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Native granule associated short chain length polyhydroxyalkanoate synthase from a marine derived *Bacillus* sp. NQ-11/A2

Nimali N. Prabhu · Maria Celisa Santimano · Suneela Mavinkurve · Saroj N. Bhosle · Sandeep Garg

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Abstract A rapidly growing marine derived *Bacillus* sp. strain NQ-11/A2, identified as *Bacillus megaterium*, accumulated 61% polyhydroxyalkanoate by weight. Diverse carbon sources served as substrates for the accumulation of short chain length polyhydroxyalkanoate. Three to nine granules either single or attached as buds could be isolated intact from each cell. Maximum activity of polyhydroxyalkanoate synthase was associated with the granules. Granule-bound polyhydroxyalkanoate synthase had a K_m of 7.1×10^{-5} M for DL-β-hydroxybutyryl-CoA. Temperature and pH optima for maximum activity were 30°C and 7.0, respectively. Sodium ions were required for granule-bound polyhydroxyalkanoate synthase activity and inhibited by potassium. Granule-bound polyhydroxyalkanoate synthase was apparently covalently bound to the polyhydroxyalkanoate-core of the granules and affected by the chaotropic reagent urea. Detergents inhibited the granule-bound polyhydroxyalkanoate synthase drastically whilst glycerol and bovine serum albumin stabilized the synthase.

Keywords Polyhydroxyalkanoate (PHA) · Granule bound PHA synthase (GBPS) · Marine derived *Bacillus* · Short chain length—PHA (SCL-PHA)

Introduction

Bioplastics have received tremendous attention in the recent years due to their high molecular weight, thermoplastic/elastomeric properties, biodegradability, biocompatibility, non-toxicity and its production from renewable carbon sources. Polyhydroxyalkanoates (PHAs) are simple macromolecules synthesized by a wide variety of Gram-positive and Gram-negative bacteria, and members of family Halobacteriaceae of the Archaea (Philip et al. 2007; Hezayen et al. 2002). The synthesis of the polymer is initiated when acetyl-CoA is restricted from entering the tricarboxylic acid cycle due to the nutrient limitation, shunting the acetyl units from the TCA cycle into the production of polyhydroxybutyrate (PHB), which is an ideal carbon and energy storage polymer. Currently more than 150 different hydroxyalkanoic acids have been identified as substrates by PHA synthases, thereby determining the type of PHA produced by the organism.

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Biosynthesis of PHB involves the condensation of two molecules of acetyl-CoA to form acetoacetyl CoA, which is reduced to (R)-3-hydroxybutyryl-CoA. This serves as a substrate for PHA synthase and acts as a direct precursor for PHA production (Rehm 2007; Valappil et al. 2007 and Volova et al. 2004).

PHA synthases have been classified into three types. Class I PHA synthases, represented by that of *Ralstonia eutropha*, utilize CoA thioesters of (R)-3-hydroxy fatty acids comprising 3–5 carbon atoms and consist of PhaC subunits with molecular weights between 61 and 73 kDa. Class II PHA synthases, represented by that of *Pseudomonas aeruginosa*, consist of PhaC subunits with molecular weights also ranging from 61 to 73 kDa and utilize CoA thioesters of (R)-3-hydroxy fatty acids comprising 6–14 carbon atoms. Class III PHA synthases, represented by that of *Allochromatium vinosum*, consist of two different types of subunits, PhaC and PhaE, which have a molecular weight of 40 kDa each. These prefer CoA thioesters of (R)-3-hydroxy fatty acids comprising 3–5 carbon atoms (Rehm 2007; Sudesh et al. 2000). *Bacillus megaterium* possesses a novel PHA synthase similar to class III containing two subunits except that the PhaE typical of class III is replaced by a 20 kDa PhaR subunit (Satoh et al. 2002; McCool and Cannon 2001). To date, PHA synthase enzymes from representatives of the genus *Bacillus* have only been characterized at a preliminary level (Valappil et al. 2007). Even though the presence of PHA as a storage compound was first discovered in *Bacillus megaterium,* only a few reports on the structural studies of PHA inclusion bodies in *Bacillus cereus* and properties of the granule associated PHA synthase from *Bacillus megaterium* are available (Lenz and Marchessault 2005; Griebel et al. 1968).

This report focuses on the isolation of PHA granules from a marine derived *Bacillus* sp. NQ-11/A2 and characterization of the granule-bound PHA-synthase (GBPS).

Materials and methods

Collection of sediment sample and bacteriological analysis

A sediment sample from Arabian Sea (Lat 19°33.949′ N, Long 71°22.041′ E), 100 km West from the Indian coast was collected aseptically using a Grab sampler at a depth of 63 m and stored at 4°C. One gram of the sediment sample was suspended in 100 ml of sterile physiological saline, in a 250 ml capacity Erlenmeyer flask and shaken for 30 min at 170 rpm (Orbitek environmental shaker). Dilutions of the sediment suspension were prepared and 100 µl was spread plated on Nutrient Agar (NA) and Luria–Bertani agar prepared with aged sea water (LBS). NA contained in g l^{-1}: beef extract- 10; peptone-10; NaCl- 5; agar agar- 20; pH 6.8. LBS contained in g l^{-1}: tryptone-10; yeast extract- 5; NaCl- 5; agar agar- 20; pH 7.2. The plates were incubated at 30°C for 48 h. Morphologically dissimilar colonies were picked and restreaked to purify the isolates. Colony characteristics were studied and the isolates were maintained at 4°C. All the chemicals used were procured either from Sigma chemicals (USA), HiMedia, Merck or Qualigens (India).

