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SHORT COMMUNICATION

Biosynthesis and characterization of polyhydroxyalkanoates in the polysaccharide-degrading marine bacterium *Saccharophagus degradans* ATCC 43961

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Abstract The marine bacterium *Saccharophagus degradans* was investigated for the synthesis of polyhydroxyalkanoates (PHAs), using glucose as the sole source of carbon in a two-step batch culture. In the first step the microorganism grew under nutrient balanced conditions; in the second step the cells were cultivated under limitation of nitrogen source. The biopolymer accumulated in *S. degradans* cells was detected by Nile red staining and FT-IR analysis. From GC–MS analysis, it was found that this strain produced a homopolymer of 3-hydroxybutyric acid. The cellular polymer concentration, its molecular mass, glass transition temperature, melting point and heat of fusion were 17.2 ± 2.7% of dry cell weight, 54.2 ± 0.6 kDa, 37.4 ± 6.0 °C, 165.6 ± 5.5 °C and 59.6 ± 2.2 J g^{-1}, respectively. This work is the first report determining the capacity of *S. degradans* to synthesize PHAs.

Keywords Marine bacterium · Polyhydroxyalkanoate · Polysaccharide-degrader · *Saccharophagus degradans*

Introduction

The marine bacterium *Saccharophagus degradans* was isolated from a decaying salt marsh grass at the Chesapeake Bay in Virginia, USA [2] and was classified as the only member of a new genus: *Saccharophagus* [4]. Because of its extraordinary metabolic capacity, *S. degradans* is considered as one of the most versatile polysaccharide-degrading bacterium reported so far. Recently, its genome was sequenced identifying more than 180 open reading frames that would encode for the synthesis of enzymes with homology to cellulases, xylanases, amylases, pectinases, alginases, agarases and chitinases. With this knowledge, *S. degradans* was successfully cultured in minimal media containing complex polysaccharides as the only source of carbon and energy, where the expected carbohydrases were expressed, and further characterized [4]. Additionally, in the genome of *S. degradans* genes that would encode for three key enzymes involved in the synthesis of polyhydroxyalkanoates (PHAs): β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase were identified [7]. Nevertheless, nowadays, its ability to synthesize PHAs has not been experimentally verified.

Polyhydroxyalkanoates are biopolyesters that are synthesized as intracellular carbon and energy reserves by a wide variety of microorganisms mainly when they are cultured under unbalanced nutrient conditions. These bioplastics have similar mechanical and thermal properties to those of plastics synthesized chemically. In contrast to classic plastics, they are fully biodegradable, biocompatible and produced from renewable materials [8]. However, there are still economical limitations to substitute petrochemical plastics by PHAs. To achieve a cost-effective PHA production scheme, the isolation of new bacterial strains able to utilize inexpensive carbon sources has become a focus of particular interest [10]. Currently, most of complex carbohydrates need a pretreatment prior to be used as a convenient source of carbon and energy for the culture of the known PHAs-producers [13, 17], which increases the cost

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of production and causes environmental problems. Consequently, new strains directly degrading complex polysaccharides are desired. Since *S. degradans* degrades non-pretreated complex polysaccharides, it is extremely interesting to confirm its ability to produce PHAs. Thus, this is a preliminary study to investigate the synthesis of PHA in this bacterium under unbalanced nutrimental conditions, using a simple medium with glucose as the sole source of carbon. Moreover, the produced PHA was physically and chemically characterized.

Materials and methods

Microorganism and culture medium

Saccharophagus degradans was bought from ATCC (43961), activated in Difco Marine Broth and stored with glycerol (15% v/v) in 1.5 ml microtubes at −20 °C. The culture medium was formulated according to the elemental composition of the bacterial biomass, the composition of sea water and the yield coefficient of biomass on glucose experimentally measured. It was composed of (g l^{-1}): glucose, 20; NH_4Cl, 5.4; KH_2PO_4, 1.4; $MgCl_2$, 0.12; Na_2SO_4, 0.24; yeast extract, 1; NaCl, 23; KCl, 0.75; $CaCl_2$, 0.13; Tris–HCl buffer 1 M (pH 7.6), 50 ml; and trace elements solution, 1 ml. The trace elements solution contained (mg l^{-1}): H_3BO_3, 13,700; $SrCl_2 \cdot 6H_2O$, 8,140; KI, 50; $NiSO_4$, 13; $ZnSO_4 \cdot 7H_2O$, 9; $MnSO_4 \cdot H_2O$, 2; $CoSO_4 \cdot 7H_2O$, 0.3; $CuSO_4 \cdot 5H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 8.4; and EDTA, 8.5. The inoculum was aseptically prepared from the frozen stock mentioned above, by adding the cells contained in a microtube (1 ml) to a 500 ml Erlenmeyer flask with 100 ml of the culture medium and incubating at 30 °C and 200 rpm for 12 h.

