001;67:5254–60.
[18] Nakamura S, Kunioka M, Doi Y. Biosynthesis and characterization of bacterial poly(3-hydroxybutyrate-co-3hydroxypropionate). Macromol Rep 1991;A28(Suppl. 1):15–24.
[19] Vreeland RH, Anderson R, Murray RGE. Cell wall and phospholipid composition and their contribution to the salt tolerance of *Halomonas elongata*. J Bacteriol 1984;160:879–83.
[20] Fendrich C. *Halovibrio variabilis* gen. nov. sp. nov, *Pseudomonas halophila* sp. nov. and a new halophilic aerobic coccoid Eubacterium from Great Salt Lake, Utah, USA. Syst Appl Microbiol 1988;11:36–43.
[21] Page WJ, Sherburne R, D'Elia L, Graham LL. Poly(β-hydroxybutyrate) extrusion from pleomorphic cells of *Azotobacter vinelandii* UWD. Can J Microbiol 1995;41(Suppl. 1):22–31.
[22] Martinez-Canovas MJ, Quesada E, Llamas I, Bejar V. *Halomonas ventosae* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. Int J Syst Evol Microbiol 2004;54:733–7.

[23] Niven DF, MacLeod RA. Sodium ion-proton antiport in a marine bacterium. J Bacteriol 1978;134:737–43.
[24] Quesada E, Béjar V, Ferrer MR, Calvo C, Llamas I, Martinez-Canovas F, et al. Moderately halophilic, exopolysaccharide-producing bacteria. In: Ventosa A, editor. Halophilic microorganisms. Berlin: Springer; 2004. p. 297–314.
[25] Steinbüchel A, Aerts K, Babel W, Folner C, Leibergesell M, Wieczorek R. Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. Can J Microbiol 1995;41(suppl. 1):94–105.
[26] Stuart ES, Tehrani A, Valentin HE, Dennis D, Lenz RW, Fuller RC. Protein organization on the PHA inclusion cytoplamic boundary. J Biotechnol 1998;64:137–44.
[27] Choi J, Lee SY. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl Microbiol Biotechnol 1999;51:13–21.