

## Isolation and identification of Polyhydroxybutyrate producing bacterial strain (*Bacillus thuringiensis* GVP) from chlorine contaminated soil

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**Abstract:** In this work a bacterial strain which is capable of accumulating polyhydroxybutyrate (PHB) was screened from chlorine contaminated soil sample using suitable staining technique. Based on morphological, biochemical and molecular characterization, the strain was identified as *Bacillus thuringiensis* GVP (GenBank accession number KC795826). PHB production was checked and was extracted by dispersion method of sodium hypochlorite and chloroform with slight modification. The amount of PHB produced was quantified by UV spectrophotometer method. The synthesised polymer was characterized by FTIR as PHB monomer.  
**Keywords:** PHB, *Bacillus thuringiensis* GVP, UV spectrophotometer, FTIR.

### Introduction

Today, synthetic polymer has become indispensable part of our life. Petrochemical materials are used to manufacture synthetic polymer and plastic, they are defiant to biodegradation which poses a grave menace to the surroundings. Environment issue due to pollution, increase in the price of petroleum and depletion of fossil fuels makes us to develop bioplastic as a substitute for petrochemical derived plastic<sup>1,2</sup>. Poly-3-hydroxybutyrate(PHB) is a widely studied biopolyhydroxyester belongs to polyhydroxyalkanoates (PHAs) family<sup>3</sup>. PHB has become substitute for petrochemical derived plastic due to similar mechanical properties. Poly-3-hydroxybutyrate is ecofriendly, biodegradable, biocompatible and microbial thermoplastic<sup>4,5</sup>. PHB is a straight chain homopolymer of D (-)- $\beta$ -hydroxybutyric acid, is synthesised and agglomerate as intracellular granules in most bacterial species. Biosynthesis of PHB starts with condensation of acetyl-CoA molecules which yield acetoacetyl-CoA. By coupling with the oxidation of NADH to NAD<sup>+</sup>, acetoacetyl-CoA is consequently reduced into hydroxybutyryl- CoA. Hydroxybutyryl- CoA is polymerised into PHB in the presence of PHA synthase (phaC). Poly 3-hydroxybutyrate synthesis occurs during exponential growth phase and transferred into important energy reserves<sup>6</sup>.

PHB is synthesised under nutrient imbalance such as limiting the concentration of essential nutrients like nitrogen or phosphorus whereas carbon source is supplied in excess<sup>7</sup>. *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Staphylococcus* and *Micrococcus* are some bacterial species which naturally produce PHB<sup>8,9</sup>. PHB from Gram-negative organisms are widely used in a variety of products but due to endotoxin in the outer membrane lipopolysaccharides (LPS), *Bacillus* species which lack LPS are extensively used in biopharmaceutical applications<sup>10</sup>. Due to wide industrial application, PHB are considered as green plastics. The popularization of bioplastics is limited because of their high production costs. Consequently, less expensive

substrates like agricultural residues, improved cultivation strategies and easier extraction techniques has to be used to bring down the cost<sup>11</sup>.

*Bacillus thuringiensis* is a sporulating, gram-positive bacteria which is capable of synthesizing PHB granules<sup>12</sup>. In the present study, we report the isolation of PHB producing *Bacillus thuringiensis* GVP strain from the soil and characterization of synthesised PHB by FTIR.

## Materials and Method

### Sample collection and isolation of bacterial strain

Soil samples were collected from chlorine spillage areas of SASTRA University, Thanjavur, India under aseptic conditions using standard practices. The strains were isolated from soil by serial dilution-pour plate technique (dilution  $10^{-6}$  and  $10^{-7}$ ) on nutrient agar media (Hi media, India). These plates were incubated at 37° C for 24 h. Morphologically distinct colonies were purified and maintained on nutrient agar slant at 4° C.

### Screening for PHB-producing bacteria

PHB producing bacteria was detected using the lipophilic stain Sudan black B (0.3% in 60% ethanol)<sup>13</sup>. The positive strain was further confirmed by Nile blue A staining. Nile blue A staining was done as per Ostle and Holt<sup>14</sup> protocol.

### Identification of PHB –producing bacteria

The isolated PHB producing bacteria GVP was identified by biochemical test as per the Bergey's Manual of Determinative Bacteriology<sup>15</sup>. 16S rRNA sequencing and phylogenetic studies were subsequently done to identify the strain. Amplification of 16S rRNA was performed using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3'). The 16S rRNA gene sequence from GVP was deposited in NCBI Genbank.