Screening of bacterial isolates for PHA production

The ability of the bacterial isolates to accumulate PHA was tested using E2 mineral medium which contained [g l^{-1} double distilled H_2O]: $KH_2PO_4 \cdot 3H_2O$- 3.7; $K_2HPO_4 \cdot 3H_2O$- 7.5; $NaNH_4HPO_4 \cdot 4H_2O$- 3.5; $MgSO_4 \cdot 7H_2O$- 10 ml (0.1 M); yeast extract- 0.5; microelement stock solution- 1 ml (Lageveen et al. 1988); glucose- 20; pH 7.0. The isolates were spot inoculated on E2 medium agar plates in triplicates and incubated at 30°C. Accumulation of PHA was monitored every 24 h for 3 days by flooding plates with 0.05% (w/v) Nile blue A in ethanol and incubating in the dark for 20 min (Kitamura and Doi 1994). The stain was decanted and plates were exposed to UV light. Bright orange fluorescence was graded and recorded.

Phenotypic characterization of the selected bacterial strain

The isolate NQ-11/A2 was phenotypically characterized using the methods described in Bergey's Manual of Systematic Bacteriology (Sneath 1986; Priest et al. 1988). The data was analyzed numerically, using the simple matching coefficient (S_{SM}). Clustering was achieved by unweighted pair group average linkage (UPGMA). The computations were performed using the Probiosys program.

Genotypic characterization of the selected bacterial strain

Genomic DNA was extracted using the protocol described in Sambrook et al. (1989). The 16S rRNA gene was amplified using isolated chromosomal DNA as the template. The primers used for the amplification were S-D-Bact-0011-a-S-17 5′-GTTTGATC CTGGCTCAG-3′ as forward and S-*-Univ-1392-b-A-15 5′- ACGGGCGGTGTGTTC-3′ as reverse primer (Alm et al. 1996). All PCR reactions were performed in a reaction mixture of 100 µl total volume using 1 µg template DNA. The reaction mixture was heated at 94°C for 3 min followed by 40 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. The final elongation step was carried out at 72°C for 5 min. The PCR product obtained was gel purified and sent for sequencing at Macrogen Inc, Korea. Sequence was obtained using the forward primer and has been deposited in GenBank under Accession number FJ392860. The sequence of the strain was compared with similar sequences of reference organisms by BLASTn 2.2.18+ and EzTaxon (Chun et al. 2007; Zhang et al. 2000). Partial sequences of the 16S rRNA gene (873 bp) including the variable region were analyzed for comparison with those of other *Bacillus* species according to Goto et al. (2000). ClustalX version 2.0.7 was used to generate multiple alignments between the selected sequences using the IUB matrix (Larkin et al. 2007). A Neighbor-joining (NJ) tree was obtained with 500 seeds and 3,000 bootstraps. The final tree obtained was rooted and drawn using MEGA4 (Tamura et al. 2007).

Further analysis of the 16S rRNA gene sequence (1,419 bp) was also carried out with the help of Chromous Biotech Pvt. Ltd (Bangalore, India). The forward (5′-AGAGTRTGATCMTYGCTWAC-3′) and reverse primers (5′ CGYTAMCTTWTTACGRCT-3′) used are specific for prokaryotic 16S rRNA genes. The sequence obtained has been deposited in GenBank under Accession number FJ392860.

Culture growth and submerged PHA production

A single colony from a 24 h culture was inoculated in a 250 ml Erlenmeyer flask containing 100 ml E2 mineral medium supplemented with glucose (20 g l^{-1}) as sole carbon source. The flask was incubated on an Orbitek environmental shaker (170 rpm) for 24 h at 30°C. This culture broth was either used as inoculum or the cells were harvested at 10,000 g for 10 min, washed twice with sodium phosphate buffer (0.1 M, pH 7.0) and used for further experiments.

Erlenmeyer flasks containing 500 ml E2 mineral medium with glucose (20 g l^{-1}) were inoculated with 5% of the inoculum. 50 ml culture broth was withdrawn every 6 h from the flask and processed for determination of biomass and PHA content.

Determination of carbon chain length of monomers using Nile red staining

The monomer chain length incorporated into the polymer was determined using Nile red O (Wu et al. 2003). Cell suspensions of the colonies growing on E2 medium agar plates containing different carbon substrates were prepared, centrifuged and used for staining with 10 µl of Nile red O solution (1 mg ml^{-1} in acetone). The acetone was allowed to evaporate and the pellets were suspended in 1 ml of distilled water. The suspensions were scanned for maximal emission wavelength from 530 to 650 nm using an excitation wavelength of 488 nm.

