Ann Microbiol
DOI 10.1007/s13213-014-0878-z

ORIGINAL ARTICLE

Biocompatible polyhydroxybutyrate (PHB) production by marine *Vibrio azureus* BTKB33 under submerged fermentation

Raghul Subin Sasidharan · Sarita Ganapathy Bhat · Muthusamy Chandrasekaran

Received: 17 July 2013 / Accepted: 17 March 2014

Abstract Polyhydroxybutyrate (PHB) is known to have applications as medical implants and drug delivery carriers and is consequently in high demand. In the present study the possibilities of harnessing potential PHB-producing vibrios from marine sediments as a new source of PHB was investigated since marine environments are underexplored. Screening of polyhydroxyalkanoate (PHA)-producing vibrios from marine sediments was performed using a fluorescent plate assay followed by spectrophotometric analysis of liquid cultures. Out of 828 isolates, *Vibrio* sp. BTKB33 showed maximum PHA production of 0.21 g/L and PHA content of 193.33 mg/g of CDW. The strain was identified as *Vibrio azureus* based on phenotypic characterization and partial 16S rDNA sequence analysis. The strain also produced several industrial enzymes: amylase, caseinase, lipase, gelatinase, and DNase. The FTIR analysis of extracted PHA and its comparison with standard PHB indicated that the accumulated PHA is PHB. Bioprocess development studies for enhancing PHA production were carried out under submerged fermentation conditions. Optimal submerged fermentation conditions for enhanced intracellular accumulation of PHA production were found to be 35 °C, pH −7, 1.5 % NaCl concentration, agitation at 120 rpm, 12 h of inoculum age, 2.5 % initial inoculum concentration, and 36 h incubation along with supplementation of magnesium sulphate, glucose, and ammonium chloride. The PHA production after optimization was found to be increased to 0.48 g/L and PHA content to 426.88 mg/g of CDW, indicating a 2.28-fold increase in production. Results indicated that *V. azureus* BTKB33 has potential for industrial production of PHB.

Keywords Marine *Vibrio* sp · Submerged fermentation · Polyhydroxyalkanoates · Polyhydroxybutyrates · Phylogenetic tree · FTIR analysis

R. S. Sasidharan · S. G. Bhat (✉) · M. Chandrasekaran
Department of Biotechnology, Cochin University of Science and Technology, Kalamassery, Cochin-22, Kerala, India
e-mail: saritagbhat@gmail.com

M. Chandrasekaran
Department of Botany & Microbiology, College of Science, King Saud University, PB No. 2455, Riyadh 11451, Kingdom of Saudi Arabia

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable materials that are accumulated to store carbon and energy in various microorganisms (Keshavarz and Roy 2010). PHAs, being biocompatible and non toxic, have extensive applications in medicine as a tissue engineering material (Chen and Wu 2005), in drug delivery, and in wound management (Simon and Martin 2002). Due to their wider range of properties, i.e., as thermoplastics to elastomers, they find several applications in the domestic (Glazer and Nikaido 1994), agriculture (Dobbelaere et al. 2001), and industrial (Chen et al. 2000) fields.

Among the candidates for biodegradable plastics, PHAs have drawn much attention due to their complete biodegradability and the similarity of their material properties to conventional plastics (Luengo et al. 2003). Polyhydroxybutyrate (PHB) is the most commonly used PHA, and the metabolic pathways of PHB have been elucidated in detail (Khanna and Srivastava 2005). The properties of PHB are similar to those of various synthetic thermoplastics such as polypropylene. Bacteria accumulate polyesters like polyhydroxyalkanoates (PHAs) under unbalanced growth conditions of carbon substrate in excess of other nutrients like nitrogen, sulfur, phosphorus, or oxygen (Madison and Huisman 1999). More than 140 different monomeric units have been identified as constituents of PHA in different bacteria (Steinbuchel 2005); this

Published online: 09 April 2014

variety being influenced by type of microorganisms, media ingredients, fermentation conditions, modes of fermentation, and the recovery process (Keshavarz and Roy 2010), all contributing to the difference in the physical and chemical characteristics of PHAs. Although low productivity was reported in several studies, the versatility of these biopolymers makes them good candidates for high-value, low-volume products. Hence, there is an increase in the need to screen large numbers of PHA producers, with diversity of monomers and high yield as desirable traits.

Accumulation of PHA reportedly enhances survival of microorganisms under adverse environmental conditions (López et al. 1995). Microbial mats with their considerably high diversity and challenging environments are potential sources for isolation of novel PHA-accumulating strains (López-Cortés et al. 2008). Until recently, there were only a few reports on marine PHA-producing microorganisms (Sun et al. 1994; Weiner 1997; Ayub et al. 2004; Rawte and Mavinkurve 2004; Chien et al. 2007; López-Cortés et al. 2008; Arun et al. 2009; Wei et al. 2011;). Vibrios are reported to be dominant among the commensally heterotrophic bacteria in Cochin backwaters and near-shore areas of the west coast of India (Chandrasekaran et al. 1984). In this context, the present study focuses on isolation of PHA-accumulating vibrios from diverse marine benthic environments of the Indian subcontinent and optimization of bioprocess variables and media constituents for enhanced production of PHA.

Materials and methods

Sample collection and isolation of vibrios

Sediment samples were collected from marine environments like estuaries, salt pans, mangroves, and coastal waters along the southwest (9.9°N 76°E) and east (8.76°N 78°E) coast regions of peninsular India. Sediment samples were serially diluted using physiological saline (0.85 % NaCl solution in sterile water). 50 μl of the diluted samples were spread plated on Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plates (Himedia, Mumbai, India) and incubated at 37°C for 24 h. The isolates obtained were identified as *Vibrio* sp. based on the morphological and biochemical characteristics outlined in Bergey's manual of determinative bacteriology (Buchanan and Gibbons 1974).

Preliminary screening on Nile blue a agar plate

Preliminary screening of PHA-producing isolates was done using a Nile Blue A agar plate assay. Bacterial polyhydroxyalkanoate (PHA) accumulation was identified by spot inoculation of cell culture onto complex nitrogen-limiting agar plates containing PHA production medium (10 g of NaC1, 3.7 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $(NH_4)_2HPO_4$, 0.2 g of $MgSO_4{\cdot}7H_20$, 2 ml of glycerol, 5 g of Bactotryptone, and 0.5 g of yeast extract per liter) outlined by Sun et al. (1994) with Nile Blue A (1 μg/ml) and agar (2 %). The plates were incubated at 37 °C for three to four days. Accumulation of lipid storage compounds including PHAs in the cells was detected from fluorescence induced by exposing the colonies to ultraviolet light (Spiekermann et al. 1999).

Secondary screening in submerged fermentation conditions

Asecondary screening of vibrios that showed PHA production on Nile Blue A agar plates was done using the complex PHA production medium (Sun et al. 1994) as detailed above with glycerol (1 %) as the carbon source (Sun et al. 1994), employing submerged fermentation conditions and a nutrient medium with 1 % NaCl as the medium for seed preparation. An aliquot of 50 ml of complex PHA production medium with glycerol (1 %) as the carbon source was placed in a 250 ml Erlenmeyer flask. The medium was inoculated with 1 % (v/v) of 12 h-old culture ($O.D._{600}$=1.00) and incubated in an environmental shaker (Orbitek, Scigenics, India) at 30 °C at 150 rpm. After 48 h, the cells were harvested by centrifugation at 8,000 rpm for 15 min at 4 °C.

