Isolation and characterization of polyhydroxyalkanoates accumulating *Vibrio* sp. strain BTTC26 from marine sediments and its production kinetics

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Sediment samples collected from various marine environments were screened for polyhydroxyalkanoates (PHAs) accumulating *Vibrio* sp. using Nile Blue A staining plate assay and 92 of the 148 *Vibrio* sp. showed positive result. High incidence (62%) of PHA accumulating vibrios were observed in this study. *Vibrio* sp. strain BTTC26 showing maximum PHA accumulation during preliminary screening by plate assay methods and spectrophotometric analysis was selected for further studies. Based on the phenotypic characterization studies and partial 16S rDNA sequence analysis, the strain BTTC26 was identified as *V. azoviridis* (99% identity). The strain BTTC26 showed multiple hydrolytic enzymes production like amylase, lipase, caseinase, gelatinase and DNase. In addition, it has the multiple antibiotic resistance (MAR) index of 0.3. The intracellular accumulation of PHAs by BTTC26 was optimized at pH - 7, with glucose as carbon source, 36h incubation time, 3% initial inoculum concentration, 37°C incubation temperature and 10% NaCl concentration. FTIR analysis showed that polyhydroxybutyrates (PHB), the smallest reported PHAs was accumulated by strain BTTC26, having wide range of industrial and medical applications. In addition, several strains of fast-growing *Vibrio* sp. accumulating intracellular PHAs were also isolated during this study.

**Keywords:** Polyhydroxyalkanoates (PHAs); *Vibrio* sp.; Marine sediments; Polyhydroxybutyrates (PHB); FTIR analysis; Optimisation studies.

**Introduction**

Plastics have successfully replaced other substances extensively in domestic, medical and industrial applications. Nearly 150 million tons of non-biodegradable plastics produced every year that cause severe pollution problems. The increasing cost and the tremendous damaging environmental impact of plastics demands an alternative, which has to be eco-friendly. Biodegradable plastics can help to overcome these associated problems to some extent. Among the candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs) have drawn much attention due to their complete biodegradability and the similarity of their material properties to conventional plastics. Bacteria under unbalanced growth conditions of carbon substrate in excess of other nutrients, like nitrogen, sulfur, phosphorus or oxygen accumulate polyesters like PHAs, which aid their survival in changing environments like marine benthic regions. Over 250 different bacteria, including gram-negative and gram-positive species, have been reported to accumulate various PHAs. Despite the common practice of exploiting the diversity of bacteria in the environment for the industrial production of novel compounds, very few reports explored the potential of industrial production of PHAs by bacteria. The difference in the physical and chemical characteristics of PHAs is attributed to their varied monomer content, which in turn is influenced by the type of microorganisms, media ingredients, fermentation conditions, modes of fermentation and the recovery processes. Hence, there is a need for screening large number of organisms that accumulate PHA with a combination of monomers, with high yielding of the desirable trait.

In this study a potential PHA accumulating *Vibrio* sp. accumulating PHB was isolated from highly diverse marine benthic environment. The genotypic and phenotypic characterization of the isolate was carried out and the conditions for enhanced PHA production were optimized. Apart from this, several fast growing *Vibrio* sp. having PHA accumulation ability was also isolated from marine benthic environments.
Materials and methods

Sample collection and isolation

Sediment samples were collected from salt pans and sandy beaches of Tutucorin (Latitude 8.81°N and Longitude 78.14°E) and Thirichendoor (Latitude 8.49°N, Longitude 78.13°E) coasts along the eastern coastal regions (Tamil Nadu) of India, using a corer of 2 cm diameter at a depth of 30 cm. For the present study a total of four sediment sampling were conducted. Sediment samples were serially diluted using physiological saline. 50µl 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 hours. The isolates were identified as *Vibrio* sp. based on their morphological and biochemical characteristics outlined in Bergey's Manual of Systematic Bacteriology12.

Preliminary screening for PHA accumulation

Poly(hydroxyalkanoates) accumulation was identified by spot inoculation of the isolates onto complex nitrogen limiting agar plates13 containing Nile Blue A (1µg/ml) and incubated at 37°C for 3-4 days. Colonies were directly examined for fluorescence by exposing to ultraviolet light in order to detect the accumulation of lipid storage compounds including PHAs14.