Determining the synthesis of PHA in *S. degradans*

The biosynthesis of PHA in *S. degradans* under nitrogen source limitation was investigated using a two-step batch culture. This experiment was carried out in 500 ml flasks containing 90 ml of sterilized medium and 10 ml of inoculum. In the first culture step, the cells were grown under nutrient balanced conditions at 30 °C and 200 rpm for 24 h. Biomass was aseptically recovered by centrifugation and washed with a sterile NaCl solution (2.3% w/v). In the second step, the washed cells were transferred to 500 ml flasks, containing 100 ml of sterilized medium without source of nitrogen (NH_4Cl and yeast extract), and incubated at 30 °C and 200 rpm for 48 h. A sample of 10 ml was taken at the end of the cultivation for the qualitative analysis of the PHA and for its quantification; meanwhile, the rest of the flask content was used for extraction and further characterization of the biopolymer. The experiments and the analysis were done by duplicate and the results are represented as the average and standard deviation from the values obtained.

Qualitative analysis of the PHA synthesized by *S. degradans*

The presence of PHAs was evidenced by the observation of cells under a microscope, and by staining the biopolymer inside the cells with Nile red as reported by Wu et al. [21]. A FT-IR analysis was also done by scanning the sample with a Perkin Elmer 1420 FT-IR spectrometer from 650 to 4,000 cm^{-1} as reported by Hong et al. [6].

Quantifying the PHA synthesized by *S. degradans*

The amount of PHA synthesized by *S. degradans* was determined by GC analysis as reported by Wu et al. [21], using a Perkin Elmer XL gas chromatograph equipped with a CP-Wax 52 CB capillary column (25 m × 0.32 mm) and a flame ionization detector. The chromatographic conditions used were: injection volume of sample, 1 μl; gas carrier, nitrogen; flow rate, 20 cm s^{-1}; injector and detector temperatures, 210 and 220 °C; temperature ramp, 50 °C for 1 min, incrementing by 8 °C min^{-1}, and 160 °C for 5 min. Methyl benzoate and polyhydroxybutyrate (P3HB) from Fluka were used as internal and external standards, respectively.

Extracting the PHA from *S. degradans* cells

The broth culture obtained at the end of the second step (90 ml) was centrifuged at 5,000 rpm for 15 min and the resulting cell pellet was washed twice with distilled water and lyophilized. The biopolymer was extracted from the lyophilized cells as reported by Hahn et al. [5].

Determining the molecular mass for the PHA

The molecular mass of the polymer was determined by gel permeation chromatography using a Waters HPLC 600, equipped with two serially connected Styragel columns (HR1 and HT 6E, 7.8 × 300 mm) and a RI detector 2410. The chromatographic conditions utilized were: injection volume of sample (1 mg ml^{-1}), 50 μl; temperature, 40 °C; mobile phase, toluene; and flow rate, 1 ml min^{-1}.

Determining the monomeric composition of the PHA

The monomeric composition of the polymer was determined by GC–MS, using a Varian Saturn 3800 gas chromatograph coupled to a Varian Saturn 2000 Mass

spectrometer, equipped with a FFAP 25 Mx capillary column (25 m × 0.32 mm). The chromatographic conditions used were: injection volume of sample, 1 µl; carrier gas, helium; flow rate, 1 ml min^{-1}; temperature of injector and detector, 230 and 275 °C; temperature ramp, 80 °C for 1 min, incrementing by 8 °C min^{-1}, and 220 °C for 12 min.

