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Effects of Culture Conditions on Poly(β-Hydroxybutyric Acid) Production by *Haloferax mediterranei*

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The halobacterium *Haloferax mediterranei* accumulates poly(β-hydroxybutyrate) (PHB) as intracellular granules. The conditions for PHB production in batch and continuous cultures have been studied and optimized. Phosphate limitation is essential for PHB accumulation in large quantities. Glucose and starch are the best carbon sources. With 2% starch, 0.00375% KH_2PO_4, and 0.2% NH_4Cl in batch culture, a production of ca. 6 g of PHB per liter was reached, being 60% of the total biomass dry weight, and giving a yield over the carbon source of 0.33 g/g. The PHB production in continuous cultures was stable over a 3-month period. Our results demonstrate that *H. mediterranei* is an interesting candidate for industrial production of biological polyesters.

Poly(β-hydroxybutyric acid) (PHB) is one of the major reserve materials found in eubacteria. It is also produced by many halobacteria, which are archaebacteria. In a previous work, *Haloferax mediterranei* was shown to accumulate more PHB than the other halobacteria did (10). PHB and other poly(β-hydroxyalkanoates) (2, 3) are of industrial interest because of their possible uses as biodegradable thermoplastics. In fact, a copolymer of β-hydroxybutyrate and β-hydroxyvalerate is being produced by ICI Ltd. under the trade name of Biopol, using the eubacterium *Alcaligenes eutrophus* (12). This organism accumulates up to 80% of its dry weight as polymer. However, the use of *H. mediterranei* to produce PHB could offer certain advantages. This organism grows on media containing high salt concentrations and simple carbon sources such as sugars or starch. Under these conditions very few organisms, if any, can develop at growth rates anywhere near as high as those reached by *H. mediterranei* (16–18). Therefore, the requirement for sterile conditions is greatly reduced, and extremely simple production systems can be developed, such as open ponds similar to those used for sewage treatment. As a result, it was interesting to study the conditions favoring PHB accumulation in this organism. Moreover, there is no information on regulation of the accumulation of reserve materials in archaebacteria. In eubacteria the synthesis of large amounts of PHB is triggered by different environmental stimuli including limitation of oxygen, nitrogen, phosphorus, and potassium individually or simultaneously depending on the organisms (8, 20). Apparently, all these stimuli act through the accumulation of reducing power and acetyl coenzyme A in the cytoplasm (8). Therefore, we have studied the effects of some of these factors, together with those of pH, salt concentration and type, and concentration of the carbon source, on PHB production by *H. mediterranei*. We have also studied PHB production in continuous culture limited by the phosphorus source. Continuous culture could be an ideal system, given the near impossibility of contamination. Therefore, the stability of PHB production during long periods of cultivation of the strain was also tested.

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MATERIALS AND METHODS

Microorganism and culture conditions. *H. mediterranei* ATCC 33500 was used for all experiments. In all batch cultures an inoculum of approximately 2% of the culture volume was used. The inoculum was prepared under the same conditions and in the same medium as in the growth experiment, but it was cultivated in an Erlenmeyer flask (500-ml volume with 100 ml of medium) with orbital shaking at 200 strokes per min.

All growth experiments were carried out in a Braun Biostat M laboratory fermentor with a working volume of 1,500 ml and equipped with pH regulation, automatic antifoam addition, and oxygen measurement.

The culture media used were based on a basal synthetic medium in which the concentrations of different components were changed to evaluate their effect on PHB production. The basal synthetic medium contained the following (percent, wt/vol): glucose, 1; NH_4Cl, 0.2; KH_2PO_4, 0.03; $FeCl_3$, 0.0005; and marine salts (SW) at ca. 25% final concentration (NaCl, 19.4; $MgCl_2$, 1.6; $MgSO_4$, 2.4; $CaCl_2$, 0.1; KCl, 0.5; $NaHCO_3$, 0.02; NaBr, 0.05) (18). The medium was sterilized by filtration through membrane filters (pore size, 0.45 μm). Unless otherwise indicated, the pH was maintained at 7.2 and the temperature was kept at 38°C. The air flow and shaking were manually adjusted to keep oxygen saturation (near 100%) in the medium. Once the culture medium had been optimized with regard to nitrogen and phosphorus source concentration and carbon source nature and concentration, the experiments involving changes of other variables (SW concentration, pH, temperature, and oxygen transfer rate) were carried out with a basal synthetic medium as above but with 2% (wt/vol) glucose and 0.00375% (wt/vol) KH_2PO_4.

