Polymer Bulletin 34, 577–584 (1995)

Polymer Bulletin

Growth behavior of *Bacillus thuringiensis* and production of poly(3-hydroxyalkanoates) on different organic substrates

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Received: 20 December 1994/Accepted: 10 January 1995

Summary

Bacillus thuringiensis is known as a bacterial species capable of the production of short side chain poly-ß-hydroxyalkanoates (PHAs). In the present work it was shown that the organism is also capable of producing longer side chain PHAs under appropriate conditions. The microorganism was grown separately on glucose, nonanoic acid, and a mixture of glucose, peptone and casein in attempts to obtain poly-ß-hydroxyalkanoates with varying lengths of the pendent alkyl group in the ß-position of the polymer repeat unit. The growth on glucose resulted in the formation of poly-3-hydroxybutyrate, (PHB), which is a very common bacterial storage polymer, while the utilization of nonanoic acid resulted in the formation of a copolymer consisting of 3-hydroxynonanoate and 3-hydroxyheptanoate units (PHN/PHH), which is the usual product of the microbial utilization of this particular carbon source by *Pseudomonas oleovorans*.

Introduction

Bacillus thuringiensis is a bacterial species which is known as, and commercially used as, an insecticide producer (1). Spore formation is employed by the microorganism as a survival mechanism (2, 3). Nickerson and coworkers (4, 5) observed, during density gradient centrifugation experiments, small black dots at a density significantly less than that exhibited by the proteinaceous crystals. These small granules were later determined to be PHB (6,7).

A broad variety of microorganisms is capable of producing internal carbon and energy storage material in the form of poly-3-hydroxyalkanoates, PHAs (8). The bacteria which are capable of producing internal polymeric storage granules, however, are divided into those which produce short chain PHAs with up to five carbon atoms in the ß-substituent group of the repeat unit, as for instance *Alcaligenes eutrophus* and *Rhodospirillum rubrum*, and those which produce PHAs with six to twelve or more carbon atoms in the ß-substituent of the repeat unit, as for instance *P. oleovorans* (9). However,

there is little known about the potential of *B. thuringiensis* to produce intracellular polymers since research efforts have mainly concentrated on the investigation of its ability of insecticide formation. The present report describes experiments in which long and short chain organic compounds were fed to the microorganism. The growth behavior of *B. thuringiensis* and polymer production on these unusual carbon sources is described.

Experimental

Fermentations

The bacterial strain under investigation, *B. thuringiensis*, was isolated from the waste treatment facilities at Rohm and Haas Company in Spring House, PA. The species was analyzed by a gas chromatographic analysis of the fatty acids extracts and determined by a data base comparison to be *B. thuringiensis* (10). The similarity index was 0.762 which represented an excellent match. The strain was maintained at 4°C on nutrient agar plates using broth as the carbon source. The cultures were cultivated applying three different media, as follows: (a) The bacteria were grown on 8 g/l broth, centrifuged and resuspended in a minimal medium which contained 5.88 g/l K_2HPO_4, 3.20 g/l NaH_2PO_4, 0.1 g/l yeast extract, 10 ml of a 0.1 M $MgSO_4$-solution, 10 ml of a 0.2 g/l $CaCl_2$ solution and 1.0 ml microelement solution (10). The latter is a 1N HCl solution containing 2.78 g/l $FeSO_4x7H_2O$; 1.67 g/l $CaCl_2x2H_2O$; 0.17 g/l $CuCl_2x2H_2O$; 0.29 g/l $ZnSO_4x7H_2O$; 1.98 g/l $MnCl_2x4H_2O$; 2.81 g/l $CoSO_4x7H_2O$. (b) GYS-Medium: The bacteria were cultivated in a medium which contained 2.0 g/l $(NH_4)2SO_4$ 2 g/l yeast extract and 0.5 g/l K_2HPO_4. After autoclaving 17 ml of a 0.1 M $MgSO_4$-solution, 0.08 g/l $CaCl_2x2H_2O$ and 0.05 g/l $MnSO_4x2H_2O$ were added (11). (c) STa Medium: The bacteria were cultivated in a medium which contained 3.0 g/l NaCl, 0.43 g/l $NH_4H_2PO_4$ and 0.77 g/l NaH_2PO_4 (12). The pH was adjusted in all cases to 7.0, and the carbon sources were added after autoclaving. The fermentations were started either by direct inoculation of bacteria from a plate (broth and GYS) or by inoculation of 1 or 10 % of pre-culture (GYS and STa). The pre-culture for growth experiments with GYS-medium was prepared using GYS-medium and 10 g/l glucose as carbon source. The pre-culture for growth experiments with STa-medium was prepared using STa-medium without the phosphate salts and 10 g/l glucose, 10 g/l peptone, 5 g/l casein and 10 g/l starch as carbon sources.