Determining the thermal properties of the PHA

The glass transition temperature (T_g), the melting point (T_m) and the heat of fusion (ΔH_m) were measured by using a Differential scanning calorimeter (Perkin-Elmer, model DSC7). The PHA samples were heated at a rate of 10 °C min^{-1} from 4 to 200 °C.

Results and discussion

The ability of *S. degradans*—one of the most versatile complex polysaccharide-degrading bacterium so far isolated—to synthesize PHA under culture conditions of nitrogen source limitation and glucose as source of carbon and energy has been demonstrated in this work. The accumulation of PHA was first investigated by the fluorescence of PHA granules bonded to the dye Nile red, furthermore when these cells were observed under the light microscope (100×), intracellular inclusions of different size were distinguished.

In order to validate the detection of PHA, cells containing the biopolymer were analyzed by FT-IR [6] and the spectrum obtained is depicted in Fig. 1. This spectrum shows a marked peak for the ester carbonyl bond at 1,742 cm^{-1} which indicated the accumulation of PHA in *S. degradans*. Moreover, the GC analysis of the samples revealed that the polymer content was 17.2 ± 2.7% of the cellular dry weight (CDW). In this context, PHA-synthesizing bacteria accumulate the polymer in different amounts, ranging from 1 to >80% of their CDW, according to the strain and its culture conditions [9]. The PHA content in *S. degradans* was similar to those observed for other wild-type bacteria growing on glucose, such as *Bacillus* sp. (25%) [22], *Caulobacter crecentus* (18%) [12], *Azotobacter macrocytogenes* (15.3%) [18], *Beijerinckia lacticogenes* (16.2%) [18], *Alcaligenes xylosoxidans* (18%) [16], *Acidovorax delafieldii* (19%) [16] and *Hydrogenophaga palleronii* (14%) [16]. On the other hand, the PHA content in *S. degradans* was low in comparison to those values obtained for genetically modified strains such as *Pseudomonas* sp. 61–3 (40%) [19], *R. eutropha* PHB⁻4 (68%) [20] and *E. coli* (80%) [8]. Nevertheless, it should be pointed out that this study was not conducted under optimal culture conditions. Work is ongoing to increase the polymer production in this strain.

Monomeric composition, molecular mass and thermal properties

The GC–MS analysis of the PHA produced by *S. degradans* indicated that the polymer was composed by 3-*β*-hydroxybutyrate units (Fig. 2). This result was consistent with the genomic prediction, concerning the synthesis of a PHA synthase Class I by *S. degradans* (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=ntsd03). The PHA synthase Class I utilizes preferentially CoA-thioesters of (*R*)-3-hydroxy fatty acids from 3 to 5 carbons [15].

Concerning the molecular mass of the PHB synthesized by *S. degradans* it was 54.2 ± 0.6 kDa, with a polydispersity of 2.76. It is well known that the PHA molecular mass is an inherent characteristic for each given strain [1]. For example, *Azotobacter* strains accumulate PHAs whose molecular masses range from 800 to 2,000 kDa, *R. eutropha* from 600 to 1,000 kDa, *Pseudomonas* sp. (AM1 strain) from 50 to 60 kDa and *Methylobacterium* sp. (B3-Bp strain) from 250 to 300 kDa [1]. In the case of biopolymer produced by *S. degradans*, the molecular mass obtained was in the low range in comparison to those of other microorganisms mentioned above. However, it was in good agreement with the range of PHAs molecular masses