Continuous culture. To study PHB production in continuous cultures under phosphate limitation, we used the same fermentor and culture conditions as in the batch cultures. Fresh medium was pumped by an LKB peristaltic pump. The medium used was the optimized synthetic medium described above, except in one set of experiments in which the phosphate concentration was reduced to 0.001875% in the reservoir. To study the stability of production of PHB over long periods in continuous culture, we prepared a small

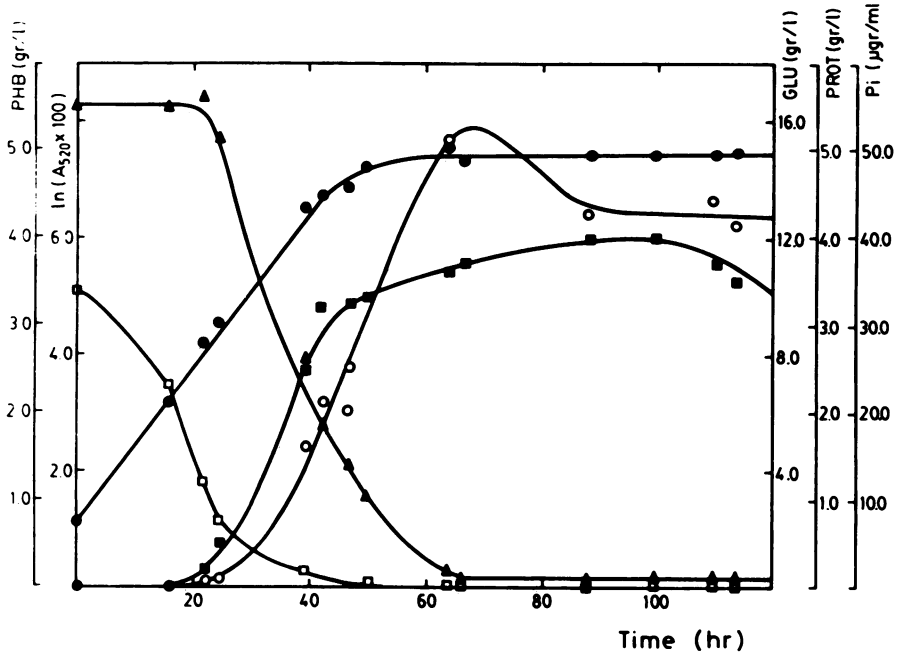

FIG. 1. Batch fermentation production curves of *H. mediterranei* grown on 2% glucose medium (see Materials and Methods) and under optimal conditions (see Results). Symbols: ●, optical density; ▲, glucose concentration (GLU); □, P_i concentration; ■, protein concentration (PROT); ○, PHB concentration.

fermentor by using a 250-ml cylindrical flask aereated by a soft humidified air flow and shaken with a magnetic stirrer. The medium used contained 0.5% (wt/vol) yeast extract (Difco Laboratories) and 25% SW. No pH regulation was provided, and antifoam was added manually at intervals. The culture was kept with a dilution rate of 0.12 h^{-1} for 3 months, after which a sample was taken from the fermentor and plated to isolate individual colonies. One of these colonies was transferred and studied in batch culture, under optimal conditions, to evaluate PHB production. In an attempt to obtain superproducing strains a continuous culture was used in which the strain of *H. mediterranei* was subjected to alternative cycles. The culture was as above, but the medium was the batch synthetic medium with 2% (wt/vol) glucose and 0.00375% (wt/vol) KH_2PO_4. Once the steady state was reached, the reservoir was changed to a medium with the same composition but with no carbon source. Then the culture was allowed to proceed until the optical density at 520 nm (OD_{520}) decreased to below 0.1, at which time the reservoir was switched again for one with 2% glucose. The dilution rate was 0.03 h^{-1}. The culture was maintained for 3 months with a total of five steady-state-plus-dilution cycles. After that, colonies were isolated and assayed for PHB production in batch culture.

