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# Isolation, Screening and Extraction of Polyhydroxybutyrate (PHB) producing bacteria from Sewage sample

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**Abstract:** Poly hydroxyl butyrate (PHB) can be used as an effective thermoplastic, and has many characteristics similar to those of standard commercial plastics like polypropylene. PHB-based plastic substitutes are less flexible than traditional plastics, they are completely biodegradable and leave behind no residue. In this present study high PHB producing strains were isolated from sewage soil sample. Six strains were showing PHB granules with Sudan Black B staining. The six strains were named as strain 2, 4, 5, 9, 11 and 16. The six strains were morphologicall and biochemically characterized. Growth profiles have been studied for all the strains and the polymer was produced after 48 hrs at 37°C. PHB producing growth medium was used for production of PHB. Strain 11 was showed high PHB production among the six strains. The medium used for PHB production showed high yield of the product was of less cost effective.

**Key words:** Polyhydroxy Butyrate, Biopolymer, Thermoplastic; Characterization.

## INTRODUCTION

Plastic materials have become an integral part in our life as a basic need but they are causing serious environmental problems due to their non biodegradability[1]. They are widely applicable in packaging films, wrapping materials, shopping and garbage bags, clothing, fluid containers, toys household and industrial products and building materials[2-3]. These materials are resistant to degradation and remains as waste on landscape for several years. The recycled plastics are more harmful to the environment than the virgin products due to mixing of color, additives, stabilizers, flame retardants etc. Due to environmental and waste management problem, alternative is the biopolymer. Biopolymers are polymers produced by living organisms. These are biodegradable and converted by microorganisms into $CO_2$, water and humic material. Mostly used

biodegradable polyesters, are poly hydroxyl alkanoates (PHAs), polylactides, aliphatic polyesters, and polysaccharides[4].

The PHA types are polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH) and polyhydroxyoctanoate (PHO)[5]. Among these types PHB is the main biodegradable polymer. This PHB can be used as an effective thermoplastic, and has many characteristics similar to those of standard commercial plastics like polypropylene. While PHB-based plastic substitutes are less flexible than traditional plastics, they are completely biodegradable and leave behind no residue. A number of bacteria producing PHAs are *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii, Rhizobium sps*[6], *Bacillus sps*[7], *methylotrophs, pseudomonads,* and recombinant *Escherichia coli*[3]. Recent advances in molecular biology, understanding metabolism and genetics of the PHA synthesizing bacteria allowed the construction of various recombinant strains that were able to synthesize more amount of PHA. In economical point of view genetically engineered plants harboring the bacterial PHA biosynthesis genes are also developed[2].

The PHAs are classified according to the number of carbon atoms in their monomers. Polyhydroxybutyrate (PHB) and polyhydroxyvalerate carbon numbers of monomers are 3 to 5. Conversely, carbon numbers in medium chain-length PHA monomers range from 6 to 16. The PHB identified in *B.megaterium* was characterized by its large accumulations of PHB[5]. PHA's are particularly important because of its good biodegradability, biocompatibility, thermoplastic nature used in drug delivery systems.

PHB production is increased by excess of carbon source and limiting the nutrients such as nitrogen, phosphorus, sulfur, magnesium, iron, oxygen, and potassium[8-9]. It is an intracellular polymer accumulated under stress conditions but with excess carbon source. It is produced by fermentation process of microorganisms such as *Bacillus megaterium* and *Ralstonia eutropha*. In this study several PHB producing bacteria were isolated from sewage soil sample and morphological and biochemical properties were identified. The growth curve, effect of pH and NaCl concentration on growth was studied and the isolates were characterized for the quantification of the PHB.

## MATERIALS AND METHODS

All chemicals were purchased from s d fine chemicals and were of analytical grade

### 1. Sample collection and isolation of pure cultures:

Sewage soil sample was collected in sterile bottle from dump yard at outscuts of Guntur, Andhra Pradesh.

One gram of soil sample is dispensed in 10ml of sterile distilled water. This is mixed vigorously and 1ml from this is taken and added to another tube with 9ml sterile distilled water to get a dilution of $10^{-1}$. This serial dilution is repeated to get dilutions of $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ and $10^{-9}$. For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient rich medium by spread plate method for the propagation of microbial growth. The plates were incubated at $30^0$c for 48 hours. Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4°C.