Determination of the monomeric composition of PHA using gas chromatograph- mass spectrometer (GC-MS)

Production of PHA using selected carbon substrates such as xylose, glucose, sucrose, lactic acid, succinic acid and citric acid was achieved by growing the isolate NQ-11/A2 under submerged conditions. These substrates were added as sole carbon source in E2 mineral medium at a final concentration equivalent to 20 g l^{-1} glucose. Inoculated flasks were incubated at 30°C (170 rpm). The polymer accumulated using the above carbon substrates was used for further analysis.

To determine the composition of PHA, the polymer was methanolyzed as per the method described by Shishatskaya and Volova (2004). Approximately 8 mg of PHA was suspended in 2 ml methanol containing 15% (v/v) concentrated sulphuric acid and 2 ml of chloroform. The reaction was carried out at 100°C for 3.5 h. After cooling, 1 ml of distilled water was added to the reaction mixture and shaken vigorously for 1 min. On phase separation, the

organic layer was transferred into a vial and used for analysis.

The methanolyzed polymer sample was analyzed using GC-MS (model QP2010, Shimadzu, Japan), equipped with a 30 m × 0.32 mm RTX-5MS (5% diphenyl and 95% dimethyl polysiloxane) capillary column. Chromatographic conditions were as follows: carrier gas helium; flow rate 1 ml/min; sample input temperature 220°C; initial temperature 70°C, programmed to 230°C at a rate of 8°C/min; interphase temperature 250°C; ion source temperature 175°C; electron impact mode 70 eV; scanning range 45–450 amu at 0.5 s/scan.

Isolation of native PHA granules

Bacterial cells (1 g wet weight) were suspended in 5 ml lysis buffer (0.1 M sodium phosphate buffer, pH 7.0 containing 1 mg/ml lysozyme) and incubated for 1 h under shaking conditions at 30°C. The lysate was centrifuged for 30 min at 10,000*g*. The pellet was suspended in sodium phosphate buffer (0.1 M, pH 7.0), centrifuged and re-suspended in minimum volume of buffer. 0.5 ml of the suspension was loaded on to sucrose density gradient consisting of 2.5 ml each of 40, 55 and 60% sucrose in 11.2 ml polyallomer Quick-seal centrifuge tubes. The sealed tubes were placed in a vertical rotor and centrifuged at 79,000*g* for 2 h at 4°C using an Ultracentrifuge (Beckman, L8-M, USA). The inclusion bodies, which banded at the interphase between 40 and 55%, were collected and suspended in 10 volumes of buffer. The suspension was centrifuged at 10,000*g* for 10 min, the pellet was re-suspended in sodium phosphate buffer and stored at 4°C.

Scanning electron microscopy of PHA granules

A drop of the granule suspension was placed on a slide, air dried and fixed with 2% (v/v) glutaraldehyde for 2 h. The slide was washed with sodium carbonate-bicarbonate buffer (0.05 M) and passed through a series of increasing acetone concentrations [50, 70, 90 and 100% (v/v)]. The slide was placed on the stub and dried using a critical point dryer. The sample was coated with a thin film of gold using a spi-module sputter coating device and observed under scanning electron microscope (JEOL, 5800LV, Japan).

Determination of PHA synthase activity

The PHA synthase activity was determined by a discontinuous spectrophotometric assay monitoring the release of CoA at 412 nm from the substrate DL-β-hydroxybutyryl-CoA (Muh et al. 1999). The final volume of the reaction mixture was made up to 1 ml and contained 50 mM NaCl, 1 mM 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB), 60 μM DL-β-hydroxybutyryl-CoA, sodium phosphate buffer (0.1 M, pH 7.0) and 100 μl of granule suspension. The reaction was carried out at 30°C, unless otherwise specified, for 8 min. The mixture was spun at 10,000 g for 2 min. The effect of varying pH was tested using citrate buffer (0.1 M, pH 4 and 5), sodium phosphate buffer (0.1 M, pH 5–8) and Bicine buffer (0.1 M, pH 8 and 9). The absorbance of the supernatant was measured at 412 nm. The concentration of CoA released was quantified using the extinction coefficient (412 nm) of 13,600 cm^{-1} M^{-1}. One unit was defined as the amount of enzyme required to convert 1 μmol of substrate in 1 min.

Analytical methods

The polymer was extracted using the sodium hypochlorite method (Rawte and Mavinkurve 2002). Twenty-five ml of the culture broth was centrifuged at 10,000*g* for 10 min. The cell pellet was suspended in physiological saline and centrifuged again. Sodium hypochlorite solution (2% available chlorine) was added to the cell pellet and incubated on an Orbitek environmental shaker (170 rpm) for 20 min. The suspension was centrifuged for 20 min. The polymer pellet obtained was suspended and washed twice with diethyl ether and dried at 80°C.

Cell biomass was estimated by drying the cell pellet at 80°C until constant dry weight was obtained.

Protein content of the samples was determined according to the method described by Lowry et al. (1951) using bovine serum albumin as a protein standard.