The cultures (1000 ml) and pre-cultures (200 ml) were autoclaved at 120°C and 18 lbs/sq in for 30 min (Amsco Laboratory Autoclave). After the culture solutions were cooled down to 30°C, bacteria were inoculated from the plate by a sterile procedure either into the pre-culture or directly into a 1000 ml culture. After 14 hours of growth on broth the cells were centrifuged in autoclaved centrifuge bottles and resuspended in the minimal medium. In order to start a fermentation in 1000 ml of the minimal medium, 1000 ml broth culture was used to pre-grow the bacteria and so to obtain the cells. The pre-culture for the

GYS medium was ready to use as an inoculum for the cultures after 15 hours of growth, and the pre-culture for the STa medium was ready to use after 24 hours. The carbon sources were not autoclaved, they were added to the culture solutions after they cooled down to 30 °C. Fermentations were carried out under aerobic conditions in 2.8 l Fernbach flasks for 1000 ml cultures. Pre-cultures were cultivated in indented 500 ml Erlenmeyer flasks. In order to guarantee a sufficient aeration of the cultures the flasks were continuously shaken in a lab-line incubator shaker at 250 rpm and 30°C. The growth of all cultures was examined by recording the optical density (O.D.) of the solution spectrometrically at 660 nm with a Spectronic 20 (Bausch and Lomb) at a layer thickness of 1 cm, referenced to distilled water.

The polymer was extracted from the lyophilized cells by refluxing in 100 to 150 ml of chloroform for 12 hours. After the cellular material was filtered, the solvent was removed by evaporation until a remaining residue of concentrated polymer solution of 1 to 5 ml was obtained. The polymer was precipitated into 50 to 70 ml of vigorously stirred methanol, collected and dried at room temperature under vacuum for 12 hours.

Polymer characterization

The polymer was characterized by using NMR. ^{1}H-NMR spectra were obtained using chloroform-d-solutions of the polymer with a Bruker AC 200 spectrometer at 200 MHz referenced to tetramethylsilane (0 ppm) and ^{13}C-NMR spectra were obtained with a Bruker AC 300 spectrometer at 75 MHz referenced to chloroform (77 ppm).

For analysis of the repeating units of the polymers by gas chromatography (GC) after methanolysis, 3 to 4 mg of the polymers, dissolved in 1.0 ml of chloroform, were converted into the methyl esters with 1.0 ml of 15 % H_2SO_4 in MeOH for 3 hours at 100°C. The solution was washed with 1.0 ml of distilled water by rapidly stirring for 20 sec. The chloroform layer was separated and analyzed by GC (Perkin Elmer 8500; D-B WAX; capillary column 15m x 0.53 mm; carrier gas; He, 20 ml/min; temp. program: 35°C for 2.0 min, ramp 15°C/min, 200°C for 3.0 min.)

Results

Growth behavior of B. thuringiensis on broth

The growth behavior of *B. thuringiensis* on broth was studied by observing the culture using a light microscope. For polymer production purposes the bacteria should be exposed to the carbon sources (i.e., transferred to the minimal medium) before the microorganism begins the sporulation process. The observations related to this behavior are described in Table 1. Based on these observations, the bacteria were centrifuged and resuspended in minimal medium after 14 hours of growth on broth in order to assure that

the sporulation had not yet started. The occurrence of crystals has not been observed in this experimental assay.