### 2. Screening of PHB producing isolates by Sudan Black B staining:

Prepare thin smear on microscope slide and thoroughly air dry. Do not heat fix. Stain with Sudan black B solution and let it stand for 10-15 minutes. Add more stain if the slide starts to dry out. Wash the slide with distilled water and counter stain with safranin for 10 seconds. Wash with distilled water and blot dry with tissue paper. Examine the slide under oil immersion microscope for PHB granules. Organism shows positive in blue-violet and shows negative in yellow-brown[10].

### 3. Characterization of PHB producing isolates

PHB producing strains were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Determinative Bacteriology[11].

**3.1 Morphological characterization:**

Morphological features were identified by growing the cultures on nutrient agar media and gram staining was performed.

**3.2 Biochemical characterization**:

Different Biochemical tests were carried out includes IMVIC tests, catalase test, urease test and starch hydrolysis[12].

**3.3 Effect of pH on Bacterial growth:**

Nutrient broth medium was prepared and the tubes were adjusted with pH 2,4,7,9 and11 respectively. Inoculate each tube with the loopful of culture. Incubate the tubes in shaking incubator at $37^0$c temperature for 24hrs. After incubation observe the growth of culture in each tube. Measure the O.D at 640 nm. Plot the graph by taking pH on x-axis and O.D on y-axis.

**3.4 Effect of salt concentration on bacterial growth:**

Nutrient broth medium was prepared and the tubes were added by Nacl with 0.1%, 0.5%, 2%, 5%, and 10% concentration respectively. Inoculate each tube with the loopful of culture. Incubate the tubes in shaking incubator at $37^0$c temperature for 24hrs. After incubation observe the growth of culture in each tube. Measure the O.D at 640 nm. Plot the graph by taking salt concentration on x-axis and O.D on y-axis.

**4. Growth curve studies of isolates**:

For the growth and production of PHB the simplified medium (g/100ml) was used. Glucose - 1g, Peptone - 0.25g, Yeast extract – 0.25g, NaCl – 0.01g, $KH_2PO_4$ – 0.05g, $MgSO_4$ – 0.02g and pH at 7. The medium containing glucose as sole carbon source was used for the growth curve studies. The medium was prepared and sterilized at 121°C and 1% inoculum was added to each flask to carry out fermentation. The flasks were incubated at 37°C for 48hrs. Samples were collected at an interval of 4 hrs and the cell biomass was measured at 600nm using spectrophotometer.

**5. Cell dry weight:**

After 48hrs incubation at $37^0$C, culture medium was collected and centrifuged at 10,000 rpm for 15min. Supernatant was discarded and the cell pellet was dried to estimate the dry cell weight (DCW) in units of g/ml[13].

**6. Extraction and Quantification of PHB**

After 48hrs incubation at $37^0$C, culture was collected and centrifuged at 10,000 rpm for 15min and lyophilized. The lyophilized pellet was digested with 4% sodium hypochlorite solution at $37^0$C for 20min. Then pellet was collected by centrifugation at 10,000 rpm for 15min, washed with water, acetone, ethanol respectively for washing and extraction. Finally polymer was dissolved in chloroform and kept for complete evaporation[14]. Dry weight of extracted PHB was estimated as g/L. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHB[15]. The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight.

Residual biomass (g/ml) = DCW (g/ml) – Dry weight of extracted PHB (g/ml)

PHB accumulation (%) = Dry weight of extracted PHB (g/ml) × 100 / DCW (g/ml)

**RESULTS AND DISCUSSION**

**1. Isolation of microorganisms:**

Microorganisms were isolated from sewage sample and independent colonies were obtained by serial dilution. A total of 17 bacterial colonies with different morphological features were selected and the numbers were given to each colony. These colonies were streaked on nutrient agar plates and preserved for further studies (Fig. 1).