### PHB production by *Bacillus thuringiensis* GVP

For the production of PHB, *Bacillus thuringiensis* GVP were inoculated in production media containing tryptone (4.0 g/l), yeast extract (2.5 g/l), sodium chloride (1.25 g/l) and glucose (1% w/v) as carbon source<sup>16</sup>. 2% (v/v) inoculum was added to 100 ml of production medium and incubated at 30° C for 48 h on a shaker at 150 rpm.

### Extraction and quantification

After 48 h, fermented broth was collected and centrifuged at 8,000 rpm for 10 min and the resulting pellets were washed with distilled water. Cell pellets were air dried to determine their weights. Chloroform and 6% sodium hypochlorite were added to cell pellet in ratio aspect of 12.5 µl chloroform to 6% sodium hypochlorite per mg of cell pellet weight; this mixture was kept at 30° C overnight and it was centrifuged at 15,000 rpm for 10 min results in three phases. The bottom phase of chloroform containing PHB was carefully removed and PHB was recovered by precipitation with 5X hexane<sup>13</sup>. The precipitate was recovered by centrifugation at 15,000 rpm for 10 min. Quantitative assay was done by UV spectrophotometer method<sup>17</sup>.

### Characterization by FTIR

2 mg of purified PHB was thoroughly mixed with KBr and dried at 100°C for 4 hr. FTIR spectrum was taken using Perkin-Elmer Fourier Transform IR Spectrometer. The spectra were recorded in range of 4000-400  $\text{cm}^{-1}$ cm.

## Result and Discussion

### Isolation of bacterial strain

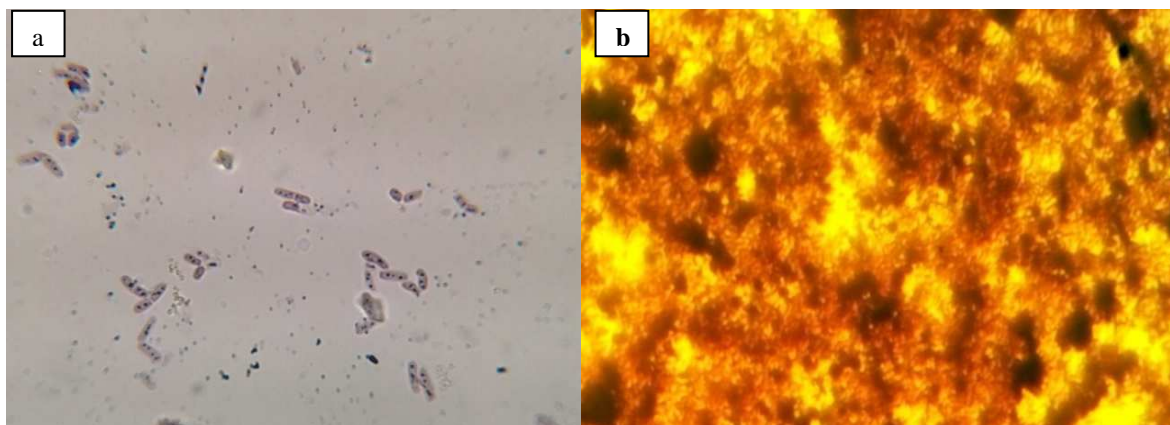
By serial dilution and pour plate technique, six different morphological strains were isolated (GAP, GCP, GHP, GKP, GSP and GVP). These dissimilar colonies were purified and preserved on nutrient agar slant at 4°C for further use.

### Screening for PHB-producing bacteria

Six strains were screened for PHB accumulation in cytoplasm by Sudan black B stain, only strain GVP showed ability for PHB accumulation which was evidenced by blue-black inclusion in cytoplasm. When stained with Nile blue A for further confirmation under fluorescence microscope, the micrograph showed golden fluorescence. The presence of golden fluorescence confirms the PHB inclusion (Fig.1). The results corroborate with findings of Masood *et al.*, 2013<sup>18</sup>.

### Identification of PHB –producing bacteria

Morphological and biochemical characteristics (Table 1) were done as per Bergey's Manual of Determinative Bacteriology<sup>15</sup>, the strain GVP was identified as gram positive *Bacillus thuringiensis*. 16S rRNA sequence followed by BLAST analysis showed *Bacillus thuringiensis* GVP has 98% homology with *Bacillus thuringiensis*. Phylogram was created using MEGA software (Fig. 2). The isolated sequence was submitted at GenBank and the accession number was obtained as KC795826.



**Fig. 1** a Micrograph of stain *Bacillus thuringiensis* GVP with Sudan black B showing PHB accumulation when counter stained with safrin using light microscope. b Nile blue A strain fluorescent micrograph of *Bacillus thuringiensis* GVP showing golden fluorescence due to PHB granules.

