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Production of poly(hydroxyalkanoates) by *Halomonas pantelleriensis* grown on glycerol from bio-diesel

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ABSTRACT

Glycerol from bio-diesel production was used in the production of poly(hydrohyalkanoates) feeding cultures of the haloalkaliphilic bacterium *Halomonas pantelleriensis*. The micro-organism grew well and bio-synthesized poly[3-hydroxybutyrate (P3HB)]. The maximum yields, 10-11% of cell dry weight, was obtained after 72 h with 0.5 % industrial glycerol and nitrogen source 0.1 g l^{-1} at 35°C and after 10 days with glycerol 1.0%. The growth with 0.1% glycerol and 0.4% valerate gave a mixed polymer P(HB-HV), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in a ratio 90:10. For quantification of glycerol and of valerate during the *H.pantelleriensis* growth, and of PHAs, ^{1}H -NMR was used. MS spectroscopy, FTIR and ^{1}H, ^{13}C-NMR were used to chemically characterize the PHAs. The thermal characteristics of polymers were obtained using a differential scanning calorimeter and the average viscosity molecular weight was assessed through specific viscosity measurements.

KEYWORDS

Poly[3-hydroxybutyrate (P3HB)-P(HB-HV)];
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate);
Haloalkaliphile;
Halomonas;
Glycerol from bio-diesel;
NMR.

INTRODUCTION

Polyhydroxyalkanoates (PHAs), a heterogeneous family of polyesters, are synthesized exclusively by prokaryotes as a storage material and are accumulated by a number of micro-organisms under restricted growth conditions[8,10,11,]. Accumulation of reserve polymers such as PHAs increases survival in changing environments. Ayub et al.[3] showed that the polymer endows bacteria with enhanced survival competition abilities and stress tolerance in conditions resembling natural environments.

The detection of PHAs in micro-organisms has also been used as a chemotaxonomic marker; among prokaryotes, some halo-tolerant micro-organisms belonging to the bacteria domain and halo-archaea are able to produce PHAs[13]. Moreover, there is interest to find biocompatible replacements for many petrochemical-based polymers because of their biocompatible and biodegradable characteristics, which make them suitable for pharmaceutical and clinical purposes.

It is well known that *Halomonas* species are able to produce PHAs[13] and, recently, Quillaguaman et al.[12] have reported the optimum production of poly(3-hydroxybutyrate) (PHB) from *Halomonas boliviensis* LC1 when the micro-organism was cultivated in shake flasks containing a maltose-based medium.

Recently, Suzuki et al.[15] reported various methods for PHA extraction and Strazzullo et al.[14] described a PHAs high yield production simplified method which is fast, more profitable and of low environmental impact. The method has been used to examine the potentialities of *Halomonas campaniensis*, a halo- and alkali-tolerant bacterium, to produce PHAs, varying the chemical-physical growth conditions.

In order to lower down the cost of production, it is essential to identify the micro-organism that utilizes cheaper carbon sources efficiently to produce PHA.

Glycerol is a co-product of many industrial processes and is generated in large quantities, thus making it a potentially attractive substrate for the bacterial production of value-added products.

In this paper, the PHA production and characterization from the haloalkaliphile *Halomonas pantelleriensis* grown on glycerol, by-product of bio-diesel, are reported. Previously reports were present in Literature about PHB production by industrial glycerol, by-product of bio-diesel[1,2,9].

EXPERIMENTAL

Glycerol recovery

Glycerol, a 10% of by-products of bio-diesel production, was kindly provided from Caffaro Srl., which produced 24,000 kg/day of bio-diesel. Glycerol was used without any treatment as substrate for the culture of *Halomonas pantelleriensis.*