Analytical techniques. Growth was monitored by measuring the OD_{520} in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc.). The salt-free dry weight was determined by drying at 105°C to constant weight and subtracting the weight of the ash remaining after heating at 400°C for 4 h. To measure total protein, 1-ml samples were spun in a microcentrifuge and after washing the pellet with 25% SW the protein present was determined by the method of Lowry et al. (14). Glucose was measured by the *o*-toluidine method (9). Starch was quantified by adding 5 ml of 0.0007 N iodine to 0.5 ml of supernatant and measuring the OD_{660}. P_i was determined as described by Chifflet et al. (6). PHB was assayed by the Law and Slepecky method (13). Cells were collected by centrifugation (4,000 × *g*) of a 5-ml sample. The pellet was suspended in 5 ml of 0.2% (wt/vol) sodium hypochlorite. After 1 h at 37°C, to allow the total lysis of the suspension, PHB granules were collected by centrifugation (2,000 × *g*). The pellet was washed with distilled water, acetone, and ethanol and the final pellet was dissolved in chloroform, with the unsoluble remains being discarded. After the chloroform had been evaporated, the residue was hydrolyzed and dehydrogenated with concentrated sulfuric acid to obtain crotonic acid, which can be quantified by its A_{235}. The standard curve was obtained by using PHB (Sigma Chemical Co.). The oxygen transfer rates under different conditions of aereation and shaking were obtained by using a salt solution as in the culture medium. This salt solution was saturated with Na_2SO_3, filtered, and then placed in the fermentor; $CuSO_4$ was added to a final concentration of 0.0001 M. After setting the conditions of aereation and shaking, 5-ml samples were regularly withdrawn and the sulfite concentration was determined by an iodometric method (4).

RESULTS AND DISCUSSION

In all growth experiments PHB production responded to a pattern that is similar to that found in eubacteria (11, 15). Figure 1 shows a typical example of curves representing growth and PHB production. PHB accumulation starts during the logarithmic phase, increasing with the biomass and reaching a peak at the beginning of the stationary phase. PHB synthesis is delayed with respect to biomass development, reaching a maximum rate of synthesis at the end of the exponential phase. Accumulation proceeds to the stationary phase, until no more carbon source is available. The effects of nitrogen source (NH_4Cl or yeast extract) concentration and phosphorus source concentration are shown in Table 1.

TABLE 1. Effect of limitation of the nitrogen and phosphorus sources on the production of PHB by *H. mediterranei*

Substrate and concn (%, wt/vol)[a]	Max OD_{520}	Generation time (h)	Max PHB concn (g/liter)	Max PHB/ protein ratio	PHB yield (g/g)
NH_4Cl					
0.40	4.80	6.40	0.62	0.51	0.062
0.20	5.25	8.88	0.73	0.70	0.073
0.10	5.40	5.50	0.28	0.32	0.028
0.05	3.78	7.53	0.02	0.04	0.002
0.025	1.72	6.19	0.04	0.01	0.004
Yeast extract					
0.30	6.70	5.50	0.69	0.87	0.069
0.075	2.00	4.08	0.24	0.58	0.024
KH_2PO_4					
0.03	5.25	8.88	0.73	0.70	0.073
0.015	9.20	5.29	0.97	0.69	0.097
0.0075	9.00	7.79	1.70	1.97	0.170
0.00375	7.50	6.86	3.09	3.95	0.309
0.0009375	2.55	14.44	1.76	2.45	0.176

[a] Unless otherwise indicated the following basal synthetic medium components were used (in percent, wt/vol): glucose, 1; NH_4Cl, 0.2; KH_2PO_4, 0.03; $FeCl_3$, 0.0005; marine salts, 25 (final concentration).