Results and discussion

Isolation and identification of the isolate

Heterotrophic bacterial counts in sediment collected from Arabian Sea were 89.2×10^4 and

14.4×10^5 cfu g^{-1} on NA and LBS, respectively. The higher counts on the medium containing sea water indicates the predominance of marine bacteria in the sediment sample. Seven and five colonially distinct bacterial isolates on NA and LBS were obtained, respectively. Among these, four isolates from NA showed accumulation of PHA. However, none of the isolates obtained on LBS exhibited PHA accumulation. PHA serves as a carbon and energy reserve. The accumulation of PHA offers a selective advantage for survival of bacteria in such sediments (Lopez et al. 1995). One of the isolates namely, NQ-11/A2 accumulated maximum PHA on glucose, detected as bright orange fluorescence when stained with Nile blue A. This isolate was Gram-positive, rod-shaped, facultatively anaerobic and an endospore former. It was able to grow at temperatures as high as 50°C and tolerate up to 10% salt. Biochemical characterization with similarity analysis and UPGMA clustering placed this isolate along with *Bacillus megaterium* and *Bacillus flexus*. S_{SM} for NQ-11/A2 were 86.0 and 83.33% with *B. megaterium* and *B. flexus*, respectively. However, the phenotypic characteristics of this isolate were in sharp contrast to *B. megaterium,* a strict aerobe, which grows only up to 37°C and in presence of a maximum of 5% salt (Priest et al. 1988). A BLAST (NCBI) search using the 16S rRNA gene sequence (873 bp) of strain NQ-11/A2 showed 90% and above homology to 30 known taxa of Bacillaceae with maximum homology to *B. megaterium* and *B. flexus*. The evolutionary relationship of the isolate with selected species of the Bacillaceae family was analyzed using the Neighbor-joining method. From the phylogenetic tree it was evident that isolate NQ-11/A2 forms a separate group with the closest relatives being *B. megaterium*, *B. flexus* and *Bacillus simplex*. Within this group, it was seen that the sequence of the isolate aligned to *B. megaterium*.

The nucleotide sequence comprising of 1,419 bp of the 16S rRNA gene was also further analyzed and revealed a S_ab (similarity score) of 1.0 when compared with that of *Bacillus megaterium* DPBS17 (NCBI accession number EU249559) indicating maximum sequence homology between the two gene sequences. The phylogenetic tree is displayed in Fig. 1. These data suggest that strain NQ-11/A2 is a representative of *B. megaterium. Bacillus* sp. NQ-11/A2 has been deposited in the National Collection of Industrial Micro-organisms (NCIM, Pune, India) with Accession Number NCIM 5334.

Bacillus sp. NQ-11/A2 grew rapidly and reached stationary phase within 24 h. PHA accumulation was initiated at 6 h and continued to be synthesized during stationary phase up to 48 h. The strain accumulated a maximum 61% of its total biomass as PHA (Fig. 2).

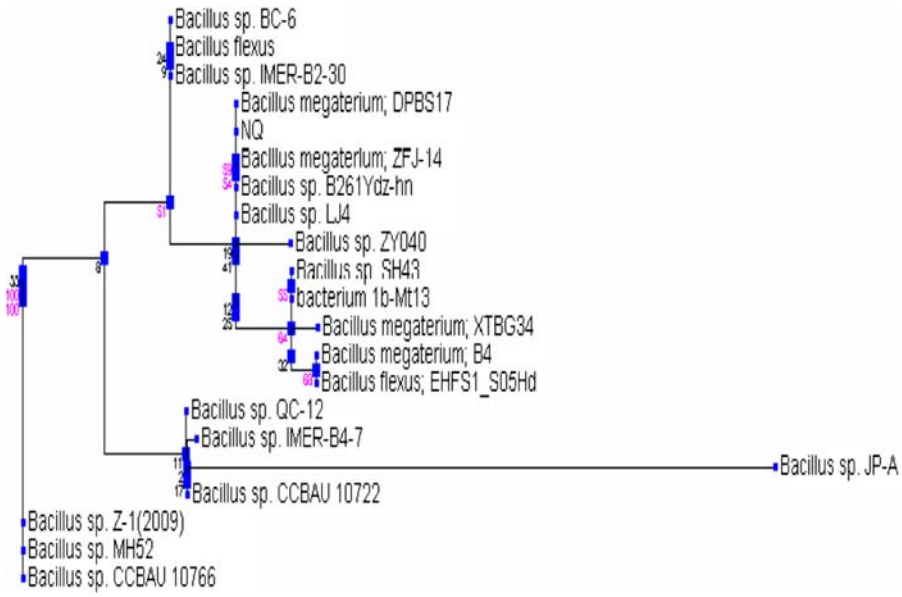

Fig. 1 Phylogenetic relationship of isolate NQ-11/A2 with closely related members of *Bacillus* based on 16S rRNA gene sequence analysis. The tree was generated using Weighbor weighted neighbor-joining method (Bruno et al. 2000). Bootstrap values with 100 replicates are displayed on the nodes