Micro-organism, culture medium

Halomonas pantelleriensis (AAP, DSM 9661^T), isolated from the hard sand of volcanic lake of Venere closed to the seashore in the island Pantelleria in the South of Sicily (Italy), was cultured as previously described[10]. *H.pantelleriensis* was usually grown in the complex medium contained the following components g l^{-1}: Na_2-citrate 3.0; KCl 2.0; $MgSO_4 \times 7H_2O$ 1.0; NaCl 100; Na_2CO_3 3.0; yeast extract 10. $MnCl_2 \times 4H_2O$ 0.36 mg l^{-1}; $FeSO_4$ 50 mg l^{-1}. The pH of medium was 9.0. NaCl and Na_2CO_3 were autoclaved separately. Growth on glycerol (0.5%-1.0%), minimal medium, was tested on liquid medium containing, g l^{-1} K_2HPO_4 7.0; KH_2PO_4 2.0; $MgSO_4 \times 7H_2O$ 0.1; $(NH_4)_2SO_4$ 1.0; NaCl 100; Na_2CO_3 3.0; biotin 500 µl/l (100 mg l^{-1}).The pH of minimal medium was 9.0. K_2HPO_4 and KH_2PO_4 were autoclaved separately.

The above described minimal medium was modulated by reducing concentration of nitrogen or phosphorus to 1/3 and 1/10 with respect to the content above described. Growth on valerate was obtained in minimal medium containing glycerol and valerate simultaneously at the following concentrations: 0.4%, 0.1%; 0.25%, 0.25%; 0.1%, 0.4 %, respectively and the nitrogen source 0.1 g l^{-1} (1/10). All growth tests were done at 35^{0}C. The cultures were grown in 1-l flasks containing 400 ml of a medium in a rotary shaker (Innova 4300) at 100 rpm, the cell growth was monitored by measuring the absorbance at 540 nm. The inoculum was 1% of the total volume. The cells grown for 24 h, 48 h and 72 h and 10 days, only for the growths with 1.0% glycerol, were extracted to obtain pure PHAs after centrifugation (12,000 g for 30 min).

More meaningful experiments were carried out in duplicate.

Glycerol and valerate content, during the growth, was quantified by ^{1}H-NMR by using a calibration curve.

PHA extraction

Humid cell pellets without any chemical pre-treatment have been used in order to obtain PHAs with a high yield production. The method we have used represents a modification[14] of the method proposed by Chen et al.[5].

The process consists in adding the cell pellets (1 up to 2 g) to 100 ml of distilled water and in using ultrasound (Ultrasonik 104 H) for 20 min until complete dispersion of the cells is obtained. SDS (Applichem) was added to the dispersion in a weight humid cells/SDS ratio of 1 and the mixture was allowed to digest with SDS for an hour at 50^{0}C in a heater (Heraeus). The mixture was then autoclaved for 20 min at 121^{0}C, cooled and centrifuged at 12,000 g for 30 min at 4^{0}C. The pellets were recovered and dried overnight at room temperature.

Chemical and physical analyses

The qualitative and quantitative of PHAs were performed by high-resolution proton nuclear magnetic resonance (^{1}H-NMR) and carbon nuclear magnetic resonance (^{13}C-NMR) using a Bruker AMX-500

spectrometer (500.13 MHz for ^{1}H-NMR and 125.75 MHz for ^{13}C-NMR). To overcome the dependency of signals intensity on the nucleus relaxation time, the NMR data were obtained by inverse-gated experiments.

The recovered fractions containing PHAs, obtained as previously described and directly dissolved in 0.75 ml of deuterated chloroform ($CDCl_3$; Aldrich), without any chemical transformation, were submitted to ^{1}H- and ^{13}C-NMR analysis. The ^{1}H, ^{13}C-NMR chemical shifts referred to $CHCl_3$ (δ = 7.26 ppm for ^{1}H and δ = 77.0 ppm for ^{13}C). For ^{1}H-NMR spectral analysis, each single PHA was identified by detecting some highly diagnostic resonances. The spectra were compared with those of authentic commercial samples of PHB, and PHV. The intensity of the same peaks was useful for their quantification. p-Hydroxy benzoic acid methyl ester (Methyl Paraben; Sigma) was used as the internal standard at a concentration of 1 mmol ml^{-1}; its very simple ^{1}H-NMR spectrum displayed signals (δ = 7.95 ppm, d, 2H ortho to COOCH, δ = 6.85 ppm, d, 2H ortho to -OH, δ = 3.88 ppm, s, 3H of the methoxy group) that fell in regions devoid of PHAs resonances. The area of diagnostic signals chosen as spectroscopic markers on CH_3 belonging to the PHAs was measured by electronic integrators and compared with the area of the methoxy group of methylparaben (3.90 ppm), used as the internal standard. An average of five integrations was utilized.