PHAs were recovered from the dry cell mass by the extraction method outlined by Dong and Sun (2000). Briefly, the cell pellet, after washing with 0.85 % NaCl solution, was initially subjected to a pretreatment step to release PHA. In this step, the biomass was treated at 100 °C for 1 min in a water bath and rapidly cooled to 55 °C, then stored at −20 °C. 1 % SDS solution was added to this pretreated pellet, which was then held at 55 °C for 15 min to effectively solubilize lipids and protein, followed by centrifugation (8,000 rpm at 4 °C for 10 min). The resultant cell pellet was then treated with 30 % NaOCl solution at 30 °C for 1 min to remove peptidoglycan and non-PHA biomass. PHA was then recovered by centrifugation (8,000 rpm at 4 °C for 10 min), washed with acetone, ethanol, and lasty, with distilled water, and then air-dried in (Law and Slepecky 1961). The extracted PHA was analyzed spectrophotometrically at 235 nm (Law and Slepecky 1961) using a Shimadzu UV-visible spectrophotometer (Japan). The cell dry weight (CDW) in grams per liter was determined. From the results obtained for cell dry weight and PHA concentration, the PHA content (mg/g of CDW) was calculated.

Characterization of Vibrio sp. strain BTKB33

Phenotypic characterization

Phenotypic characterization of the *Vibrio* sp. BTKB33 was performed using Hi-Vibrio identification systems (HiMedia,

Mumbai, India) and involved 12 biochemical tests: Voges Proskauers's test, arginine dihydrolase test, 1 % salt tolerance test, ONPG test, citrate utilization test, ornithine, mannitol, arabinose, sucrose, glucose, salicin, and cellobiose utilization tests; extracellular enzyme profiling by various plate assay methods (Kazanas 1968; Zierdt and Golde 1970; Hankin et al. 1971; Furniss et al. 1978; Nautiyal 1999; Kasing et al. 2000; Anuradha et al. 2007) was also performed.

Genotypic characterization

A portion of the 16S rDNA was amplified using a primer pair for 16S rDNA (Reddy et al. 2000) using the isolated and purified Genomic DNA (Sambrook et al. 2000). The PCR amplification products were purified by Clean Genei Kit (Bangalore Genei Pvt. Ltd., Bangalore, India), and the nucleotide sequence was determined by ABI Prism 310 Genetic analyzer using the BigDye Terminator kit (Applied Biosystems, USA). The sequence obtained was submitted to the GenBank database, and an accession number was assigned. The identity of the sequence was determined by comparing the 16S rDNA sequence with the sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and the BLAST (Basic Local Alignment Search Tool) algorithm. The sequences were aligned and the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0.

Fourier transform infrared analysis

The extracted polymer was dissolved in boiling chloroform, and the polymer film was subjected to FTIR analysis after evaporation of the chloroform (Hong et al. 1999) in order to identify the exact molecule of PHA. A standard PHB purchased from Sigma-Aldrich, USA was also subjected to FTIR analysis. The spectrum was recorded using a spectrometer (Thermo Nicolet, Avatar 370) between wave numbers of 4,000 and 400 $cm^{-1.}$

Optimization of bioprocess variables and media constituents

Various bioprocess parameters and media constituents were optimized for maximizing production of PHA under submerged fermentation using the medium described above. PHA accumulation at different levels of pH (3, 4, 5, 6, 7, 8, 9 and 10), incubation temperature (25, 30, 35,40, 45 and 50 °C), sodium chloride concentrations (1, 2, 3, 4, 5, 10, 15 and 20 %), agitation (0, 80, 100, 120 and 150 rpm), age of preinoculum (6, 9, 12,15,18, 21 and 24 h), and initial inoculum concentrations (1, 2, 3, 4, 5, 10, 15 and 20 %) were studied. Optimum incubation time was determined by incubating the production medium for a total period of 96 h and analyzing the samples at regular intervals of 6 h.

The effect of an inorganic N source on PHA production, PHA content, and CDW was studied by the addition of ammonium sulphate, ammonium nitrate, ammonium hydrogen phosphate, ammonium chloride, ammonium ferrous sulphate, sodium nitrate, and potassium nitrate individually at 0.05 % (w/v) level, replacing the inorganic N source of the PHA production medium described earlier. A medium without any inorganic N source was used as a control. The effect of inorganic salts was studied by the addition of $MgSO_4$, $CaCl_2$, $ZnSO_4$, KCl, $FeSO_4$, $MgSO_4$, $MgCl_2$, and $CoCl_2$ individually at a 0.02 % (w/v) level, replacing the inorganic salt source of the PHA production medium described earlier; a medium without any inorganic salts served as a control. The effect of various C sources was studied by the addition of glucose, glycerol, lactose, mannitol, sodium acetate, starch, and sucrose individually at a level of 1 % (w/v), replacing the C source of the PHA production medium described earlier; a medium without any C source was used as a control. PHA production (g/L), PHA content (mg/g of CDW), and CDW (g/L) were determined for all the samples of the different experiments, which were conducted in triplicate.

Results

From among the 828 isolates obtained from marine sediments identified by routine biochemical tests as *Vibrio* sp., 513 (62 %) isolates emitted pink fluorescence on exposure to UV light after the fluorescent Nile blue sulphate staining method, indicating that they were able to accumulate PHA. Hence, they were segregated as potential PHA producers. Based on the preliminary plate assay and quantitative spectrophotometric analysis of fermented broths, *Vibrio* sp. strain BTKB33, which recorded maximum PHA production of 0.21 g/L and PHA content of 193.33 mg/g of CDW, was selected as the potential strain for further optimization studies towards maximizing PHA production under a submerged fermentation process.

Characterization of *Vibrio* sp. strain BTKB33

The biochemical characteristics of the *Vibrio* sp. BTKB33 strain are as represented in Table 1. The strain produced several industrially important hydrolytic enzymes which included caseinase, amylase, lipase, gelatinase, and DNase. On comparson with partial sequences of 16S rDNA of other vibrios in the NCBI database, the *Vibrio* sp. BTKB33 strain recorded maximum identity with *V. azureus* strain VPMP45 (Accession no. 663915). The phenotypic characterization also confirmed the identification of strain BTKB33 as *V. azureus* (Yoshizawa et al. 2009). The nucleotide sequence was

Table 1 Phenotypic characterization of *Vibrio azureus* BTKB33

Trait	Result	Trait	Result
Colony colour	Green	Salicin	−
Oxidase test	+	Cellobiose	−
MOF test	Fermentative	Hemolytic test	−
Voges Proskaur's	−	Caseinase	+
Arginine	+	Amylase	+
Salt tolerance	+	Lipase	−
ONPG	−	Pectinase	−
Citrate	+	Cellulase	−
Ornithine	−	Xylanase	−
Mannitol	−	Alginase	−
Arabinose	−	DNase	+
Sucrose	−	Gelatinase	+
Glucose	+	Phosphatase	−

+=positive reaction; −=negative reaction

deposited in the GenBank database with accession number HM346671. The phylogeny based on partial 16S rDNA sequences of *Vibrio* sp. BTKB33 and related *Vibrio* sp. is shown in Fig. 1. In the tree, the strain BTKB33 is grouped with *V. azureus* strains to form a major clade, but within the clade, it grouped with *V. azureus* strain VPMP45, showing divergence from the rest.