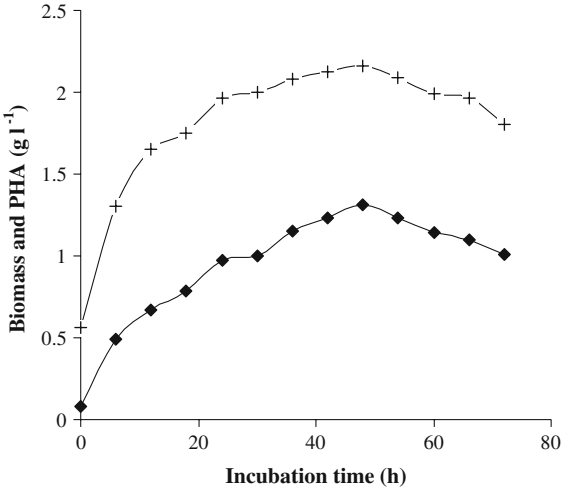

Fig. 2 Accumulation of biomass (*plus*) and PHA (*filled diamond*) in shake flask of *Bacillus* sp. NQ-11/A2

Usually, PHA accounts for 11–37% of cellular mass in *Bacillus* spp. (Shamala et al. 2003). Recently, *Bacillus thuringiensis* R1 and an anoxic *Bacillus* sp. CL1 have been reported with high PHA content (Bordoloi et al. 2007; Rohini et al. 2006; Full et al. 2006). *Bacillus* sp. NQ-11/A2 accumulated PHA using diverse carbon substrates (Table 1). Except for a few carbon sources, all served as substrates for PHA production. Nile red O fluorescence spectra of the intracellular PHA indicated that the polymer is of the short chain length (SCL) type.

The polymer accumulated by isolate NQ-11/A2 using various carbon substrates such as xylose, glucose, sucrose, lactic acid, succinic acid and citric acid was analyzed by GC-MS. The total ion current chromatogram (TIC) of the methanolyzed products of the polymer accumulated by isolate NQ-11/A2 when grown using xylose is shown in Fig. 3a and the mass spectra of the dimers of beta-hydroxybutyric acid methyl ester is shown in Fig. 3b. GC-MS analysis confirmed that the same profile was obtained when isolate NQ-11/A2 was grown on succinic acid and lactic acid as sole carbon sources (Online Supplementary Figs. 1 and 2). In all the cases, the peak with retention time 7.9 min matched with the mass spectrum of the dimer of beta-hydroxybutyric acid methyl ester from the MS library (WILEY7). This confirmed that beta-hydroxybutyric acid was the major momomeric unit incorporated into the PHA polymer by the isolate grown using the above carbon substrates.

Table 1 PHA accumulation in *Bacillus* sp. NQ-11/A2 using diverse carbon substrates

Substrates	Fluorescence intensity	Emission peak (nm)
Arabinose	+++	590
Ribose	–	–
Xylose	++	589
Glucose	+++	594
Galactose	+++	593
Mannose	–	–
Fructose	+++	593
Rhamnose	–	–
Sorbose	–	–
Lactose	+++	591
Sucrose	+++	593
Maltose	+++	588
Cellobiose	+++	590
Mellibiose	+++	589
Trehalose	+++	588
Glycerol	+++	588
Erythitol	+++	586
Adonitol	++	588
Xylitol	–	–
Dulcitol	+++	585
Mannitol	+++	589
Sorbitol	+	585
Acetic	+	590
Glycolic	–	–
Pyruvic	+	591
Lactic	++	590
Propionic	–	–
Butyric	–	–
Maleic	+	586
Tartaric	++	585
3-hydroxybutric	++	589
Succinic	++	594
Malonic	++	586
Pentanoic	–	–
Citric	++	592

Key: (–) no PHA accumulation; (+, ++, +++) degree of fluorescence

The genus *Bacillus* has been demonstrated to accumulate SCL-PHA (Tajima et al. 2003). The type of polymer synthesised by the organism is dependent

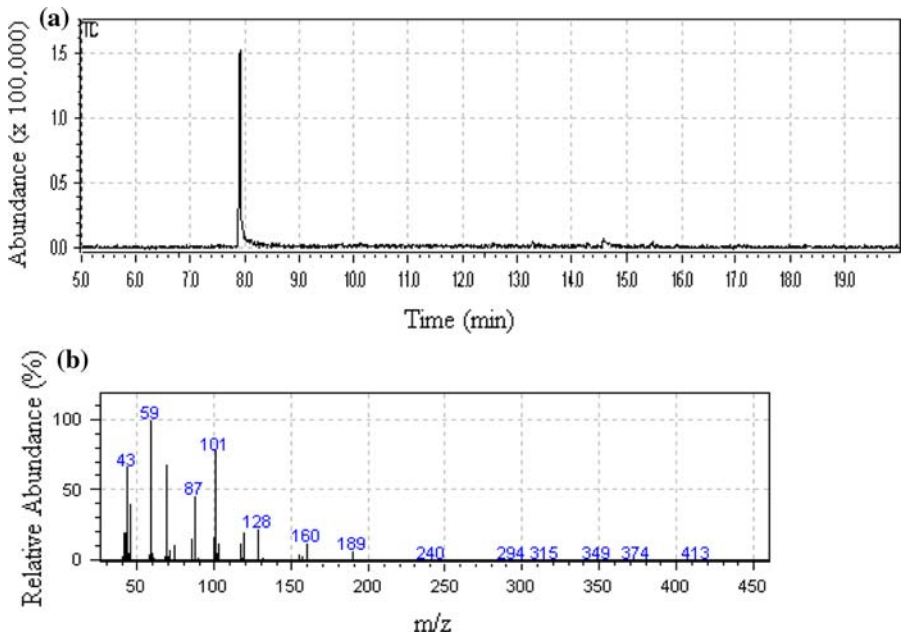

Fig. 3 **a** The total ion current chromatogram (TIC) of the methanolyzed products of the polymer accumulated by isolate NQ-11/A2 grown on xylose and **b** mass spectrum of the dimer of beta-hydroxybutyric acid methyl ester from isolate NQ-11/A2