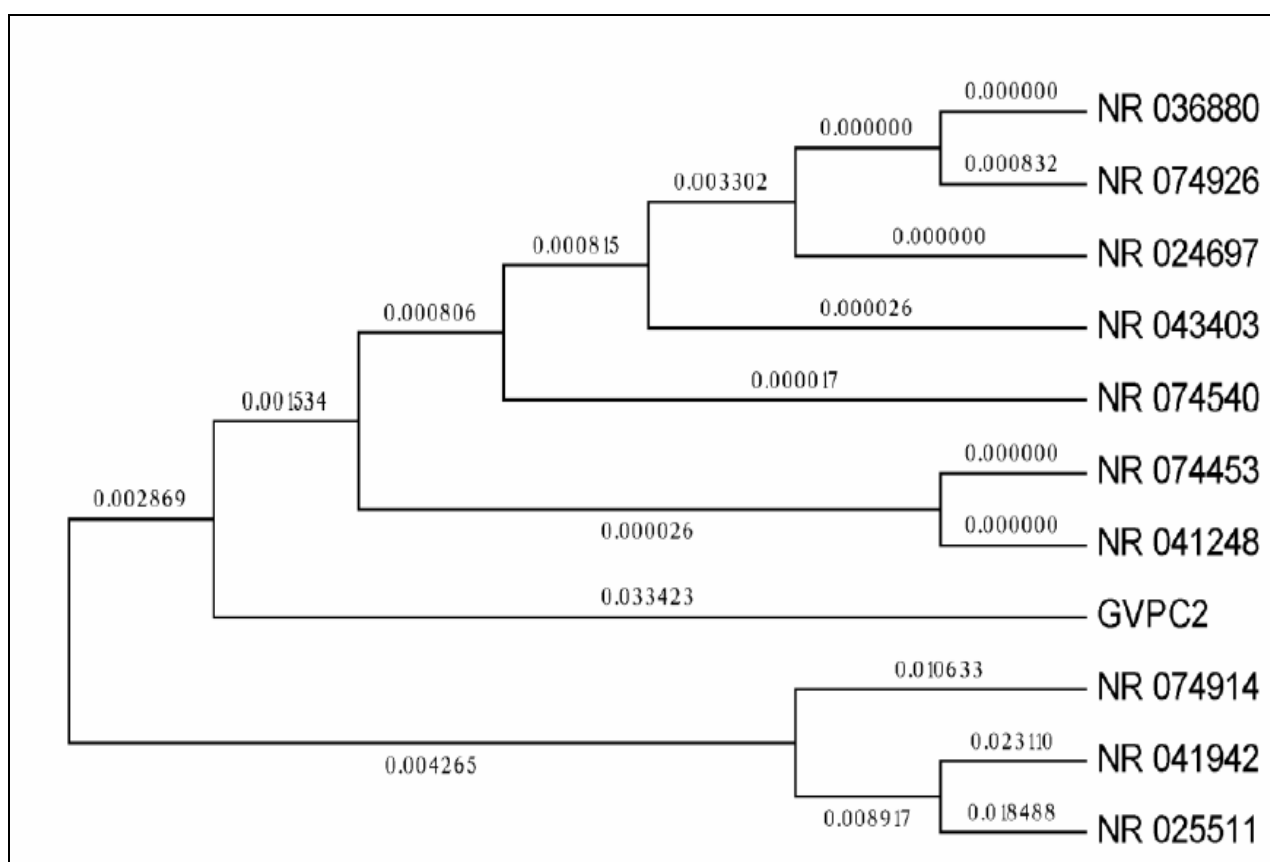
### PHB extraction and characterization by FTIR

The isolate GVP was checked for PHB production in the production media and then the inclusions from the cells were extracted (Table 2). *Bacillus thuringiensis* GVP was able to produce PHB of 0.73 g/l and 31.26 %. The extracted PHB was purified by dissolving it in chloroform and again precipitating by 5X hexane and then characterized by FTIR. FTIR spectroscopy recorded for extracted PHB gives peak at 1724.04  $\text{cm}^{-1}$  and 1280.69  $\text{cm}^{-1}$  which corresponding to C=O and C-O stretching group respectively. The bands for  $-\text{CH}_2$ ,  $\text{CH}_3$ , C-O-C and terminal OH group occurred at 1458.15  $\text{cm}^{-1}$ , 1381.71  $\text{cm}^{-1}$ , 1184.33  $\text{cm}^{-1}$  and 3444.40  $\text{cm}^{-1}$  (Fig. 3) The FTIR spectrum of extracted polymer corroborates with peaks reported by Oliveira *et al.*, 2007<sup>5</sup>