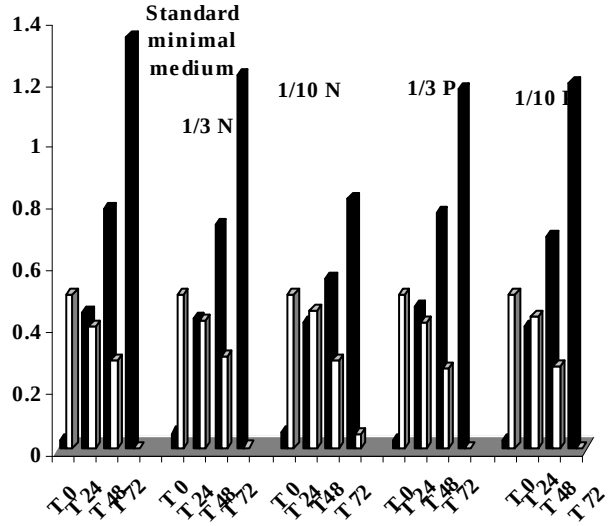

Figure 1: Total glycerol utilized (%) by Halomonas pantelleriensis (DSM 9661) as a function of initial glycerol concentration and growth of micro-organism as optical absorbance at 540 nm (A540 nm). Note: T0, T24, T48 and T72 referred to the incubation time of cultures. 1/3 N, 1/10 N, 1/3 P and 1/10 P referred to the amount of nitrogen or phosphorus used in the cultures (see material and methods). A540nm (■), % glycerol (□)

The amount of each PHA in the solution was evaluated as previously reported[14]. The amounts of polymer were expressed in terms of monomeric unit weight: 3-hydroxy butyric acid weight equivalent (HBA) for the PHB and HBA and 3-hydroxyvaleric acid (HVA) for the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(HB-HV)]. The ^{13}C-NMR spectral analysis showed resonances that were consistent with data reported in literature for butyrate and valerate[4,6]. The assignments of the signal were as previously reported[14].

The monomer unit types were also identified by GC-MS analysis performed with an HP5890 series II plus-5989B instrument, according to Strazzullo et al.[14], and by FTIR using a Perkin-Helmer Paragon 500 instrument.

Thermal analysis and average viscosity molecular weight determination

Thermal analysis and average viscosity molecular weight determination were made according to Strazzullo et al.[14].

RESULTS AND DISCUSSION

Effect of growth conditions on micro-organism growth and PHA synthesis

All growth conditions tested for PHA synthesis sustained the growth of *H.pantelleriensis* with a similar biomass yield, with the exception when the growth was done in the presence of 1/10 of nitrogen content, in this case the biomass yield was 30% lower than other growth conditions above reported (Figure 1).

The decrease of glycerol during the growth paralleled the growth itself. The average rate of glycerol utilization was similar in all experiments done and it was linear between 24 and 48 h of incubation, while was three fold higher between 48 and 72 h of growth. Glycerol residue ranged, after 72 h, between 0.2 and 10% of the initial concentration, this last value was observed when the growth was carried out in the presence of 1/10 of nitrogen content (Figure 1). In

TABLE 1: PHA produced (mg) by *Halomonas pantelleriensis* on minimal medium after 48 h, 72 h and 240 h of incubation

AAP	T_{48} PHB (mg) PHV (mg)	T_{72} PHB (mg) PHV (mg)	T_{240} PHB (mg) PHV (mg)
Glycerol 0.5%	3.18	5.58	-
	-	-	-
Glycerol 0.5% - 1/3 N	1.28	6.98	-
	-	-	-
Glycerol 0.5% - 1/10 N	1.71	9.61	-
	-	-	-
Glycerol 0.5% - 1/3 P	4.92	5.60	-
	-	-	-
Glycerol 0.5% - 1/10 P	6.44	4.04	-
	-	-	-
Glycerol 1.0%	-	-	21.1
	-	-	-
Glycerol 1.0% - 1/10 N	-	-	7.07
	-	-	-
Valerate 0.4% and glycerol 0.1% - 1/10 N	1.46	4.40	-
	0.18	0.59	-
Valerate 0.25% and glycerol 0.25% - 1/10 N	1.69	1.96	-
	0.19	0.25	-
Valerate 0.1% and glycerol 0.4% - 1/10 N	1.40	3.74	-
	0.04	0.09	-

contrast, biomass yield, when growth was carried out with glycerol and valerate, was ca. one half of that obtained in absence of valerate and the yield was similar in any experiments carried out with valerate as one of the carbon source. Probably, this compound could have inhibitor effect on the growth.