on the carbon substrate. The use of some carbon substrates as precursors for production of new types of PHA is mainly limited by their cost and availability (Rehm 2003). Alternatively, screening for bacteria metabolizing cheap carbon sources and synthesizing these precursor substrates endogenously can help to overcome the problem (Steinbuchel and Valentin 1995).

Native PHA granules of *Bacillus* sp. NQ-11/A2 formed a white band at the interphase between 40 and 55% sucrose concentration on sucrose density centrifugation. PHA granules were spherical and intact in shape (Fig. 4). These granules were 0.51 ± 0.074 μm. There were three to nine granules in the cells. These granules were either single or in budding groups. McCool et al. (1996) have reported the presence of inclusion bodies in numbers of 2–8 which also appeared dividing or budding.

The specific activity of the GBPS was 11.625 U mg^{-1} whereas specific cytosolic PHA synthase was only 0.056 U mg^{-1}. This granule associated specific activity was higher than that reported for *Pseudomonas oleovorans* but lower than reported in *B. megaterium* and halophillic archaeon (De Roo et al. 2000; Griebel et al. 1968; Hezayen et al. 2002). The GBPS of this isolate showed a requirement for sodium ions in the reaction mixture (Table 2). Interestingly, potassium ions had a negative effect on the enzyme. This could be due to the

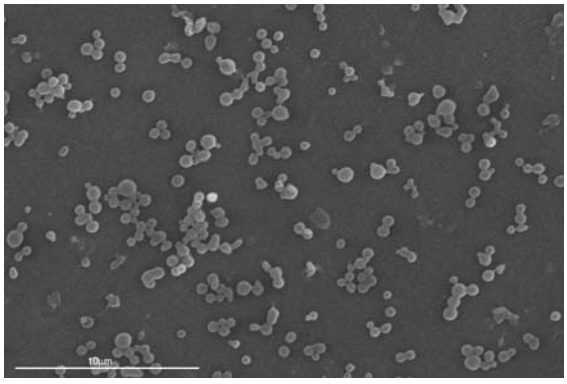

Fig. 4 Scanning electron micrograph of native PHA granules

Table 2 Effect of sodium and potassium ions on granule bound PHA synthase

Salt (50 mM)	Buffer (0.1 M, pH 7.00)	
	Sodium phosphate	Potassium phosphate
NaCl	19.217	5.390
KCl	4.050	2.498

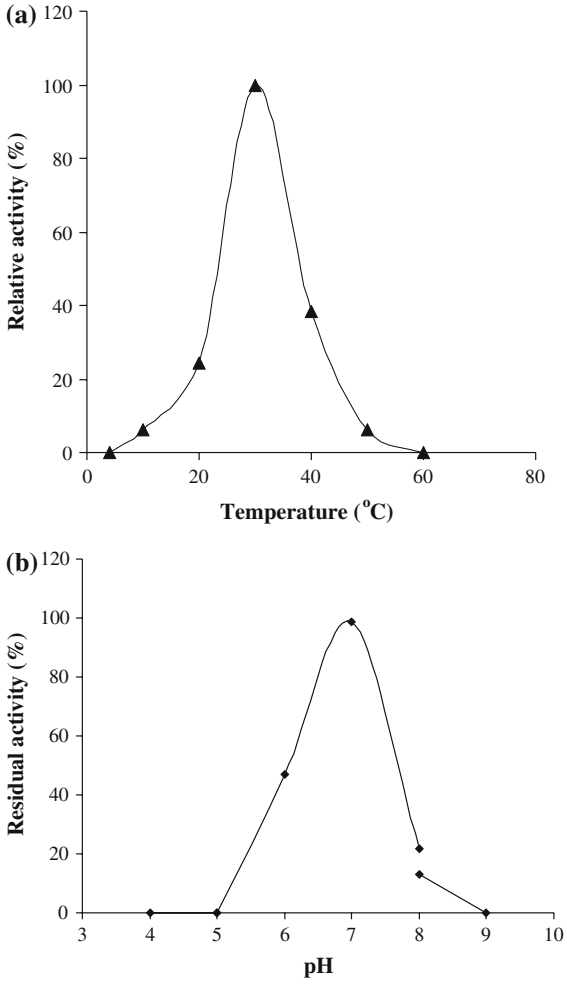

Fig. 5 **a** Effect of temperature on the activity of granule bound PHA synthase. **b** Effect of pH on the activity of granule bound PHA synthase