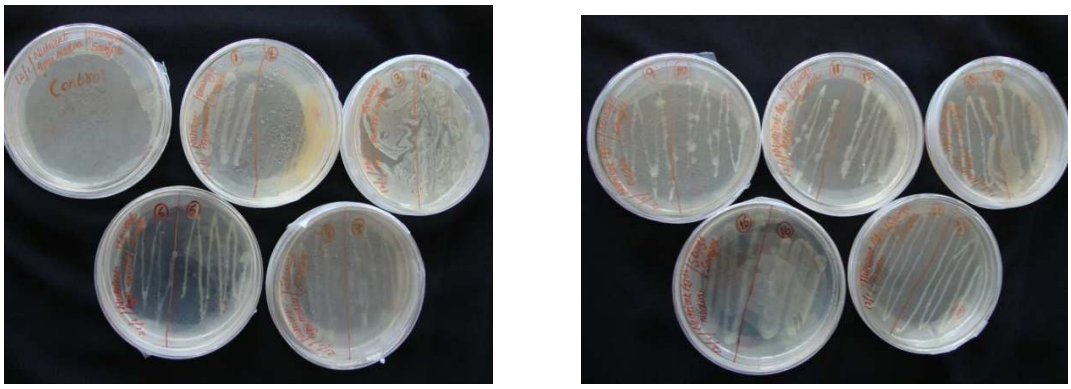

**Fig 1: Isolated colonies were streaked on nutrient agar plates**

## 2. Screening of PHB Producing Bacteria:

Among 17 colonies, 6 colonies showed positive for Sudan Black B staining. These 6 colonies are 2, 4, 5, 9, 11 and 16. They were named as strain 2, strain 4, strain 5, strain 9, strain 11 and strain 16. All the strains except 2 are showing high color intensity with Sudan black B. The Microscopic pictures were depicted in **figure 2.**