TABLE 2. Maximum PHB production in relation to the nature of the carbon source and glucose concentration

Carbon source (concn)[a]	Max OD_{520} reached	Generation time(h)	Max PHB concn (g/liter)	Max PHB/ protein ratio	PHB yield (g/g)
Saccharose (1%)	3.92	13.75	0.64	0.73	0.063
Glutamate (1%)	1.59	10.05	0.13	0.37	0.013
Butyrate (1%)	0.33	9.76	0.03	2.45	0.003
Acetate (1%)	0.72	10.19	—[b]	—	—
Glycerol (1%)	0.22	41.51	0.01	0.03	0.001
Methanol (1%)	0.15	45.84	0.01	0.02	0.001
Glucose (1%)	7.50	6.86	3.09	3.95	0.309
Glucose (2%)	8.90	8.30	4.16	5.13	0.208
Glucose (5%)	9.40	9.21	3.28	2.01	0.066
Glucose (10%)	6.40	6.33	3.52	2.03	0.035
Starch (2%)	12.40	6.89	6.48	4.08	0.324

[a] For these experiments, the concentration of phosphate that gave the highest PHB production was used: 0.00375% (wt/vol) KH_2PO_4. Otherwise, except for the carbon source, the composition of the media was as in the basal synthetic medium.
[b] —, Undetectable amounts.

The concentration of the nitrogen source had similar effects on PHB and biomass production, both of which were decreased at lower concentrations. The use of a complex nitrogen source such as yeast extract did not significantly affect PHB production, giving only a slight stimulation coupled to an increase in the biomass production. Decreasing the nitrogen source also decreased the PHB/protein ratio and the PHB yield. However, limiting the phosphorus concentration to 0.00375% (wt/vol) stimulated both total PHB production and the yield with respect to the carbon source (3.09 g/liter and 0.3 g/g, respectively). Lower phosphate concentrations gave poorer PHB production and a corresponding decrease in growth. Phosphate limitation is a powerful stimulant of PHB accumulation in this microorganism. Under these conditions (0.00375% phosphate in the medium), the biomass contained four times more PHB than protein. However, other substrates that often have strong effects in eubacteria, such as the nitrogen source or oxygen, had no stimulating effects.

For industrial production, the cost of the carbon source is important. *H. mediterranei* uses a wide range of compounds as sole sources of carbon and energy (17). Therefore, we tested different carbon sources which are cheaper than glucose: sucrose, methanol, and starch. Acetate and butyrate were also tested because they are precursors of PHB and could therefore stimulate production (7). Glutamate and glycerol were also used as carbon sources. The results are shown in Table 2. Glucose gave the highest PHB concentration and yield. Acetate and butyrate gave very poor production, which also corresponded to low biomass production. Sucrose gave the best productivity after glucose, but still the yield was much lower. This phenomenon has been noticed in a previous study under less controlled conditions (10). In that study cellobiose, lactose, and sucrose were also shown to be very unsuitable substrates for PHB production. The effect of the carbon source concentration in the medium is also shown in Table 2. Of four concentrations used, 2% (wt/vol) was the one giving the best production, although the yield was somewhat lower than with only 1%. *H. mediterranei* is one of the few halobacteria able to use starch, being very strongly amylolytic (17). In fact, it grows with starch as rapidly as with glucose (generation time, 6.89 h) and the production of PHB and yield were slightly higher even than with glucose (Table 2). The ability to use starch, a cheap and abundant substrate, as the carbon source is an important advantage. The maximum yield of PHB with respect to the carbon source is very high in this organism, comparable with that found for *A. eutrophus* under optimal conditions (0.33 g/g) (7). This yield is remarkable if we consider that PHB is not the sole biopolymer produced by *H. mediterranei*; an extracellular polysaccharide is also produced under similar conditions (1), and a joint yield (weight of PHB plus polysaccharide produced per gram of substrate) of 0.5 g/g has been registered (data not shown).