Secondary screening by spectrophotometric assay

The PHA production medium used was complex medium with glycerol (1%) as carbon source13. Nutrient medium having 1% NaCl was used for seed medium. Complex medium inoculated with sixteen hours old culture (1% v/v inoculum at O.D.600 = 1.00) was incubated on an environmental shaker at 150 rpm at 30°C for 48 h. The cells were harvested by centrifugation at 8000 rpm for 15 min. at 4°C. The polymers were extracted15 and the quantitative analysis of PHA was evaluated spectrophotometrically16 using Shimadzu UV-visible spectrophotometer (Japan).

Phenotypic characterization of isolates

Biochemical tests were performed using the Hi-*Vibrio* identification system (Himedia, Mumbai, India) involving 12 biochemical tests (Voges Proskauer's test, arginine dihydrolase test, 1% salt tolerance test, ONPG test, citrate utilization test, ornithine, mannitol, arabinose, sucrose, glucose, salicin and cellobiose utilization tests). The results of the biochemical tests were used for identification of the isolates17.

Exoenzyme production - The screening for extracellular enzymes was done by plate assays for amylase18, caseinase19, lipase18, cellulase20, pectinase21, xylanase22, alginate18, DNase23, gelatinase18 and phosphatase24. The antibiotic susceptibility tests were done using the Kirby-Bauer disc diffusion method25. The antibiotics discs used were - Vancomycin (V, 30mcg/disc), Ampicillin (Am, 10mcg/ disc), Co-Trimoxazole (Co, 25mcg/disc), Carbenicillin (Cb, 100mcg/disc), Tetracycline (T, 30mcg/disc), Trimethoprim (Tr, 5mcg/disc), Azithromycin (At, 30mcg/disc), Ciprofloxacin (Cf, 5mcg/disc), Rifampicin (R, 5mcg/disc) and Gentamicin (G, 10mcg/disc). The results were interpreted as resistant, intermediate or sensitive based on the size of the inhibition zones around each disc as per instructions provided by the manufacturer (Himedia, Mumbai, India)26.

Genotypic characterization

Genomic DNA was isolated and purified27; a portion of the 16S rDNA was amplified using a primer pair for 16S rDNA28-31. The products after PCR amplification were purified by gene clean kit (Bangalore Genei, Bangalore, India) and the nucleotide sequence was determined by the ABI Prism 310 Genetic analyzer using the big dye terminator kit (Applied Biosystems, USA). The identity of the sequences were determined by comparing the 16S rDNA sequence with the sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool) algorithm12. The sequences were aligned and the phylogenetic tree was constructed using the neighbor-joining method13 using MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0.

Optimization studies

Parameters like initial pH, carbon sources, incubation time, initial inoculum level, temperature and NaCl conc. were optimized by shake flask culture method for PHA accumulation. The medium used was described earlier. The PHA accumulation at different pH values (3, 4, 5, 6, 7, 8, 9 & 10), carbon sources (glucose, glycerol, lactose, mannitol, sodium acetate, starch & sucrose at a level of 10 g/L), different time intervals (0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90 & 96h), different initial inoculum concentrations (1, 2, 3, 4, 5, 10, 15 & 20%), temperatures (4, 30, 37 & 45°C) and sodium chloride concentrations (1, 2, 3, 4, 5, 10, 15 & 20%) were studied.
Fourier Transform Infra Red Analysis (FTIR)

The extracted PHAs were dissolved in boiling chloroform and after evaporation of chloroform, the polymer films were subjected to FTIR analysis. FTIR spectra were recorded using spectrometer (Thermo Nicolet, Avatar 370) between wave number of 4000 and 400 cm$^{-1}$.

Results and Discussion

The marine environment provides an untapped resource for novel bacteria and possibly the polymers they produce. Occurrences of PHAs from highly diverse and challenging environments like mangroves, marine sediments and antartic areas have been reported. In the present study, a total of 148 strains were isolated from the salt pans and sandy beach benthic environment and the isolates were segregated as Vibrio sp. employing routine biochemical tests. The preliminary screening by fluorescent Nile Blue sulphate staining method showed 92 of 148 isolates (62%) to be PHA accumulators (Fig. 1). Incidence of PHA accumulation in Vibrio sp. have already been reported previously and at 62%, incidence of PHA accumulation in Vibrio sp. was higher in the present study. The outcome of the present study and previous reports clearly indicate marine benthic environments to be a good source of PHA accumulators.

From the preliminary screening employing plate assay and spectrophotometric assay, four strains were selected accumulating more than 0.16 g/L of PHAs i.e. Vibrio sp. strain BTTC27, BTTC26, BTTR39 and BTTC10 accumulated 0.17, 0.20, 0.16 and 0.16 g/L respectively of PHA and were further identified. The highest PHA accumulator among these isolates was Vibrio sp. strain BTTC26 and was selected for production studies.