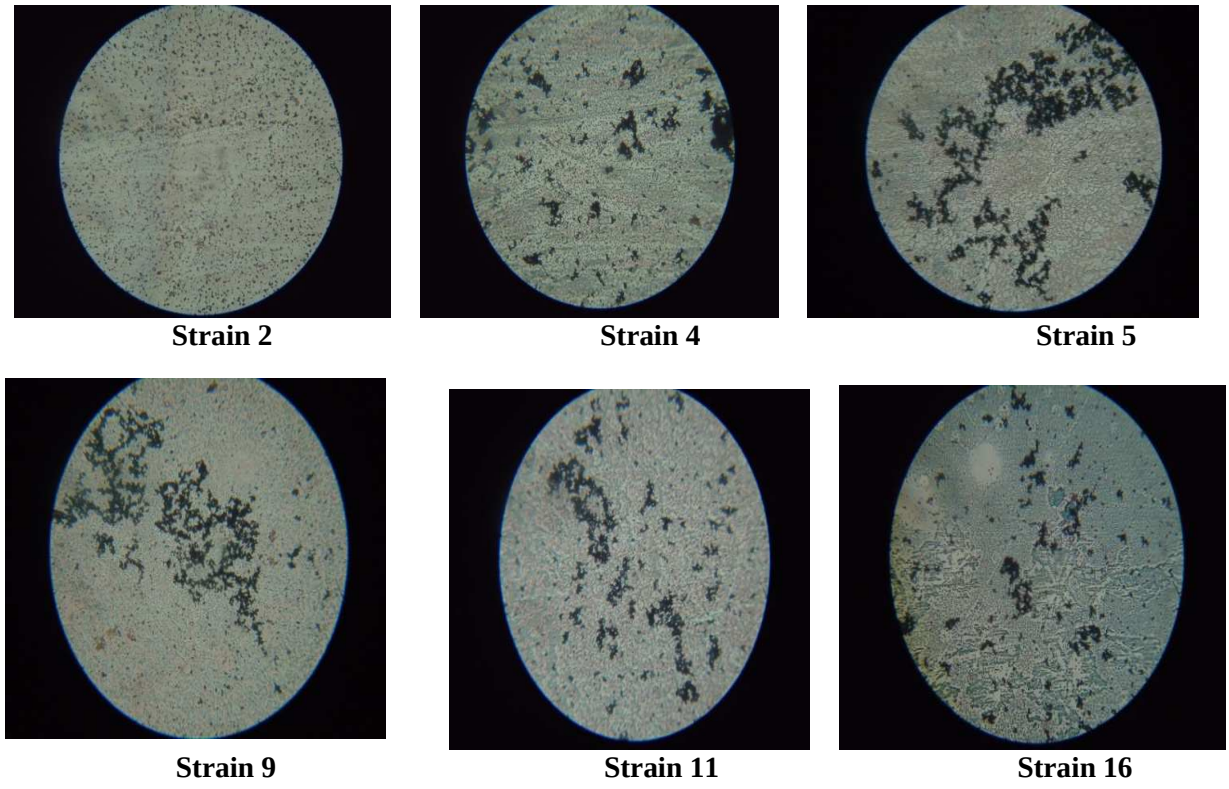


**Fig 2: Strains 2, 4, 5, 9, 11 and 16 showing PHB granules with Sudan Black B staining**

## 3. Characterization of PHB producing isolates

### 3.1 Morphological and Biochemical characteristics

Morphological features were observed for PHB producing strains. Strain 4 and 11 has bacillus in shape and strain 2, 5,9,16 were cocci in shape. Strain 2 is yellow in color and the remaining strains 4,5,9,11,16 were Creamish white in color. Strain 4 is Gram positive and the remaining strains 2,5,9,11 and 16 were Gram negative to gram staining. Morphological features were represented in Table 1.

Different Biochemical tests have been performed for PHB producing strains. Strain 9 and 16 are positive to indole and the remaining are negative to indole test. Strain 5 is negative to MR and positive to VP test. Strains 2, 4, 9, 11 and 16 are positive to MR and negative to VP test. Only strain 2 is negative to citrate utilization test and the remaining strains are positive to citrate utilization test. Strain 2and 4 are negative to starch hydrolysis test and the remaining strains are positive to starch hydrolysis test. Biochemical tests were depicted in Table 1.

**Table 1: Morphological and Biochemical characteristics of PHB isolates**

| | Strain 2 | Strain4 | Strain5 | Strain 9 | Strain 11 | Strain 16 |
|---|---|---|---|---|---|---|
| Morphological characteristics | | | | | | |
| Shape | Cocci | Bacillus | Cocci | Cocci | Bacillus | Cocci |
| Color | Yellow | Creamish white | Creamish white | Creamish white | Creamish white | Creamish white |
| Gram staining | Gram -ve | Gram +ve | Gram -ve | Gram -ve | Gram -ve | Gram -ve |
| Biochemical tests | | | | | | |
| Indole production | Negative | Negative | Negative | Positive | Negative | Positive |
| MR | Positive | Positive | Negative | Positive | Positive | Positive |
| VP | Negative | Negative | Positive | Negative | Negative | Negative |
| Citrate utilisation | Negative | Positive | Positive | Positive | Positive | Positive |
| Catalase | Negative | Negative | Negative | Negative | Positive | Negative |
| Starch hydrolysis | Negative | Negative | Positive | Positive | Positive | Positive |