FTIR analysis The FTIR spectrum of purified PHA extract from *Vibrio* sp. BTKB33 is represented in Fig. 2a. The FTIR spectrum of the purified PHA extracted from *Vibrio* sp. BTKB33 was compared with that of standard PHB (Sigma-Aldrich, USA) as represented in Fig. 2b and with previous reports. It was inferred that there was maximum resemblance between the spectrum of *Vibrio* sp. BTKB33 and that of polyhydroxybutyrate (PHB).

Optimization of bioprocess variables for PHA production by *Vibrio azureus* BTKB33

Effect of initial pH and temperature From the results presented in Fig. 3a, it was observed that *V. azureus* BTKB33 could accumulate PHA over a wide range of pH from 6 to 9, though maximal PHA was recorded with pH 7 (0.22 g/L). Cell dry weight measurement and PHA content at pH 7 was 1.13 g/L and 193.03 mg/g of CDW, respectively. Little or no PHA production was observed at pH levels<6 and>pH 9. Results presented in Fig 3b for the effect of temperature on PHA production in *V. azureus* BTKB33 indicated that the optimum temperature for maximal PHA production was between 30–35 °C. PHA production at 35 °C was recorded as 0.25 g/L. The cell dry weight and PHA content of the strain were also maximum at 35 °C (1.39 g/L and 177 mg/g of CDW respectively).

Effect of sodium chloride concentration and agitation With respect to the effect of NaCl concentration in the medium, the results are as documented in Fig. 3c; it was inferred that PHA concentration (0.23 g/L) and PHA content (183.45 mg/g of CDW) were increased up to 1.5 % of NaCl, while maximal cell dry weight (1.3 g/L) was noted at 2 % NaCl concentration. It was evident from the results presented in Fig. 3d that agitation is essential for enhanced PHA production in *V. azureus* BTKB33 since there was little production in stationary cultures. PHA production (0.24 g/L) and PHA content

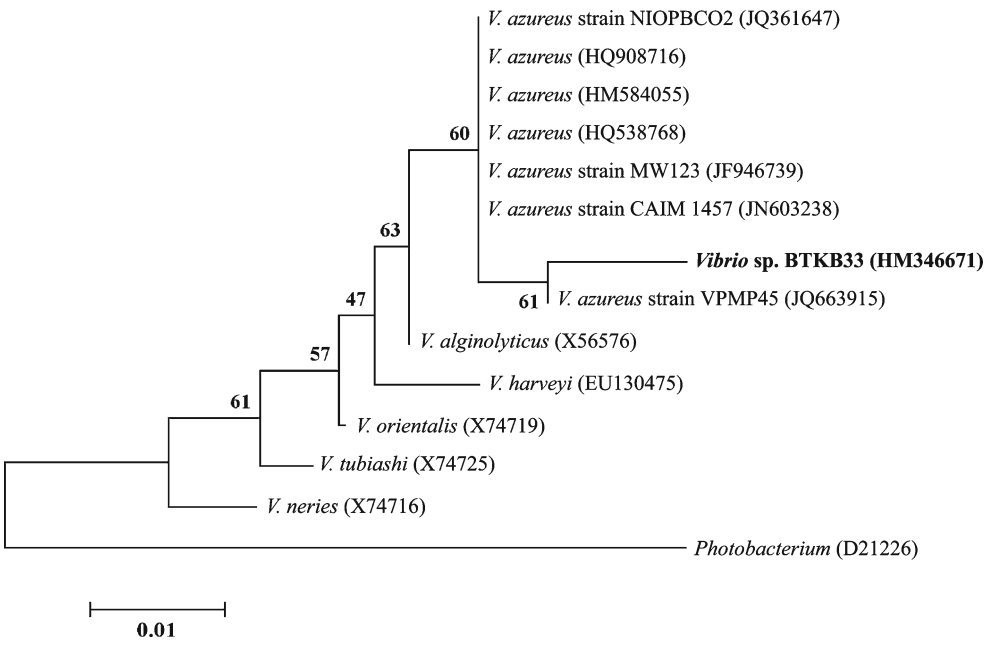

Fig. 1 Phylogenetic relationship based on partial 16S rDNA sequences of *Vibrio* sp. strain BTKB33 with related taxa. The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar=10 % sequence divergence. *Photobacterium* (D212261) was used as an out group