Phenotypic characterization of Vibrio sp. BTTC26

The results of phenotypic characterization studies of Vibrio sp. strain BTTC26 are represented in Table 1. Strain BTTC26 also produced biotechnologically important hydrolytic exoenzymes like caseinase, amylase, lipase, gelatinase and DNase. The presence of these extracellular hydrolytic enzymes points towards its capability in utilizing diverse and complex organic material as C-source in industrial production of PHAs. The isolate showed MAR index of 0.3 showing resistance towards vancomycin, rifampicin and ampicillin. Higher MAR indices are indicative of the stressful environments inhabited by these microbes in the marine environments.

Genotypic characterization studies

The 16SrDNA sequence analysis and biochemical tests helped to identify strain BTTC26. On comparing the partial sequences of 16S rDNA with those in NCBI database, the strain BTTC26 showed maximum identity with V. azureus (99% identity). The nucleotide sequence has been deposited in the GenBank database under accession number HM346664. The phylogeny based on
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Partial 16S rDNA sequence of Vibrio sp. BTTC26 and related Vibrio sp. is shown in Fig. 2. This is the first report of PHA accumulation in V. azureus. Strains BTTC27, BTTR39 and BTTC10 were also characterised genotypically and were identified as V. alginolyticus. The GenBank accession numbers of these strains are GU904006, GU904005 and HM030802.

**Effect of cultural conditions on PHA accumulation in Vibrio sp. strain BTTC26**

Physical and chemical parameters – different pH, various C sources, incubation time, initial inoculum concentrations, temperature and different NaCl concentrations were tested in order to increase the yield of PHA accumulation in Vibrio sp. strain BTTC26. Here PHAs accumulation were dramatically reduced at acidic pH, while alkaline pH supported PHA accumulation. Maximum PHAs production (0.20 g/L) was observed at an initial pH of 7 (Fig. 3) and similar observations were reported earlier in the studies of PHA accumulation in Vibrio sp.\(^{37,38}\). These results showed that PHA production is highly influenced by pH of cultivation medium and similar results were reported in Cupriavidus taiwanensis\(^{39}\) and Haloferax mediterranei\(^{40}\). Glucose is found to be ideal carbon source for PHA accumulation in Vibrio sp. BTTC26 from the optimization studies of different C-sources and is represented in Fig. 4. PHA accumulation was found to be maximum (0.26 g/L) with glucose as C source followed by glycerol (0.18 g/L). Optimization of suitable carbon source is very important for high production of PHA\(^{41}\). Glucose as an ideal C
source in PHA accumulation in *Vibrio* sp. has already been reported in previous optimization studies\(^{37,38}\). *Alcaligenes eutrophus* and *Alcaligenes latus* were also reported to accumulate PHA in high concentration in medium with glucose as carbon source. But in some cases like in *P. oleovorans* ATCC 29347 higher yields of PHA were obtained with n-alkanes as C source for PHA production\(^{41}\). The PHA accumulation in the strain BTTC26 was found to be 0.18 g/L while using glycerol as C source. This result point towards the usage of cheap industrial byproduct like glycerol in industrial production of PHAs from *Vibrio* sp. strain BTTC26 in future. Glycerol was reported as an ideal C source for PHA production in vibrios isolated from sediments\(^{36}\).

Maximum accumulation of PHAs in *Vibrio* sp. strain BTTC26 was at 36 h incubation (0.27 g/L) and is represented in Fig. 5. It was observed that PHA accumulation decreased with increase in incubation time. After 36h increase, there is a sudden decrease in accumulation of PHA in strain BTTC26. PHA accumulated by organisms are utilized as a carbon source for their survival and can thus cause lowering of PHA accumulation at longer incubation periods\(^{41,42}\). The influence of different inoculum concentrations on PHA accumulation is represented in Fig. 6. The yield was found to be highest at 3% initial inoculum concentration (0.28 g/L), beyond 3%, there is a gradual decrease in PHA accumulation. One of the reasons for this affect may be that higher inoculum of bacterial cells rapidly utilized the already accumulated intra-cellular PHA granules as carbon and energy source\(^{41,42}\). This may be the reason for strain BTTC26 for not accumulating more PHAs with increase in initial inoculum concentration beyond 3%. In some organisms like *Alcaligenes eutrophus*, *Alcaligenes latus* and *Pseudomonas oleovorans*, PHAs accumulation was lower at high concentration (5%) of inoculum\(^{42}\). In some cases like in *Bacillus* sp. higher initial inoculum concentration (10%) was required to produce PHA\(^{43}\).