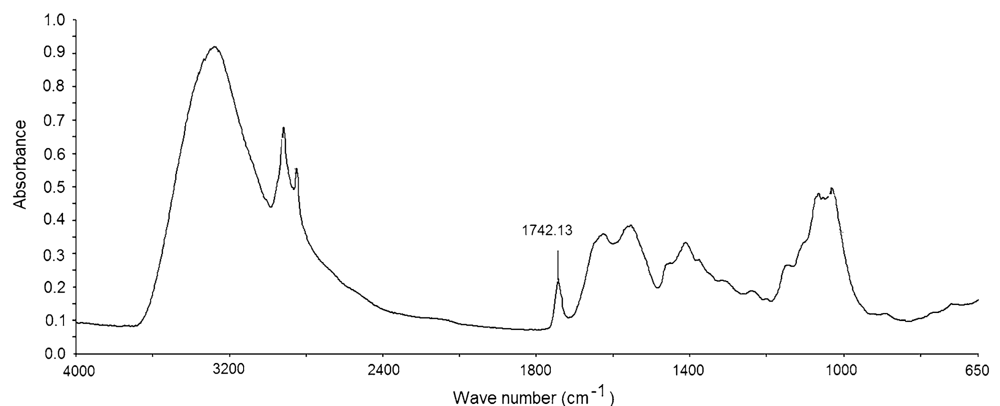

Fig. 1 FT-IR spectrum of PHA-accumulating *S. degradans* cells. The peak at 1742.13 cm^{-1} corresponds to the ester-carbonyl bond, characteristic for PHAs

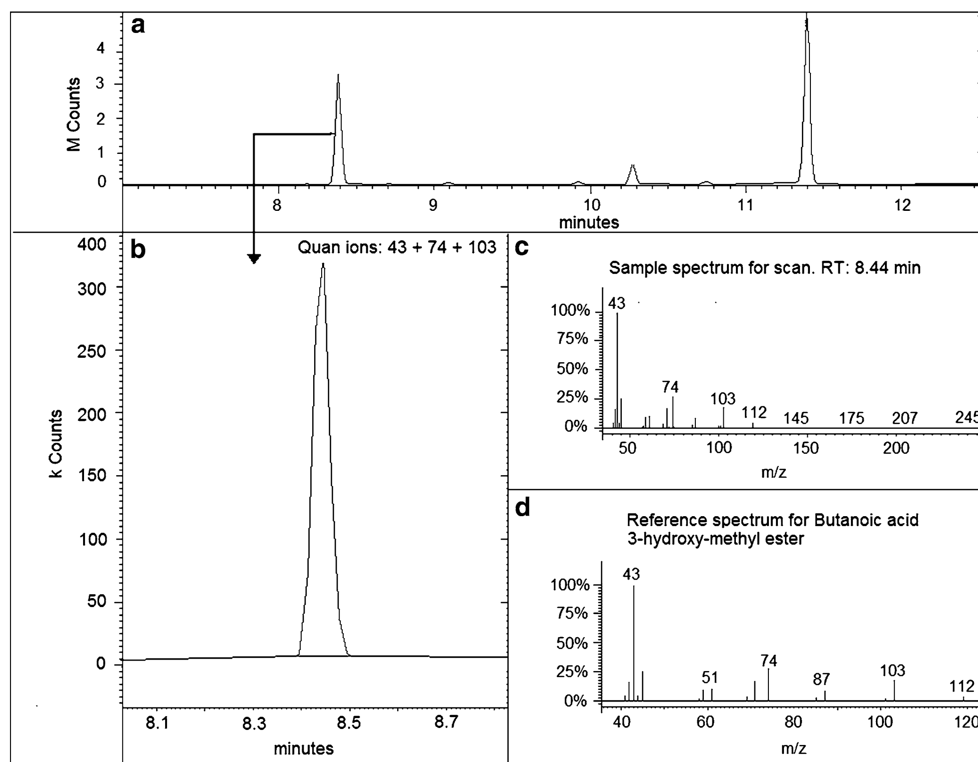

Fig. 2 GC–MS analysis of the extracted polymer. **a** Total ion chromatogram. The peaks at 8.44 min and 11.45 min correspond to 3-hydroxybutanoic acid methyl ester (3HB-Me) and the internal standard (methyl benzoate). **b** Zoom of the peak at 8.44 min showing the characteristic ion fragments for 3HB-Me. **c** Mass spectrum for the sample. **d** Reference mass spectrum for 3HB-Me from the MS library. The occurrence of the ions 43, 74 and 103 in the sample indicates the synthesis of PHB by *S. degradans*

obtained by wild-type bacteria [8, 11]. It is also worth noting that the molecular mass of PHA is influenced by the extraction technique employed; indeed, neutral solvents extraction yields higher values than alkaline hypochlorite treatment [3]. Since the second technique was used in this study, the extraction procedure could have affected the molecular mass. Regarding the thermal properties of the polymer, the values for T_g, T_m and ΔH_m were 37.4 ± 6.0 °C, 165.6 ± 5.5 °C and 59.6 ± 2.2 J g^{-1}, respectively. The T_g value obtained in this work was higher than those values reported for PHB synthesized by many other bacteria (4–20 °C) [1], which confers a high brittleness to this biopolymer. On the other hand, the T_m and ΔH_m values where in the range of those values reported for PHB produced by other marine bacteria: 162.3–177.0 °C and 32.4–65.8 J g^{-1}, respectively [14].