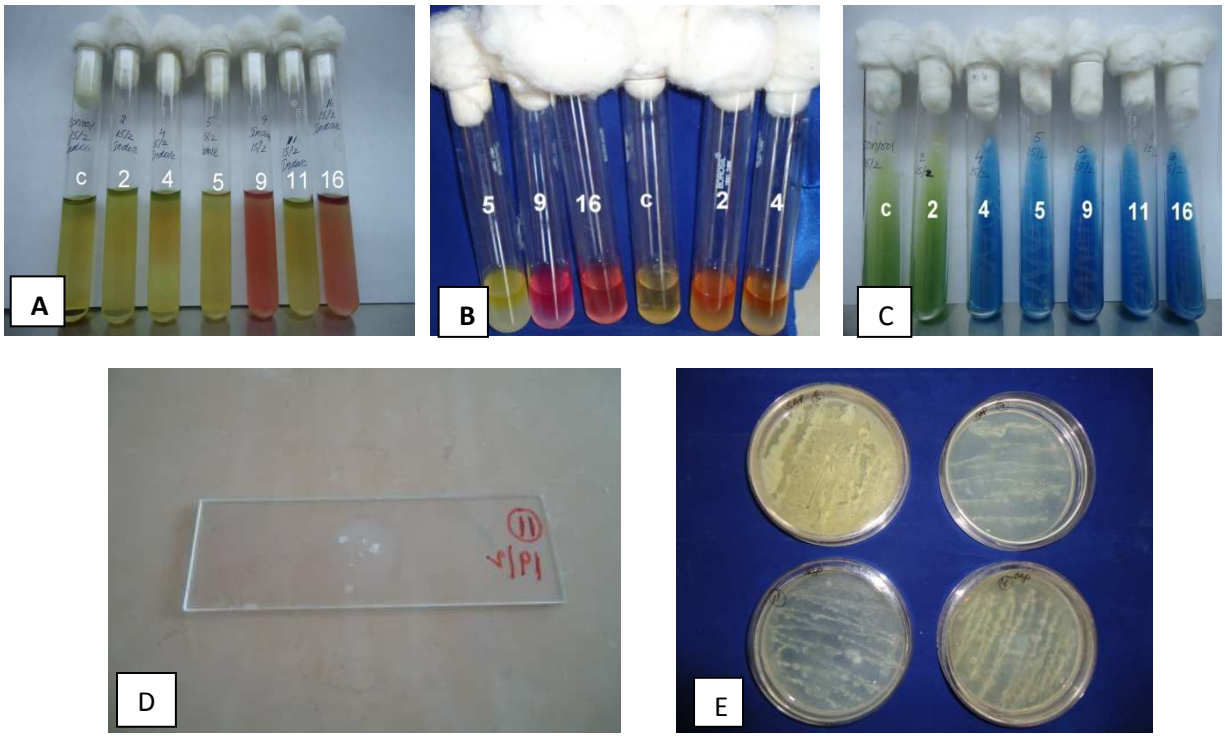


**Fig: 3 Images of results of various biochemical tests: A) Indole test:** Strains 9 and 16 showing positive for indole and strains 2, 4, 5, 11 showing negative to indole. **B) Methyl red:** Strain 5 showing negative to methyl red and 2,4,9,11,16 showing positive to MR. **C) Citrate utilization test:** Strain 2 is negative to citrate utilization**. D) Catalase test: S**train 11 is positive to catalase test. **E) Starch hydrolysis test:** Strains 5,9,11 and 16 showing positive to starch hydrolysis.

### 3.2 Effect of pH on Bacterial growth

A pH variation from acidic to alkaline was carried out for the influence on growth. The pH 5 was showing optimum pH for all the strains and the cell biomass was maximum at this pH for all the strains. In the alkaline pH the cell growth and cell concentration was decreased compared with pH 5. The pH profiles were depicted in Fig. 4.

### 3.3 Effect of NaCl concentration on growth

Different NaCl concentrations from 0.1% to 10% were used to check for growth of all the PHB isolates. 0.1% NaCl concentration was suitable for growth of all the PHB isolates and increasing concentration of NaCl resulted in the decrease of the growth. The NaCl concentration profiles were shown in Fig. 5.