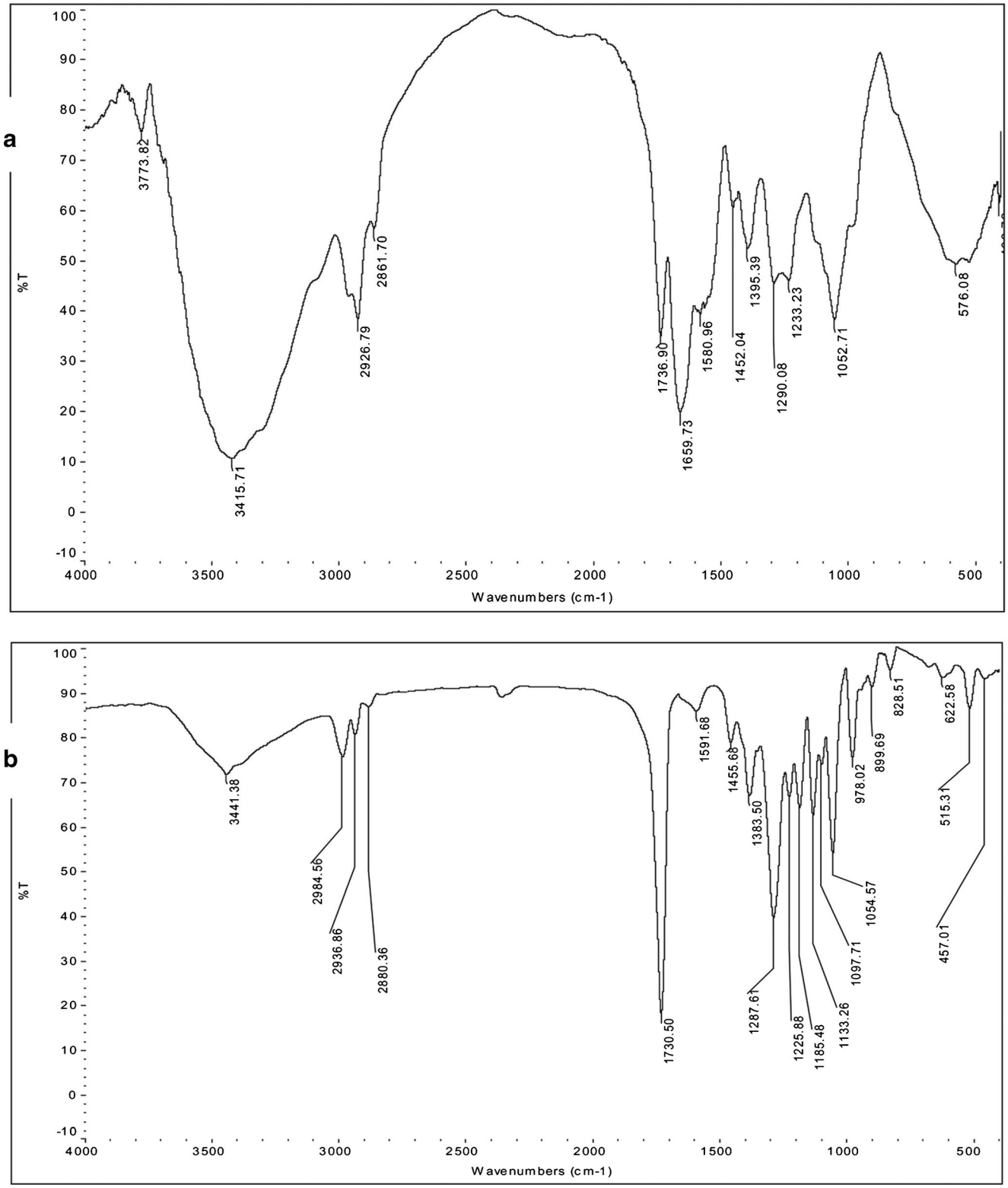

Fig. 2 **a**. FTIR spectrum of purified extracted PHAs from *Vibrio azureus* BTKB33; **b**. FTIR spectrum of standard polyhydroxybutyrate (Sigma-Aldrich, USA)

(180.87 mg/g of CDW) were highest at 120 rpm. The PHA production and PHA content were almost similar in all tested agitation rates. The cell dry weight increased with increased agitation speed, and CDW was 1.47 g/L at 150 rpm.

Effect of inoculum age and inoculum concentration Results presented in Fig. 3e showed that the PHA accumulation varied in response to variation in age of the primary inoculum. Specifically, maximal PHA production (0.31 g/L) was

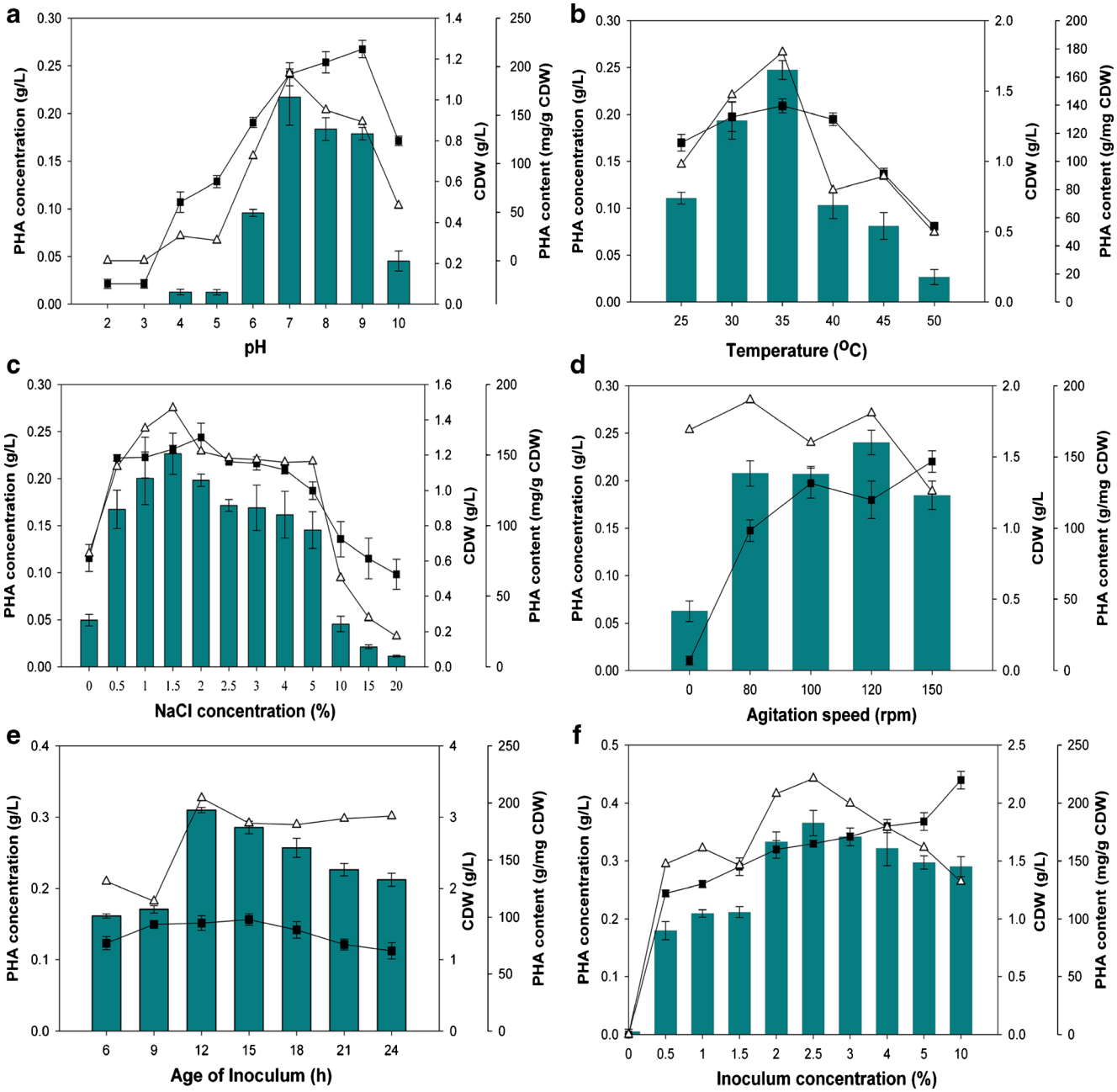

Fig. 3 Optimization of conditions for PHA accumulation, PHA content, and CDW by *Vibrio azureus* BTKB33 under submerged fermentation. **a**. Initial pH; **b**. Incubation temperature; **c**. NaCl concentrations; **d**. Agitation; **e**. Inoculum age; **f**. Inoculum concentration. The cultivation was monitored with respect to CDW (filled square), PHA content (filled triangle), and PHA concentration (filled bar). Data points are the arithmetic mean of triplicates, while error bars denote the standard deviation of the average

recorded with 12 h inoculum, and there was reduced PHA accumulation with other inoculum ages. The PHA content and CDW were also at enhanced levels when primary inoculum of 12 h was used (204.39 mg/g of CDW and 1.5 g/L). The inocula with lesser age (6 and 9 h) yielded relatively lower levels of PHA. With respect to initial concentration of inocula, it was observed that 2.5 % inoculum concentration supported maximum PHA production (0.37 g/L) and PHA content (221.45 mg/g of CDW) compared to other levels (Fig. 3f). However, the CDW increased along with increased inoculum concentration, and 10 % inoculum yielded maximum CDW for the bacterium (2.2 g/L).