Table 1. Observation of the growth behavior of *B. thuringiensis* on broth

Growth Time [h]	O.D.	Observation
9.5	1.3	Single cells
11.5	1.3	Formation of chains consisting mostly of 4 single cells
14.0	1.45	Almost all cells were arranged in 4-membered chains
17.0	2.0	Chains broke down into 2-membered chains or single cells Less than 10 % of the cells contained spores
19.5	1.9	A few chains still existed, most of the single cells were arranged in cell clusters, 10 % of the cells, especially those in the clusters, contained spores
33.5	2.05	Almost all of the cells, which were arranged mostly in 2-membered chains, contained spores Many released spores were observable
42.5	1.5	No changes

Growth of B. thuringiensis on glucose

Table 2 shows the results of the growth on glucose. As expected *B. thuringiensis* produced PHB homopolymer when grown on glucose as the sole carbon source, and the micoorganisms incorporated up to 29 % of its dry cell weight. The polymer incorporation in the two-step procedure, with a pre-growth on broth and the subsequent resuspension of the cells in minimal medium, was very poor and reached only a 3.3 % of dry cell weight after the cells had been suspended for 48 hours in the minimal medium with glucose as the carbon source. The optical density increased slightly when the cells remained for 72 hours in this medium; however, the amount of incorporated polymer decreased significantly. Most likely the production of δ-endotoxin crystals had started and the PHB was involved in the crystal formation.

Table 2. Growth of *B. thuringiensis* on 10 g/l glucose

Growth time [h]	O.D.	Medium	Biomass [g/l]	Polymer [mg/l]	Incorp. [%][d]	Polymer[c]
24	1.95	broth[a]	0.74	11.5	1.55	PHB
48	2.2	broth[a]	0.63	21.0	3.3	PHB
72	2.3	broth[a]	0.67	4.2	0.6	PHB
16	3.45	GYS[b]	2.76	309.0	11.2	PHB
24	5.3	GYS[b]	2.99	280.1	9.3	PHB
48	6.5	GYS[b]	2.05	598.0	29.2	PHB

a) 4 x 250 ml broth solution for pre-growth in order to assure better aeration
b) direct inoculation of bacteria from a plate into the culture
c) analyzed by ^{1}H NMR
d) based on dry cell weight

The GYS medium proved to be considerably more efficient for the fermentation of glucose and production of PHB than the two-step procedure with broth. Whether the cultures were started directly with bacteria from a plate or by inoculation with a pre-culture did not influence the growth behavior or polymer formation. The growth started immediately without a lag time. An increase in the growth time from 16 to 48 hours resulted in an increase of the optical density from 3.4 to 6.5 indicating that the bacteria were continuously growing on this rich medium. The amount of incorporated polymer increased during the same time as well, from 11 % to 29 % based on dry cell weight, Table 2. The polymers were determined to be pure PHB. The primary polymer characterization was carried out by ^{1}H-NMR. For verification purposes methanolysis gas chromatography and ^{13}C-NMR were employed. The results of both techniques confirmed the polymer to be PHB.

The PHB yield could be increased by feeding the bacteria a mixture of 10 g/l glucose, 10 g/l peptone and 5 g/l casein applying the STa medium. Wakisaka and coworkers (12) pointed out that the addition of phosphate salts supported the formation of PHB. Therefore, in all experiments 0.43 g/l $NH_4H_2PO_4$ and 0.77 g/l NaH_2PO_4 were added to the medium in order to force the polymer formation. As the data in Table 3 show,

a polymer incorporation of up to 38 % after a 72 hours of growth could be achieved. The polymer was analyzed by ^{1}H-NMR and by methanolysis gas chromatography and determined to be pure PHB. The optical density could not be used as a measure for the cell density in this experimental, since the solutions were turbid from the beginning.