The maximum yield of PHB, 9.6 mg (TABLE 1), was obtained when the micro-organism was grown with 1/10 of nitrogen in growth medium and it content represented about 10% of dry cell weight. This value was 25 fold higher than that obtained when the micro-organism was grown in complex medium (0.37 mg; data not shown). *H. pantelleriensis* biosynthesized between 4.0-6.98 mg of PHB in other growth conditions tested.

The phosphorus limitation did not affect both the growth and the PHB production. In fact, in the minimal medium the yield of PHB was 5.6 mg, value similar to those obtained by limiting phosphorus in the range 1/3 and 1/10 with nitrogen lowered to 1/3 (TABLE 1). Generally, limiting growth conditions such as low presence of nitrogen, phosphorus and/or sulphate acted on PHB biosynthesis[12,13].

By increasing the glycerol concentration at 1% in a standard medium the biomass increased, after 10 days, of 1.3 fold with respect to the medium with glycerol concentration at 0.5% and the PHB yield increased up to 4 fold. By lowering nitrogen content to 1/10 in the medium with 1% glycerol the growth was lower as well as the PHB yield (TABLE 1).

When *H.pantelleriensis* was grown with a different ratio glycerol/valerate, the best PHA yield (4.40 mg) was obtained when valerate and glycerol were present in the medium in the ratio 4:1. The minimum PHA yield was when glycerol and valerate were present in the medium in the ratio 1:1 (TABLE 1). Recently, Ashby et al.[2] reported that in the presence of purchased glycerol mixed culture of *Pseudomonas* accumulated higher amount of PHA.

In the present paper we firstly reported the re-use of a by-product of bio-diesel production for obtaining value-added polymers by a non-pathogenic extremophile micro-organism.

PHA chemical and physical characterization

The polymers produced by *H. pantelleriensis* were analyzed for their monomeric composition by FTIR and MS spectroscopy. An intensive absorption band of the polymer in the IR spectrum at 1,750-1,630 cm and at 1,282 cm corresponding to the C = O and C-O stretching group, respectively indicated that the stain was a PHA producer.

MS spectra of hydrolysed polymers exhibited the main diagnostic peaks, one at *m/z* 119, which is the protonated molecular ion MH+, a peak at *m/z* 101 which is the main fragment due to the loss of the water molecule from the molecular ion and a peak at *m/z* 87 due to the loss of methanol molecule from the protonated molecular ion. The peak at *m/z* 69 represented the ion due to the combined loss of both water and methanol. Finally, the same fragmentation pattern was observed for PHB, the shift being of -14 for each peak, depending on the monomer length.

Polymers were fully characterized and quantified by NMR analyses. Only when valerate was employed in the medium as a carbon source together with glycerol, a co-polymer was obtained, which was composed of valerate and butyrate units in a relative molar ratio ranging from 10:90 to 3:97 depending upon the glycerol: valerate ratio. The lower value of copolymer was obtained when the growth was carried out in the presence of glycerol: valerate ratio 4:1 (Figure 2).

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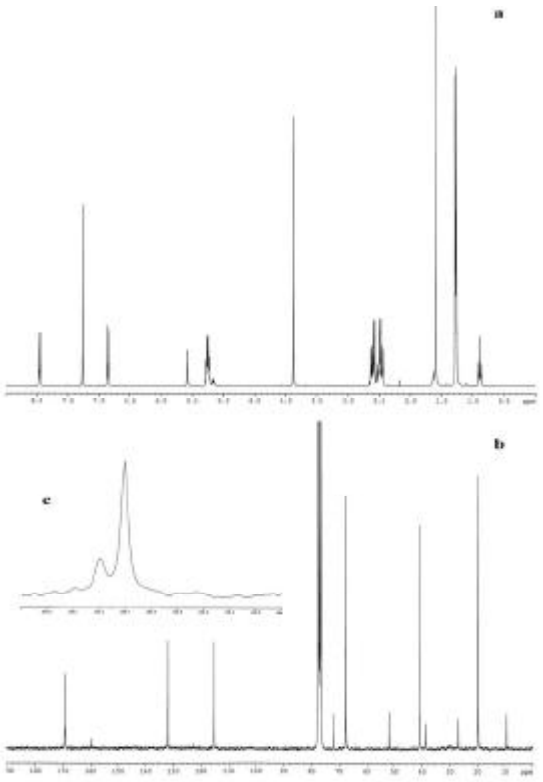