Effect of incubation time Results from studies on the effect of incubation period on PHA accumulation in *V. azureus* strain BTKB33 are represented in Fig. 4a. It was noted that maximum PHA accumulation occurred at 36 h incubation (0.21 g/L), although PHA accumulation increased along with an extended incubation period up to 36 h and then gradually declined on further incubation. The PHA content was at maximum at 12 h

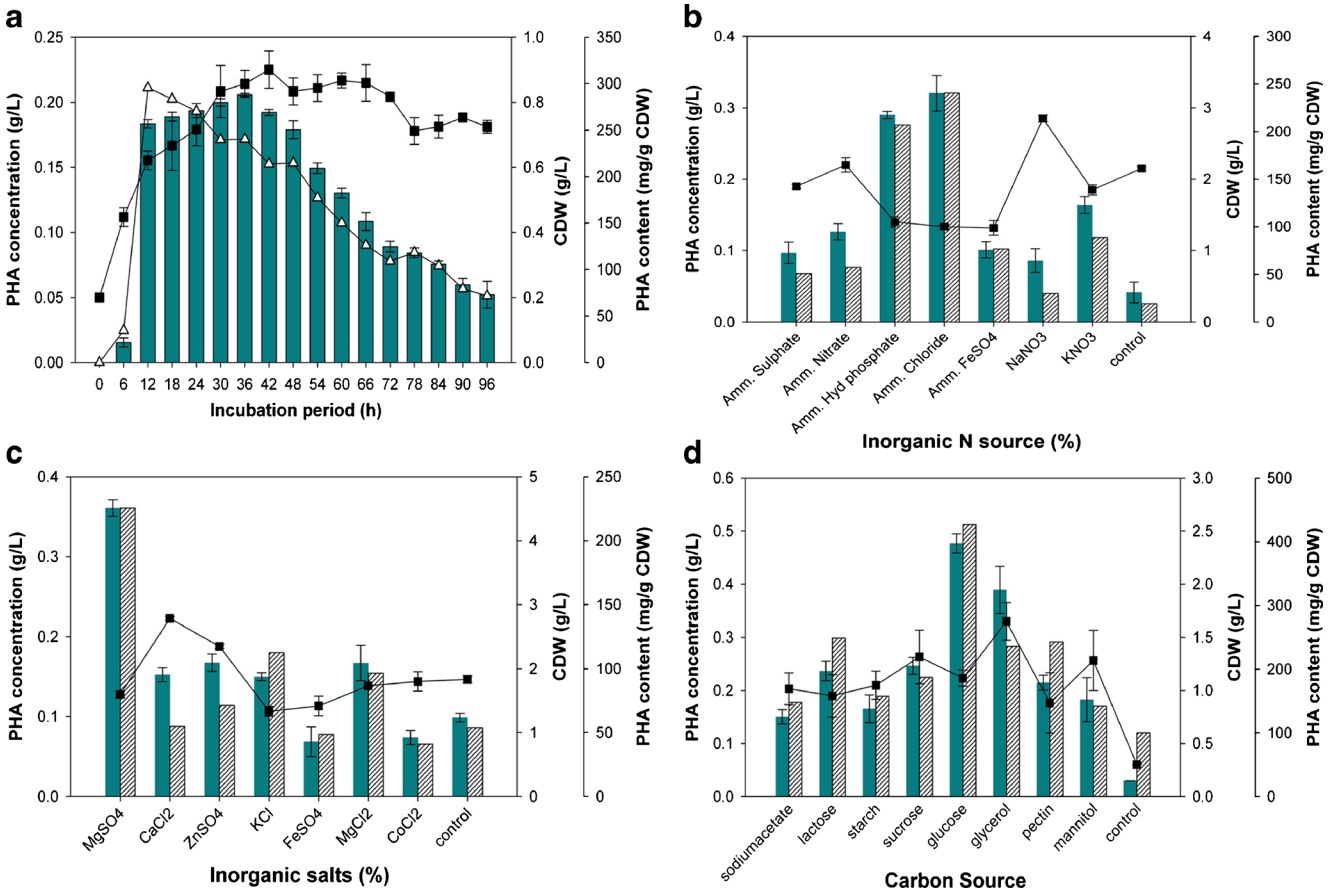

Fig. 4 Optimization of conditions for PHA accumulation, PHA content, and CDW by *Vibrio azureus* BTKB33 under submerged fermentation. **a**. Incubation period; **b**. Various inorganic N source; **c**. Various inorganic salts; **d**. Various C sources. The cultivation was monitored with respect to CDW (filled square), PHA content (filled triangle), and PHA concentration (filled bar) in Fig. 4a. The cultivation was monitored with respect to CDW (filled square), PHA content (striated bar), and PHA concentration (filled bar) in Fig. 4b, c and d. Data points are the arithmetic mean of triplicates, while error bars denote the standard deviation of the average

incubation (295.46 mg/g of CDW) but decreased drastically with further increases in incubation time. The optimum incubation time for maximum cell dry weight was 42 h (0.9 g/L).

Effect of inorganic nitrogen sources, inorganic salts, and carbon sources With respect to the impact of inorganic nitrogen sources in the production medium, it was found that there was an enhanced PHA production, PHA content, and cell dry weight compared to control in the presence of all the nitrogen sources tested (Fig. 4b). It was observed that PHA production (0.32 g/L) and PHA content (240.14 mg/g of CDW) were maximum in the presence of ammonium chloride as the inorganic nitrogen source, and CDW was highest with sodium nitrate (2.85 g/L). It was evident from the experiments that use of inorganic salts like $MgSO_4$, $MgCl_2$, $ZnSO_4$, $CaCl_2$, and KCl had a positive effect on PHA production (Fig. 4c). Maximum PHA production and PHA content (225.52 mg/g of CDW) were supported by $MgSO_4$ (0.36 g/L), while CDW was highest in the presence of $CaCl_2$ (2.8 g/L). In the case of different carbon sources tested for their effect on PHA production, it was noted that glucose as a carbon source in the minimal medium led to maximal PHA production (0.48 g/L) and PHA content (426.88 mg/g of CDW) (Fig. 4d). The CDW was higher when glycerol was used as a C source (1.38 g/L). From the "one-factor-at-a-time" optimization method, a 2.28-fold increase in PHA production was observed.

Discussion

The microorganisms of marine environments remain an untapped resource, although it has immense potential to provide not only industrially important biomaterials such as biopolymers but also several other biomaterials besides novel genes and novel enzymes. It may be noted that occurrence of PHAs in highly diverse and challenging environments like mangroves (Rawte et al. 2002), marine sediments, (Chien et al. 2007) and antarctic areas (Ayub et al. 2004) has been previously reported. In the present study, it was also noted that 62 % of the isolates of *Vibrio* sp. obtained from marine benthic environments, which are a rich source for several biomaterials, were identified as PHA producers. In fact, several species of the genus *Vibrio* have been reported to produce PHA

(Sun et al. 1994; Rawte and Mavinkurve 2004; Chien et al. 2007; Arun et al. 2009; Wei et al. 2011). Our present study indicates higher incidence of PHA accumulation in *Vibrio* sp. inhabiting marine sediments.