**Table 1:** Physiobiochemical characterization of the isolate *Bacillus thuringiensis* GVP

Test	Observation
Gram's stain	Gram positive
Spore staining	Positive
Cell shape	Rod
Colony character	White, raised, irregular
Motility	Positive
Starch hydrolysis	Positive
Oxidase	Negative
Indole	Negative
Methyl Red	Negative
Voges-Proskauer	Negative

Citrate Utilization	Positive
Catalase	Positive
Casein	Positive
Urea hydrolysis	Negative
Growth at 50° C	Positive
Growth in 10 % NaCl	Negative
TSI	Acid slant/Acid butt, gas, no H2S
<b>Sugar utilization</b>	<b>Result( Acid/Gas)</b>
Glucose	Positive / Negative
Sucrose	Positive / Negative
Lactose	Positive / Negative
Starch	Negative / Negative
Xylose	Negative / Negative



**Fig. 2** Unrooted phylogenetic tree obtained by neighbour-joining of 16S rRNA sequence of the isolated *Bacillus thuringiensis* GVP strain

**Table 2:** Isolate showing PHB production

Isolate	Habitat	PHB (g/l)	Yield (%)
GVP	Chlorine contaminated soil	0.723	31.26

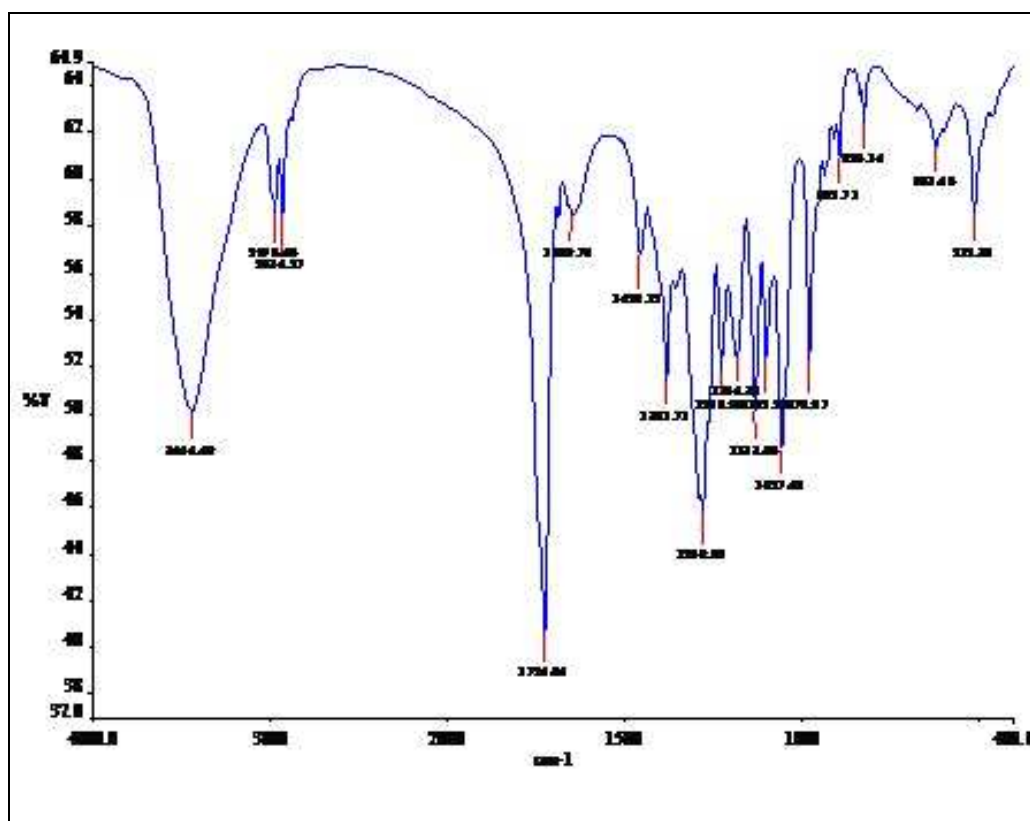


Fig.3 FTIR spectra of extracted PHB from *Bacillus thuringiensis* GVP

## Conclusion

From the studies it was concluded that soil isolate GVP which showed ability to accumulate PHB. Based on morphological and taxonomical features, the isolate was found to be *Bacillus thuringiensis* GVP. *Bacillus thuringiensis* GVP was able to produce 0.73 g/l PHB and yield was found to be 31.26 %. FTIR spectra of the synthesised PHB reveal similar spectra with commercial PHB and also it confirms the isolated strain *Bacillus thuringiensis* GVP as an active PHB producer.

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