Figure 2: ^{1}H-NMR spectra of PHB/PHV from H. pantelleriensis; (a) ^{13}C-NMR spectrum of PHB/PHV from *H.pantelleriensis* (b); the enlarged spectrum of carboxyl groups is reported in the insert (c) (see results and discussion)

TABLE 2: T_ms from DSC analyses and average viscosity molecular weight (MW) of PHA produced by *Halomonas pantelleriensis* on minimal medium after 72 h of incubation

AAP	1° run Tm (°C)	Crystallization (°C)	2° run crystallization peak (°C)	2° run Tm (°C)	MW (Da)
PHB (Sigma)	172.24	87.82	-	168.70	1.0×10^5
PHB (Glycerol 0.5% - 1/10 N)	178.93	-	36.74	173.60	8.4×10^5
PHB/PHV (Sigma)	160.36	-	76.02	162.78	1.6×10^5
PHB/PHV (Valerate 0.4% and glycerol 0.1% - 1/10 N)	170.83	-	43.97	169.38	2.0×10^5
PHB/PHV (Valerate 0.1% and glycerol 0.4% - 1/10 N)	168.92	40.98	38.76	167.11	8.2×10^5

Ashby et al.[2], reported that mixed *Pseudomonas* cultures in the presence of glycerol accumulated PHB and PHA with medium-chain-length, consisting of 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid.

Thermal analysis and average viscosity molecular weight determination

The MW and T_m values of commercial PHB, commercial PHB/PHV, PHB synthesized in minimal medium with 1/10 of nitrogen and containing 0.5% glycerol and copolymers PHB/PHV obtained in minimal medium with 1/10 of nitrogen when valerate and glycerol were present in the medium in the ratio 4:1 and 1:4 respectively, were determined using a Cannon Ubbeholede viscometer and a Mettler TA11C differential scanning calorimeter[7].

When the growth of *Halomonas pantelleriensis* was carried out in minimal medium with 1/10 of nitrogen and containing 0.5% glycerol, a "high molecular weight" PHB type was synthesized whose value was 8.5×10^5 Da (TABLE 2).

The MW (Da) of PHB/PHV bio-synthesized by *Halomonas pantelleriensis* in minimal medium with 1/10 of nitrogen and containing 0.4% valerate and 0.1% glycerol does not vary significantly from the commercial PHB/PHV (2.0×10^5 Da and 1.6×10^5 Da, respectively), while the MW of PHB/PHV synthesized using minimal medium with 1/10 of nitrogen and containing 0.1% valerate and 0.4% glycerol was similar to commercial PHB (8.2×10^5 Da and 8.4×10^5 Da, respectively).

The measured T_ms (°C) of five PHAs were reported in TABLE 2. These values depended on the purity grade of each PHA used for DSC analyses. In the first and the second run of calorimetric analysis we note that T_ms of our samples were higher then those of commercial polymers. This can be attributed to high MW of synthesized of sample with respect of commercial PHB and commercial PHB/PHV.

CONCLUSIONS

In the present paper we reported the re-use of a by-product of bio-diesel production without any treatment to obtain added-value products, such PHA, by using a haloalkaliphilic *Halomonas* species. The methods utilised for PHA extraction was simple and rapid without solvents. Moreover, the PHA

biosynthesised by *Halomonas pantelleriensis* showed high MW with respect to commercial PHB and commercial PHB/PHV.

In addition the glycerol assay, usually carried by an expensive kit, was performed by ^{1}H-NMR analyses of culture medium without any pre-treatment.

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