The 16S rDNA sequence analysis and biochemical tests helped to identify *Vibrio* sp. strain BTKB33 as *V. azureus*. There are several reports on PHA accumulation in *Vibrio* sp. like *V. harveyi* (Sun et al. 1994), *V. nereis* (Rawte and Mavinkurve 2004), *V.natriegens* (Chien et al. 2007), *V.fischeri* (Boyandin et al. 2008), *Vibrio* sp. strain MK4 (Arun et al. 2009), and *Vibrio* sp. BM-1 (Wei et al. 2011). Phylogenetic tree analysis suggests the interspecies variation of the 16S rDNA sequence of *Vibrio* sp. strain BTKB33 with the related taxa. The negative hemolytic screening test and tests for virulence genes like *ctxB*, *tox R*, and *zot* in the strain BTKB33 (data not shown) showed that the isolate is nonpathogenic. *Vibrio* sp. strain BTKB33 produced multiple hydrolytic extracellular enzymes. This ability may serve the bacterium when cheap, diverse, and complex organic material may be used as a carbon source in PHA production.

FTIR spectral analysis of extracted PHA from BTKB33 indicated the presence of functional groups of PHB. The presence of a band at 1,736.90 cm^{-1} in FTIR spectroscopy of extracted PHAs from *Vibrio* sp. BTKB33 indicated the presence of C=O grou,ps and this result confirmed the presence of PHAs in this strain (DeSmet et al. 1983; Hong et al. 1999; Kansiz et al. 2000). In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Madison and Huisman 1999). The typical spectra of methyl and methylene groups in the purified PHAs could be observed in the regions between 3,415.71 cm^{-1} and 2,861.70 cm^{-1}. The smaller bands obtained at 2,926.79 $cm^{-1,}$ which indicated the presence of a methyl group, also implied the presence of PHB in the strain BTKB33 (Hong et al. 1999). In the FTIR spectrum, stretching between 3,415.71 and 1,452.04 cm^{-1} indicated C-H stretching and bending (Desmet et al. 1983), and the band at 1,395.39 cm^{-1} indicated the presence of a methyl group (DeSmet et al. 1983; Castillo et al. 1986). The presence of a spectral band at 1,290.08 cm^{-1} is characteristic for PHB (Hong et al. 1999). The bands at 1,233.23 and 1,052.71 cm^{-1} may be contributed by the presence of polyhydroxybutyrate (PHB) within them (Kansiz et al. 2000). The FTIR spectra of the extracted PHAs (Fig. 2a) from *Vibrio* sp. BTKB33 was compared with the FTIR spectrum of standard PHB (Fig. 2b). Based on the comparison with standard PHB and from previous reports (DeSmet et al. 1983; Castillo et al. 1986; Hong et al. 1999; Kansiz et al. 2000), it was inferred that the FTIR spectra of PHA extracted from *Vibrio* sp. BTKB33 was polyhydroxybutyrate (PHB). In their studies on PHA accumulation in vibrios from marine sediments, Chien et al. (2007) reported that *Vibrio* sp. harbored only PHB and no other type of PHA, and pointed out that the diversity of PHAs produced in marine environments may be not as versatile as found in other environments.

In this study, PHA accumulation was dramatically reduced at acidic pH, while pH≥7 up to pH 9 supported PHA accumulation. A pH of 7 was optimal for PHA production, and similar observations have been previously reported in certain *Vibrio* sp. (Rawte and Mavinkurve 2004; Arun et al. 2009), clearly indicating the influence of pH on PHA accumulation. Similar results have also been reported in *Haloferax mediterranei* (Lillo and Valera 1990), *Alcaligenes latus* (Palleroni and Palleroni 1978), and *Bacillus sphaericus* NCIM 5149 (Ramadas et al. 2009). The effect of incubation temperature on PHA production is known to vary among genera and is a critical control parameter. In the present study 37 °C was found as optimal for PHA production (0.36 g/L) in *V. azureus* BTKB33. Whereas the optimal temperature for PHA production in *Cupriavidus taiwanensis*was 30 °C (Wei et al. 2011), a higher temperature of 45 °C was favorable for PHA production in *Haloferax mediterranei* (Lillo and Valera 1990).

Agitation is clearly a requirement for PHA production in *V. azureus* BTKB33 since there was low PHA production in stationary cultures. In the present study PHA production was observed to be increased with increases in agitation speed. This observation may be attributed to increases in oxygen transfer and mass transfer rates due to increased surface area of contact among media components. However, the decrease in PHA production observed at 150 rpm may be due to shearing stress on the organism due to greater agitation. There are reports that application of stress during fermentation induces PHA accumulation (Ayub et al. 2004; Obruca et al. 2010). The ability of *Rhizobium* DDSS-69 for PHB accumulation under NaCl stress has been pointed out (Natarajan et al. 1995). PHA production by *V. azureus* BTKB33 was observed to be at maximum at 1.5 % NaCl. This observation can be attributed to the marine origin of the isolate. These results were in agreement with PHA production in *Vibrio* sp. BM-1 reported earlier (Wei et al. 2011), where PHA production increased with NaCl concentration up to 1.8 %. In fact NaCl in the medium has enhanced PHA production in *Vibrio* sp. (Chien et al. 2007; Arun et al. 2009), and a maximum was recorded at 30 % NaCl (Arun et al. 2009). The present study confirmed the crucial role of NaCl concentration in the PHA accumulation process in vibrios. This finding reflects the necessity of controlling the salinity of the medium during cultivation within a proper range to prevent high osmotic stress and its effect on polymer production (Wei et al. 2011).

PHA production in the current study varied widely with differences in preinoculum age, and this obviously is also an important factor to be considered in production studies while preparing the seed inoculum samples for PHA production. 12-h-old preinoculum gave maximum PHA accumulation in the present study. Mid-log cultures could probably efficiently employ the PHA synthetic machinery, mediated by PHA

synthase genes, when compared to other stages of culture. In a similar fashion in the case of *Bacillus* sp., 16-h-old preinoculum was optimal (Ramadas et al. 2009). Santhanam and Sasidharan (2010) pointed out that optimization of the initial microbial load is paramount to PHA production studies. PHA production by *V. azureus* BTKB33 increased with initial inoculum concentration up to 2.5 %, while concentrations above 2.5 % did not result in further increases. One reason for this effect may be that higher volumes of bacterial cells rapidly utilized the already accumulated intracellular PHA granules as a carbon and energy source (Yamane et al. 1996; Santhanam and Sasidharan 2010). In organisms like *Alcaligenes eutrophus*, *Alcaligenes latus,* and *Pseudomonas oleovorans*, PHA accumulation was low at high concentration (5 %) of inoculum (Yamane et al. 1996). Similar observations were reported in *Bacillus sphaericus* NCIM 5149 (Ramadas et al. 2009). In *Bacillus* sp. CFR 256, 1 % initial inoculum was reportedly favorable for PHA production (Vijayendra et al. 2007), and for certain *Bacillus* sp. a higher 10 % inoculum concentration was desirable (Valappil et al. 2007).