Table 3. Growth of *B. thuringiensis* on a mixture of glucose, peptone and casein

Growth time [h]	Dry Cell Yield [g/l]	Polymer Yield [mg/l]	Polymer Incorp. [%][b)]	Polymer[a)]
24	3.63	629	17.3	PHB
72	2.58	986	38.2	PHB

a) analyzed by ^{1}H NMR and methanolysis gas chromatography
b) based on dry cell weight

Growth of B. huringiensis on nonanoic acid

Nonanoic acid was used in a concentration of 25 mMol as a sole carbon source for the growth of *B. thuringiensis* using broth and GYS media. *B. thuringiensis* was capable of utilizing this organic compound for bacterial growth and of transforming it into an internal storage material. During the uptake and conversion of this substrate to form PHAs, the molecular size of the substrate was principally retained so that the polymer obtained consisted mainly of poly(3-hydroxynonanoate-co-heptanoate). This copolyester is the usual product of the biosynthesis when *P. oleovorans*, a bacterium known for the production of medium and long chain PHAs, is fed with nonanoic acid (9). The cells have an ability to remove or add two carbon units during their metabolic utilization of substrates resulting in the formation of copolymers with repeat units which contain two or four carbon atoms less or more, respectively, than the original substrate (13). The same manner of substrate consumption was observed for *B. thuringiensis*.

The results of these experiments are summarized in Table 4. The utilization of this organic compound required a longer time because the growth and/or utilization of the carbon source did not start immediately; however, a lag time was observed. When the bacteria, which were pre-grown on broth, were exposed for 50 hours to nonanoic acid in the minimal medium (O.D. 1.9), they incorporated 17 % copolymer based on their dry cell weight, which was equivalent to 0.75 g/l. After remaining for 70 hours in the minimal medium with 25 mMol nonanoic acid the optical density, which is in general a measure for the cell density, reached a value of 5.8. This value is about three times higher than the

amount for the growth time of 50 hours, and accordingly the amount of biomass produced was determined to be 3.08 g/l. The amount of incorporated polymer was 20 %, which is similar to the previous result. Prolonging the growth time resulted in an increase of the biomass obtained; however, it did not lead to an increase of the amount of polymer. This result might indicate that the polymer formed contributes, in a later state of the bacterial growth, to the sporulation and formation of the proteinaceous crystal within the cell, and no further polymer accumulation occurs. The addition of nonanoic acid to the culture changed its density which led to difficulties in the centrifugation of the cells. It was only possible to obtain the total amount of cells after multiple dilutions of the culture solutions.

Table 4. Growth of *B. thuringiensis* on nonanoic acid

Growth time [h]	O.D.	Medium	Biomass [g/l]	Polymer [mg/l]	Incorp. [%]	Polymer[c)]
50	1.9	Broth	3.08	632	20.5	PHN/PHH
70	5.8	Broth	0.75	129	17.2	PHN/PHH
163	4.5	GYS[a)]	1.67	43	2.6	PHN/PHH
176	2.55	GYS[b)]	1.70	58	3.4	PHN/PHH

a) direct inoculation from the plate into the flask

b) 15 hours Growth on pre-culture, 10 % inoculum

c) analyzed by ^{1}H NMR

Table 5 shows the compositions of the polymers obtained when nonanoic acid was used as the sole carbon source. The product obtained was a copolymer consisting of 68 to 69 % of poly-3-hydroxynonanoate and 25 % of poly-3-hydroxyheptanoate. These main components were accompanied by small amounts of poly-3-hydroxyvalerate and poly-3-hydroxyundecanoate. During the conversion of the carbon source, in the bacterial metabolic cycle, removal and addition processes of two-carbon units occur which result in the formation of copolymers with repeating units which differ by two carbon atoms.

Table 5. Compositions of polymers produced with nonanoic acid as carbon source

Growth time [h]	O.D.	Medium	Polymer Composition [%] C_5	C_7	C_9	C_{11}	other
50	1.9	Broth	2.0	24.6	69.3	-	3.1
163	4.5	GYS	-	25.6	68.4	2.1	3.9

Acknowledgments

We gratefully acknowledge the financial support from the Materials Research Laboratory at the University of Massachusetts, funded by the National Science Foundation grant number NSF-DMR-9023848, and from the National Science Foundation grant number MCB-9202419, and the support provided to Dr. Carmen Scholz by the Feodor Lynen-Fellowship Program of the Alexander von Humboldt-Foundation, Germany. The authors wish to acknowledge the technical support provided by Dr. Frank Küchenmeister.

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