The influence of temperature of incubation on PHA accumulation is represented in Fig. 7. Maximum PHAs production was found to be at incubation temperature of 37°C (0.36 g/L). It was found that PHA production decreased drastically at higher temperatures. The effect of incubation temperature on PHA production varies from one genus to another. The optimum temperature for PHA production in *Cupriavidus taiwanensis*\(^{39}\) was found to be 30°C, while high temperature of 45°C favoured PHA production in *Haloferax mediterranei*\(^{40}\). The effect of NaCl concentration on PHA accumulation is represented

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**Fig. 5**—Optimisation of PHA production (g/L) of *Vibrio* sp. BTTC26 at different incubation time periods (h). The values are mean of three replicates ± standard deviations

**Fig. 6**—Optimisation of PHA production (g/L) of *Vibrio* sp. BTTC26 at different inoculum concentrations (%). The values are mean of three replicates ± standard deviations

**Fig. 7**—Optimisation of PHA production (g/L) of *Vibrio* sp. BTTC26 at different incubation temperatures (in degree celsius). The values are mean of three replicates ± standard deviations
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The present study clearly showed that higher NaCl concentration favored PHA accumulation in *Vibrio* sp. strain BTTC26. In medium having 10% NaCl, maximum PHA accumulation (0.4 g/L) was observed. Since the bacterium was isolated from marine environments, NaCl concentration may play an important role in its PHA production. It was already reported in the previous studies that PHA production in *Vibrio* sp. is enhanced by an increase in the concentration of NaCl in medium$^{36,37}$. In certain other studies, PHA production in *Vibrio* sp. was reported to be maximum at 30% NaCl concentration$^{37}$. After optimization studies of various parameters, there was almost 2 fold increase in PHA accumulation ie, from 0.2 to 0.4g of PHA per litre of culture medium.

**Fourier Transform Infra Red (FTIR) spectroscopic analysis**

FTIR spectrum of purified PHA extract from *Vibrio* sp. BTTC26 are represented in Fig. 9. The bands stretching between 3000 and 1460 cm$^{-1}$ indicate C-H stretching and bending$^{44}$. The functional groups of the extracted PHA was C=O group indicated by the presence of bands at 1722 cm$^{-1}$ and these results confirmed the presence of PHAs$^{34}$. The presence of bands at 1376 cm$^{-1}$ indicates the presence of methyl group$^{44,45}$. The presence of bands at 1279, 1228, 1135,
1105 and 519 cm\(^{-1}\) confirmed the presence of polyhydroxybutyrate (PHB) in them\(^{14,41,45}\). On comparing with the FTIR spectrum of standard PHB and from previous reports, the PHAs in the 
*Vibrio* sp. BTTC26 was identified as polyhydroxybutyrates (PHB). In the studies of Chien *et al.* \(^{36}\) in the PHA accumulation of vibrios from marine sediments, all the *Vibrio* sp. isolated were harbouring PHB and and no other types of PHAs were detected in their studies. These findings indicate that PHAs synthesized by *Vibrio* sp. were not as diverse like other microbes.

**Conclusion**

The study has revealed that 62% of the strains tested accumulated PHAs, indicating the stressful environment in which these bacteria survive. Studies have shown that accumulation of reserve polymers like polyhydroxyalkanoates increases survival in changing environments like benthic environments\(^{7}\). In this study several strains of fast-growing *Vibrio* sp. were identified, that were easy to cultivate and which produced intracellular PHAs. Fast growth and high PHA production are important factors in industrial production of PHA. The strain selected for submerged fermentation was *Vibrio* sp. strain BTTC26 identified as *V. azureus*. From the FTIR analysis it was found that PHAs in the isolate BTTC26 were Polyhydroxybutyrate (PHB), the smallest known PHA which displays a similar degree of crystallinity and Tm as polystyrene and main candidate among PHAs having wide range of industrial and medical applications. The various conditions optimized for the intracellular accumulation of PHAs by strain BTTC26 were glucose as carbon source, pH 7, sodium chloride concentration - 10%, initial inoculum concentration - 3%, incubation time - 36h and incubation temperature – 37°C. The production of PHB by this marine isolate (*Vibrio* sp. strain BTTC26) by labscale fermentation is currently under investigation.

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**References**