For the next step of the study the culture media used contained glucose and phosphate at the optimum concentrations found (2% and 0.00375%, respectively). The optimum conditions for PHB production with regard to the physicochemical conditions studied corresponded without exception to the optimal growth conditions: SW concentration, 25%; temperature, 45°C; pH 7.2, and the highest oxygen transfer rate used (Table 3).

The PHB production in continuous culture was also studied. Continuous culture offers many advantages for industrial production, provided that contamination is avoided and the stability of the strain is guaranteed. The advantages include simplicity of culture control, homogeneity of the production, and constancy of culture conditions (19, 21). Two sets of chemostat conditions were used. In both, the medium in the reservoir contained 2% (wt/vol) glucose as the carbon source. In one set the phosphate concentration was 0.00375% (the same as in the optimum medium batch), and in the other it was reduced to half (0.001875%). The culture conditions used were as follows: SW, 25%; temperature, 45°C; pH 7.2; oxygen transfer rate, 0.4 mmol of O_2/liter per h. In both cases the medium in the fermentor was grown until an OD_{520} of 4.80 was reached, and then the flow of fresh medium started. Once the steady state was reached, a new dilution rate was established. This way, five different dilution rates were studied for each culture medium. The samples taken in the steady state were analyzed for PHB, biomass, and glucose and phosphate concentrations. Some

TABLE 3. Production and yield of PHB in relation to varying the physical parameters and total marine salt concentration[a]

Parameter assayed at 520 nm	Max OD_{520} reached	Generation time (h)	Max PHB concn (g/liter)	Max PHB/ protein ratio	PHB yield (g/g)
SW (%)					
15	12.62	7.59	2.70	0.81	0.135
20	6.80	11.63	0.57	0.45	0.028
25	8.90	8.30	4.16	5.13	0.208
30	7.20	15.80	1.83	5.23	0.091
Fermentation temp (°C)					
30	0.44	23.34	0.05	0.77	0.003
38	8.90	8.30	4.16	5.13	0.208
45	18.75	4.69	5.13	1.42	0.256
50	8.20	4.12	3.80	3.20	0.190
55	5.10	7.73	1.32	1.58	0.066
pH					
6.5	7.50	11.55	1.41	0.64	0.071
7.2	8.90	8.30	4.16	5.13	0.208
OTR[b] (mmol of O_2/liter per h)					
0.02	6.70	12.91	0.96	0.83	0.048
0.07	8.80	7.27	3.17	2.85	0.158
0.40	8.90	8.30	4.16	5.13	0.208

[a] For these experiments the media used always contained phosphate at the concentration found optimal for PHB production (0.00375% [wt/vol] $K_2H_2PO_4$) and the carbon source was 2% (wt/vol) glucose. Otherwise, the composition of the media was as in the basal synthetic medium, except when different salt concentrations (SW%) were used.

[b] OTR, Oxygen transfer rate.

of these results are summarized in Fig. 2. The yields of both PHB and protein were higher with 0.00375% phosphate in the medium than with the other concentration tested, but they were always lower than those obtained in batch culture. The best results obtained in these chemostats was a PHB concentration of 1.5 g/liter at a dilution rate of 0.02 h^{-1}, corresponding to 42% of the salt-free cell dry weight. At low dilution rates (0.04 and 0.02 h^{-1}) the amount of PHB was double that of protein. In the chemostats with 0.001875% phosphate the amount of PHB produced was small, reaching only 30% of the salt-free dry weight at a dilution rate of 0.04 h^{-1}. The concentration of phosphate in the chemostats with 0.00375% phosphate behaved as expected for the limiting substrate (21); in the other set of chemostats the concentration of phosphate was so low that it could not be detected in the fermentor (Fig. 2). The carbon source present, measured by adding the amounts of starch and glucose, was always high (more than 0.0062%, wt/vol); i.e., the limiting nutrient was not the carbon source. However, the yield obtained in chemostat cultures was much lower than that obtained in batch cultures. Still, the concentration of the carbon source was not optimized, and that parameter was essential for a high yield in batch culture.