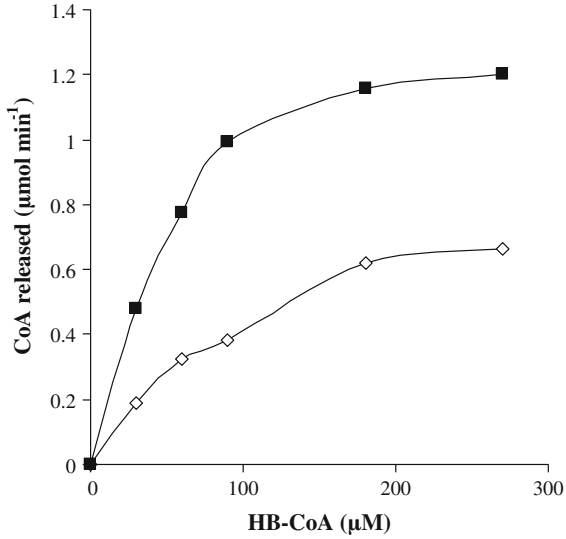

Fig. 6 Effect of substrate concentration (HB-CoA) on the activity of the granule bound PHA synthase. 100 µl of granules (*filled square*), 50 µl of granules (*empty diamond*)

marine origin of *Bacillus* sp. NQ-11/A2. Sodium was also found to be necessary for the PHA synthase activity in a halophillic archaeon (Hezayen et al. 2002). The GBPS was active over a narrow temperature and pH range with optima at 30°C and 7.0, respectively (Fig. 5a, b). Either side of the optima for temperature and pH, the GBPS activity dropped drastically, unlike the GBPS activity reported for the haolophillic archaeon (Hezayen et al. 2002). The substrate saturation constant of the GBPS was 7.1×10^{-5} M for 3-hydroxybutyryl CoA (Fig. 6) and therefore twenty percent better than reported previously for *B. megaterium* (Griebel et al. 1968).

Detergents adversely affected GBPS of NQ-11/A2 (Fig. 7). This effect was maximal with CTAB buffer and minimal with Triton-X-100. Ren et al. (2000) have reported complete inhibition of GBPS activity with Triton-X-100. These detergents were expected to stabilize the amphiphilic enzymes. However, GBPS was inhibited. The loss in activity could be due to the interference of the detergent with protein–protein interactions of GBPS and other proteins in the granule membrane. GBPS was not affected by $MgCl_2$, similar to the observations made with GBPS from an archeal halophile (Hezayen et al. 2002). This was further confirmed as EDTA did not have any effect on GBPS. Only BSA and glycerol showed positive effects on the enzyme. This could be due to the stabilizing effect of BSA and glycerol as they are known to bind to the PHA granules and interact directly with PHA synthases (De Roo et al. 2000). Lysozyme, a known inhibitor of GBPS (Liebergesell et al. 1992), reduced the activity by 40%. PHA synthase in the granules of *Bacillus* sp. NQ-11/A2 was apparently covalently linked to the PHA-core and therefore addition of the chaotropic agent urea reduced the activity.

To our knowledge, this is the first report on marine derived *Bacillus* sp. growing rapidly and

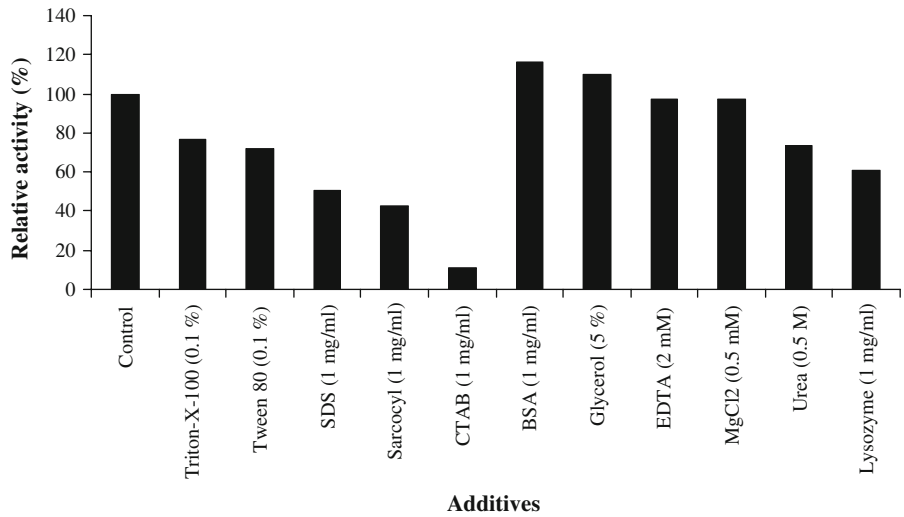

Fig. 7 Effect of various additives on granule-bound PHA-synthase activity

accumulating large amounts of SCL-PHA. Our studies indicate a large amount of polymer accumulation inside the granule by GBPS in vitro with minimum maintenance requirement.