Although all the inorganic nitrogen sources enhanced production by *V. azureus* BTKB33, ammonium chloride optimized production. In fact, the suitability of ammonium chloride as an inorganic nitrogen source has been reported earlier for *Alcaligenes eutrophus* (Koutinas et al. 2007), *Rhodopseudomonas palustris* KU003 (Ramachander et al. 2010), and *Sinorhizobium fredii* (Liangqi et al. 2006), while ammonium sulphate was the preferred inorganic nitrogen source for PHA accumulation in certain *Vibrio* sp. (Arun et al. 2009) and in *Pseudomonas* sp. LDC-5 (Saranya and Shenbagarathai 2010).

Inorganic salts play an important role in PHA accumulation (Lee et al. 2007). Magnesium sulfate followed by $MgCl_2$, $ZnSO_4$, and $CaCl_2$ supported PHA production by the *V. azureus* BTKB33 strain. Inorganic salts like $MgSO_4$ and $MgCl_2$ affect monomer composition in PHAs, wherein concentration of Mg^{2+} affected the biosynthesis of P (3HB-co-4HB) in *Delftia acidovorans* by impacting glucose uptake from the culture medium (Lee et al. 2007). A positive influence of $CaCl_2$ supplementation in PHA production was also reported earlier (Saranya and Shenbagarathai 2010).

Glucose was found to be an ideal carbon source for maximizing PHA accumulation in *V. azureus* BTKB33. Previous studies in *Vibrio* sp. (Rawte et al. 2002; Arun et al. 2009), *Alcaligens eutrophus* (Santhanam and Sasidharan 2010), and *Alcaligens latus* (Santhanam and Sasidharan 2010) have also reported high PHA concentration with glucose as a carbon source. Additionally, in this study, glycerol as a C source also supported PHA production and has been reported for PHA production in certain other *Vibrio* sp. (Chien et al. 2007; Wei et al. 2011). These observations suggest the possible utilization of cheap industrial byproducts like glycerol in industrial production of PHAs from *V. azureus* BTKB33 in the future. Of course in the present study, all the substrates used as the sole carbon source were found to enhance PHA production, although glucose supported maximal PHA production.

Incubation time is an important factor that influences enhanced accumulation of PHA in bacteria and consequent industrial production of PHAs. In the present study, PHA production was observed to commence after 12 h of incubation and peaked at 36 h, although with a decline upon further incubation. This may be due to utilization of accumulated PHAs by the organism (Nam and Ryu 1985; Benoit et al. 1990). PHAs may be utilized as a carbon source for survival, causing a reduction of PHA accumulation at higher incubation periods (Yamane et al. 1996; Santhanam and Sasidharan 2010). Reports indicate that PHA accumulation in marine vibrios begins at the late log phase, reaching maximum at the stationary phase of growth (Rawte and Mavinkurve 2004). However, in *Rhodopseudomonas palustris* KU003 (Ramachander et al. 2010), the maximum accumulation of PHAs was during the exponential phase. *Ralstonia eutropha* accumulated PHB at the stationary phase (Madison and Huisman 1999). In the studies of Ramadas et al. (2009) on PHA accumulation in *Bacillus sphaericus* NCIM 5149, incubation up to 28 h favored PHA accumulation, which declined thereafter. In the present study, however, *V. azureus* BTKB33 recorded maximal PHAs during the mid-log phase, unlike other vibrios. The fact that the organism can accumulate maximal PHA could have a positive impact when utilizing the organism for industrial production of PHAs, particularly in achieving economic production, since incubation time directly influences production cost.

Conclusion

Vibrio azureus BTKB33 with the potential for accumulating PHB was isolated from marine benthic environments. The various bioprocess parameters and media constituents were optimized for enhanced PHB production under submerged fermentation conditions. The strain was found to elaborate several industrially important exoenzymes in addition to accumulating PHB internally. Based on the results, it is concluded that the strain can be further manipulated in the future for possible utilization of diverse substrates as a C source for industrial production of PHB, which has biomedical applications besides use in bioplastics. It is also speculated that the marine benthic environment could serve as a mine for recovering several PHA-accumulating strains, which may include other species besides *Vibrio* sp.

Acknowledgement This study was supported by project grants from the Department of Ocean Development, Government of India (No. DOD/11 – MRDF/1/29/06) and the Centre for Marine Living Resources & Ecology, Ministry of Earth Sciences, Government of India (MOES/10-MLR/2/2007) given to Dr. Sarita G. Bhat, Dept. of Biotechnology, Cochin University of Science and Technology, Kochi, India.

References

Anuradha P, Vijayalakshmi K, Prasanna ND, Sridevi K (2007) Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields. Curr Sci 92:1283–1286

Arun A, Arthi R, Shanmugabalaji V, Eyini M (2009) Microbial production of poly-beta-hydroxybutyrate by marine microbes isolated from various marine environments. Bioresour Technol 100:2320–2323

Ayub ND, Pettinari MJ, Ruiz JA, Lopez NI (2004) A polyhydroxybutyrate-producing *Pseudomonas* sp. isolated from Antarctic environments with high stress resistance. Curr Microbiol 49:170–174

Benoit TG, Wilson GR, Baygh CL (1990) Fermentation during growth and sporulation of *Bacillus thuringiensis* HD-1. Lett Appl Microbiol 10:15–18

Boyandin AN, Kalacheva GS, Medvedeva S, Rodicheva EK, Volova TG (2008) Luminous bacteria as producers of polyhydroxyalkanoates. Macromol Symp 269:17–22

Buchanan R, Gibbons N (1974) Bergey's manual of determinative bacteriology. Baltimore, Maryland

Castillo RF, Valera FR, Ramos JG, Berraquero FR (1986) Accumulation of poly (hydroxybutyrate) by halobacteria. Appl Environ Microbiol 51:214–216

Chandrasekaran M, Lakshmanaperumalsamy P, Chandramohan D (1984) Occurrence of vibrios during fish spoilage. Curr Sci 53(1):31–32

Chen GQ, Wu Q (2005) Microbial production and applications of chiral hydroxyalkanoates. Appl Microbiol Biotechnol 67:592–599

Chen G, Wu Q, Zhao K, Yu P (2000) Functional polyhydroxyalkanoates synthesized by microorganisms. Chin J Polym Sci 18:389–396

Chien CC, Chen CC, Choi MH, Kung SS, Wei YH (2007) Production of poly-betahydroxybutyrate (PHB) by *Vibrio* spp. isolated from marine environment. J Biotechnol 132:259–263

DeSmet MJ, Eggin G, Witholt KB, Kingma J, Wynberg H (1983) Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J Bacteriol 154:870–878

Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Vanderleyden J, Dutto JP (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Aust J Plant Physiol 28:871–879

Dong Z, Sun X (2000) A new method of recovering polyhydroxyalkanoate from *Azotobacter chroococcum*. Chin Sci Bull 45:252–255

Furniss AL, Lee JV, Donovan TJ (1978) The Vibrios, Public Health Laboratory Service Monograph Series no 11. London

Glazer AN, Nikaido H (1994) Microbial Biotechnology - Fundamentals of Applied Microbiology. Freeman WH, New York