Some halobacteria are known to have a high genomic instability (5). One of the problems that can arise when continuous culture is used for industrial production is the stability of the strain used (19), since nonproducer mutants are often favored and can displace the producer strain. To test the stability of PHB production by *H. mediterranei* we set a chemostat that was maintained in the steady state for 3 months with a dilution rate of 0.12 h^{-1}, which roughly corresponds to 370 generations. Afterwards the production of the strain isolated from the fermentor was evaluated in batch culture under optimal conditions with 2% starch as the

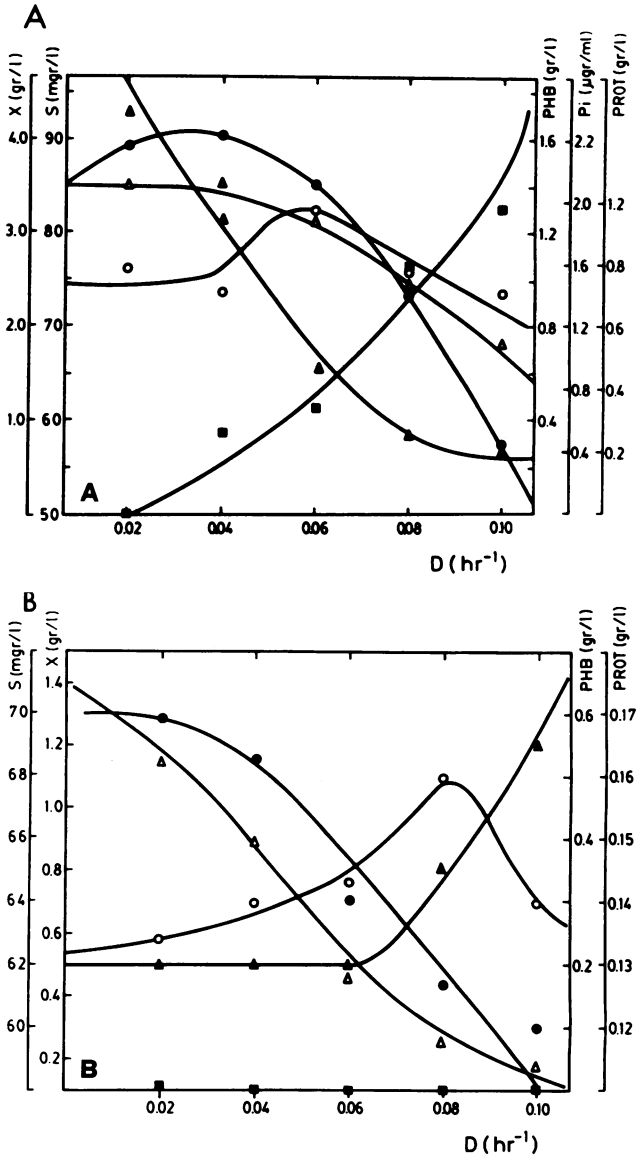

FIG. 2. Effect of the phosphate limitation in continuous culture on biomass, protein, and PHB production by *H. mediterranei*. (A) Concentration of phosphate, 0.00375%. (B) Concentration of phosphate, 0.001875%. Symbols: △, biomass (x); ○, protein (PROT); ▲, carbon source (s); ■, P_i; ●, PHB. D, Dilution rate.

carbon source. The total PHB production, yield, etc., were not notably different from those of the original strain (6.45 g of PHB per liter). Therefore, the stability seemed remarkable and suitable for the use of continuous culture for industrial production. On the other hand, in the chemostat with alternating cycles of carbon source starvation, which theoretically would favor overproducing strains, the strain tested at the end of the experiment also had the same characteristics with regard to PHB production (maximum PHB concentration, 6.29 g/liter).

A requirement in the evaluation of *H. mediterranei* for industrial production of polyesters is the possible production of copolymers, including acids of different chain length as in the case of *A. eutrophus*. This is very important in obtaining polymers of adequate physical properties (12). We are now investigating this aspect, and some preliminary data indicate

that polyhydroxyalkanoates of different monomer composition can also be obtained from *H. mediterranei*.

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