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References

Alm WE, Oerther DB, Larsen N, Stahl DA, Raskin L (1996) The oligonucleotide probe database. Appl Environ Microbiol 62:3557–3559

Bordoloi M, Borah B, Thakur PS, Nigam JN (2007) Process for the isolation of polyhydroxybutyrate from *Bacillus mycoides* RLJ B-017. US Patent 7,273,733

Bruno WJ, Socci ND, Halpern AL (2000) Weighted neighbor joining: a likelihood-based approach to distance-based phylogeny reconstruction. Mol Biol Evol 17:189–197

Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 57:2259–2261

De Roo G, Ren Q, Witholt B, Kessler B (2000) Development of an improved in vitro activity assay for medium chain length PHA polymerases based on coenzymeA release measurements. J Microbiol Meth 41:1–8

Full TD, Jung DO, Madigan MT (2006) Production of poly-β-hydroxyalkanoates from soy molasses oligosaccharides by new, rapidly growing *Bacillus* species. Lett Appl Microbiol 43:377–384

Goto K, Omura T, Hara Y, Sadaie Y (2000) Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J Gen Appl Microbiol 46:1–8

Griebel R, Smith Z, Merrick M (1968) Metabolism of poly-beta-hydroxybutyrate. I. Purification, composition, and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry 7:3676–3681

Hezayen FF, Steinbüchel A, Rehm BHA (2002) Biochemical and enzymological properties of the polyhydroxybutyrate synthase from the extremely halophilic archaeon strain 56. Archives Biochem Biophy 403:284–291

Kitamura S, Doi Y (1994) Staining method of poly(3-hydroxyalkanoic acid) producing bacteria by Nile Blue. Biotechnol Tech 8:345–350

Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G, Witholt B (1988) Formation of Polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl Environ Microbiol 54:2924–2932

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and clustal X version 2.0. Bioinformatics 23:2947–2948

Lenz RW, Marchessault RH (2005) Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. Biomacromolecules 6:1–8

Liebergesell M, Schmidt B, Steinbüchel A (1992) Isolation and identification of granule-associated proteins in *Chromatium vinosum* D. FEMS Microbiol Lett 99:227–232

Lopez NI, Floccari ME, Steinbuchel A, Garcia AF, Mendez BS (1995) Effect of poly(3-hydroxybutyrate) (PHB) content

on the starvation of bacteria in natural waters. FEMS Microbiol Ecol 16:95–102

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

McCool GJ, Cannon MC (2001) PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. J Bacteriol 183:4235–4243

McCool GJ, Fernandez T, Li N, Cannon MC (1996) Polyhydroxyalkanoate inclusion-body growth and proliferation in *Bacillus megaterium*. FEMS Microbiol Lett 138:41–48

Muh U, Sinskey AJ, Kirby DP, Lane WS, Stubbe J (1999) PHA synthase from *Chromatium vinosum*: cysteine 149 is involved in covalent catalysis. Biochemistry 38:826–837

Philip S, Keshavarz T, Roy I (2007) Polyhydroxyalkanoates: biodegradable polymers with a range of applications. J Chem Technol Biotechnol 82:233–247

Priest FG, Goodfellow M, Todd C (1988) A numerical classification of the genus *Bacillus*. J Gen Microbiol 134: 1847–1882

Rawte T, Mavinkurve S (2002) A rapid hypochlorite method for extraction of polyhydroxyalkanoates from bacterial cells. Indian J Exp Biol 40:924–929

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33

Rehm BHA (2007) Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Curr Issues Mol Biol 9:41–62

Ren Q, De Roo G, Kessler B, Witholt B (2000) Recovery of active medium-chain-length-poly-3-hydroxyalkanoate polymerase from inactive inclusion bodies using ion-exchange resin. Biochem J 349:599–604

Rohini D, Phadnis S, Rawal SK (2006) Synthesis and characterization of poly-β-hydroxybutyrate from *Bacillus thuringiensis* R1. Indian J Biotechnol 5:276–283

Sambrook J, Maniatis T, Fritsch EF (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, New York, USA

Satoh Y, Minamoto N, Tajima K, Munekata M (2002) Polyhydroxyalkanoate synthase from *Bacillus* sp. INT005 is composed of PhaC and PhaR. J Biosci Bioeng 94:343–350

Shamala TR, Chandrashaekar A, Vijayendra SVN, Kshama L (2003) Identification of polyhydroxyalkanoate (PHA)-producing *Bacillus* spp. using the polymerase chain reaction (PCR). J Appl Microbiol 94:369–374

Shishatskaya EI, Volova TG (2004) A comparative investigation of biodegradable polyhydroxyalkanoate films as matrices for in vitro cell cultures. J Mater Sci Mater Med 15:915–923

Sneath PHA (1986) Endospore-forming Gram-positive rods and cocci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn. Williams and Wilkins, Baltimore, pp 1104–1129

Steinbuchel A, Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Letts 128: 219–228

Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 25:1503–1555

Tajima K, Igari T, Nishimura D, Nakamura M, Satoh Y, Munekata M (2003) Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil. J Biosci Bioeng 95:77–81

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Valappil SP, Boccaccini AR, Bucke C, Roy I (2007) Polyhydroxyalkanoates in Gram-positive bacteria: insights from the genera *Bacillus* and *Streptomyces*. Antonie van Leeuwenhoek 91:1–17

Volova TG, Kalacheva GS, Gorbunova OV, Zhila NO (2004) Dynamics of activity of the key enzymes of Polyhydroxyalkanoate metabolism in *Ralstonia eutropha* B5786. Appl Biochem Microbiol 40:170–177

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

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214