Hankin L, Zucker M, Sanda DC (1971) Improved solid medium for the detection and enumeration of pectolytic bacteria. Appl Microbiol 22:205–209

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

Kansiz M, Billman JH, Mcnaughton D (2000) Quantitative determination of the biodegradable polymer poly (hydroxybutyrate) in a recombinant *Escherichia coli* strain by use of mid-infrared spectroscopy and multivariative statistics. Appl Environ Microbiol 31:3415–3420

Kasing A, Jong BC, Salleh MA (2000) Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste. J Gen Appl Microbiol 46:263–267

Kazanas N (1968) Proteolytic activity of microorganisms Isolated from freshwater fish. Appl Microbiol 16:128–132

Keshavarz T, Roy I (2010) Polyhydroxyalkanoates: bioplastics with a green agenda. Curr Opin Microbiol 13:321–326

Khanna S, Srivastava AK (2005) Recent advances in microbial polyhydroxyalkanoates. Proc Biochem 40:607–619

Koutinas AA, Xu Y, Wang R, Webb C (2007) Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. Enzym Microb Technol 40:1035–1044

Law JH, Slepecky RA (1961) Assay of poly-β-hydroxybutyric acid. J Bacteriol 82:33–36

Lee WH, Azizan MNM, Sudesh K (2007) Magnesium affects poly(3-hydroxybutyrate-co-4-hydroxybutyrate) content and composition by affecting glucose uptake in *Delftia acidovorans*. Malays J Microbiol 3:31–34

Liangqi Z, Jingfan X, Tao F, Haibin W (2006) Synthesis of poly (3 hydroxybutyrate co-3- hydroxyoctanoate) by a *Sinorhizobium fredii* strain. Lett Appl Microbiol 4:344–349

Lillo JG, Valera FR (1990) Effects of culture conditions on poly(3-hydroxybutyric acid) production by *Haloferax mediterranei*. Appl Environ Microbiol 56:2517–2521

López NI, Floccari ME, Steinbüchel A, García AF, Méndez BS (1995) Effect of poly(3-hydroxybutyrate) content on the starvation-survival of bacteria in natural waters. FEMS Microb Ecol 16:95–101

López-Cortés A, Lanz-Landázuri A, García-Maldonado JQ (2008) Screening and Isolation of PHB-Producing Bacteria in a Polluted Marine Microbial Mat. Microb Ecol 56:112–120

Luengo JM, García B, Sandoval A, Naharro G, Olivera ER (2003) Bioplastics from microorganisms. Curr Opin Microbiol 6:251–260

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Nam DH, Ryu DDY (1985) Relationship between butirosin biosynthesis and sporulation in *Bacillus circulans*. Antimicrob Agents Chemother 27:789–801

Natarajan K, Kishore L, Babu CR (1995) Sodium chloride stress results in increased poly-beta-hydroxybutyrate production in *Rhizobium* DDSS-69. Microbios 82:95–107

Nautiyal SC (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Obruca S, Marova I, Stankova M, Mravcova L, Svoboda Z (2010) Effect of ethanol and hydrogen peroxide on poly(3-hydroxybutyrate) biosynthetic pathway in *Cupriavidus necator* H16. World J Microbiol Biotechnol 26:1261–1267

Palleroni NJ, Palleroni AV (1978) *Alcaligenes latus*, a new species of hydrogen-utilizing bacteria. Int J Syst Bacteriol 28:416–424

Ramachander M, Girisham S, Reddy SM (2010) Production of PHB by *Rhodopseudomonas palustris* KU003 under nitrogen limitation. Int J Appl Biol Pharm Technol 1:676–678

Ramadas NV, Singh SK, Soccol CR, Pandey A (2009) Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. Braz Arch Biol Technol 52:17–23

Rawte T, Mavinkurve S (2004) Factors influencing polyhydroxyalkanoate accumulation in marine bacteria. Indian J Mar Sci 33:181–186

Rawte T, Padte M, Mavinkurve S (2002) Incidence of marine and mangrove bacteria accumulating polyhydroxyalkanoates on the mid-west coast of India. World J Microbiol Biotechnol 18:655–659

Reddy GSN, Aggarwal RK, Matsumoto GI, Shivaji S (2000) *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. Int J Syst Evol Microbiol 50:1553–1561

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Fritsch EF, Maniatis T (2000) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NewYork

Santhanam A, Sasidharan S (2010) Microbial production of polyhydroxyalkanotes from *Alcaligens* spp. and *Pseudomonas oleovorans using* different carbon sources. Afr J Biotechnol 9:3144–3150

Saranya V, Shenbagarathai R (2010) Effect of Nitrogen and Calcium Sources on Growth and Production of PHA of *Pseudomonas* sp. LDC-5 and its Mutant. Curr Res J Biol Sci 2:164–167

Simon F, Martin DP (2002) Applications of PHAs in medicine and pharmacy. In: Doi Y, Steinbuchel A (eds) Biopolymers, 4th edn. Wiley-VCH, Weinham, pp 91–103

Spiekermann P, Rehm BHA, Kalscheuer R, Baumeister D, Steinbüchel A (1999) A sensitive, viable-colony staining method using nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 171:73–80

Steinbuchel A (2005) Non-biodegradable biopolymers from renewable resources: perspectives and impacts. Curr Opin Biotechnol 16:607–613

Sun W, Cao JG, Teng K, Meighen EA (1994) Biosynthesis of poly-3-hydroxybutyrate in the luminescent bacterium, *Vibrio harveyi* and regulation by the lux autoinducer N-(3-hydroxybutanoyl) homoserine lactone. J Biol Chem 269:20785–20790

Valappil SP, Misra SK, Boccaccini AR, Keshavarz T, Bucke C, Roy I (2007) Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised *Bacillus cereus* SPV. J Biotechnol 132:251–258

Vijayendra SVN, Rastogi NK, Shamala TR, Anil Kumar PK, Kshama L, Joshi GJ (2007) Optimization of polyhydroxybutyrate production by *Bacillus* sp. CFR 256 with corn steep liquor as a nitrogen source. Indian J Microbiol 47:170–175

Wei YH, Chen WC, Wu HS, Janarthanan OM (2011) Biodegradable and biocompatible biomaterial, polyhydroxybutyrate, produced by an indigenous *Vibrio* sp. BM-1 isolated from marine environment. Mar Drugs 9:615–624

Weiner RM (1997) Biopolymers from marine prokaryotes. Trends Biotechnol 15(10):390–394

Yamane T, Chen XF, Ueda S (1996) Polyhydroxyalkanoate synthesis from alcohols during the growth of *Paracoccus denitrificans*. FEMS Microbiol Lett 135:207–211

Yoshizawa S, Wada M, Kita-Tsukamoto K, Ikemoto E, Yokota A, Kogure K (2009) *Vibrio azureus* sp. nov., a luminous marine bacterium isolated from seawater. Int J Syst Evol Microbiol 59:1645–1649

Zierdt CH, Golde DW (1970) Deoxyribonuclease-Positive *Staphylococcus epidermidis* strains. Appl Microbiol 20:54–57