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References

1. Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472
2. Andrykovitch G, Marx I (1988) Isolation of a new polysaccharide-digesting bacterium from a salt marsh. Appl Environ Microbiol 54:1061–1062
3. Braunegg G, Lefebvre G, Genser KF (1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. J Biotechnol 65:127–161
4. Ekborg NA, Gonzalez JM, Howard MB, Taylor LE, Hutcheson SW, Weiner RM (2005) *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. Int J Syst Evol Microbiol 55:1545–1549
5. Hahn SK, Chang YK, Kim BS, Chang HN (1994) Optimization of microbial poly(3-hydroxybutyrate) recovery using dispersions of sodium hypochlorite solution and chloroform. Biotechnol Bioeng 44:256–261
6. Hong K, Sun S, Tian W Chen GQ, Huang W (1999) A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl Microbiol Biotechnol 51:523–526
7. Kalia VC, Chauhan A, Bhattacharyya G, Rashmi (2003) Genomic databases yield novel bioplastic producers. Nat Biotechnol 21:845–846
8. Khanna S, Srivastava AK (2005) Recent advances in microbial polyhydroxyalkanoates. Process Biochem 40:607–619
9. Kim YB, Lenz RW (2001) Polyesters from microorganisms. Adv Biochem Eng Biotechnol 71:51–79
10. Koller M, Bona R, Braunegg G, Hermann C, Horvat P, Kroutil M, Martinz J, Neto J, Pereira L, Varila P (2005) Production of polyhydroxyalkanoates from agricultural waste and surplus materials. Biomacromolecules 6:561–565
11. Luengo JM, Garcia B, Sandoval A, Naharro G, Olivera ER (2003) Bioplastics from microorganisms. Curr Opin Microbiol 6:251–260
12. Qi Q, Rehm BH (2001) Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase. Microbiology 147:3353–3358
13. Quillaguamán J, Hashim S, Bento F, Mattiasson B, Hatti-Kaul R (2005) Poly(beta-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. J Appl Microbiol 99:151–157

14. Rawte T, Mavinkurve S (2002) Characterization of polyhydroxy alkanoates—biodegradable plastics from marine bacteria. Curr Sci 83:562–564
15. Rehm BH (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33
16. Renner G, Haage G, Braunegg G (1996) Production of short-side-chain polyhydroxyalkanoates by various bacteria from the rRNA superfamily III. Appl Microbiol Biotechnol 46:268–272
17. Silva LF, Taciro MK, Michelin Ramos ME, Carter JM, Pradella JG, Gomez JG (2004) Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. J Ind Microbiol Biotechnol 31:245–254
18. Stockdale H, Ribbons DW, Dawes A (1968) Occurrence of poly-β-hydroxybutyrate in the azotobacteriaceae. J Bacteriol 1798–1803
19. Taguchi S, Doi Y (2004) Evolution of polyhydroxyalkanoate (PHA) production system by enzyme evolution: successful case studies of directed evolution. Macromol Biosci 4:146–156
20. Tsuge T, Yano K, Imazu S, Numata K, Kikkawa Y, Abe H, Taguchi S, Doi Y (2005) Biosynthesis of polyhydroxyalkanoate (PHA) copolymer from fructose using wild-type and laboratory-evolved PHA synthases. Macromol Biosci 5:112–117
21. Wu HA, Sheu DS, Lee CY (2003) Rapid differentiation between short-chain-length and medium-chain-length polyhydroxyalkanoate-accumulating bacteria with spectrofluorometry. J Microbiol Methods 53:131–135
22. Wu Q, Huang H, Hu G, Chen J, Ho KP, Chen GQ (2001) Production of poly-3-hydroxybutyrate by *Bacillus* sp. JMa5 cultivated in molasses media. Antonie Van Leeuwenhoek 80:111–118