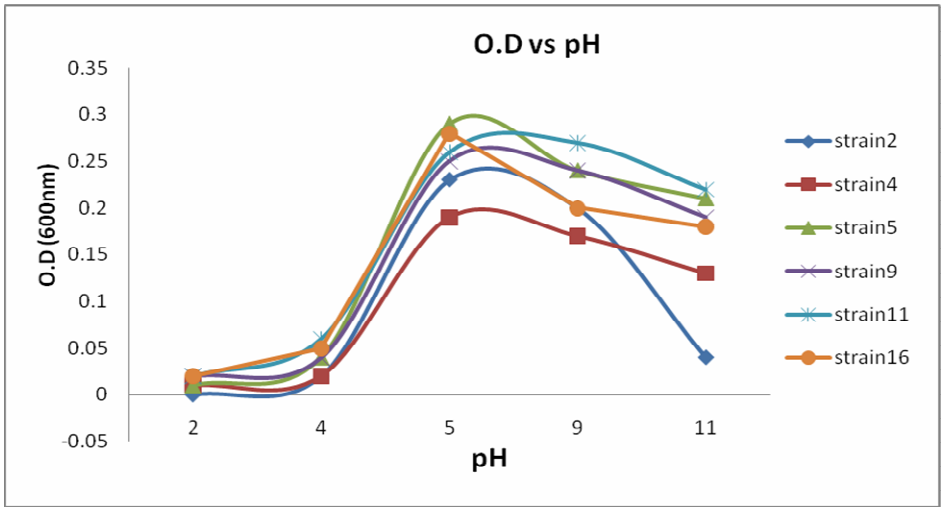


**Fig 4: Effect of pH on Bacterial growth**

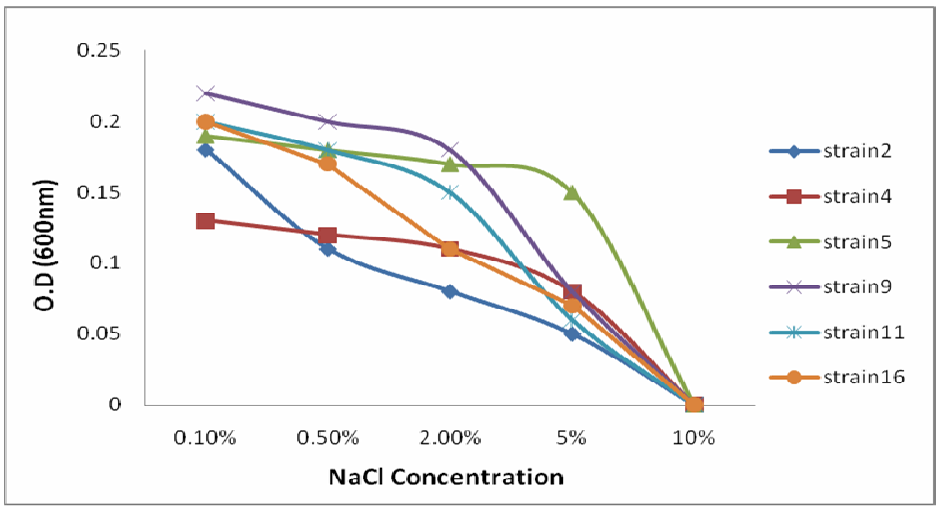


**Fig 5: Effect of NaCl Concentration on Bacterial growth**

### 4. Growth curve studies for PHB isolates

The PHB isolates were growned in biopolymer producing media for 48hrs at $37^0$C. The optical readings were taken at 640nm for different time intervals. Growth curve was plotted by taking time on X-axis and O.D on Y-axis and the graphs were represented in figure 6.

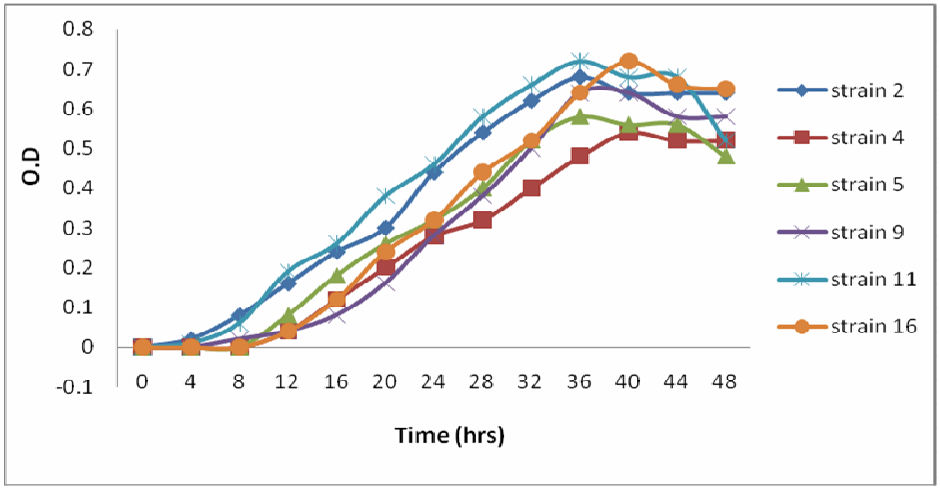


**Fig 6: Growth curves for PHB producing isolates**

## 5. Quantification of PHB

The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry cell weight. Residual biomass was estimated as difference between the dry cell weight and dry extract of PHB. The results were depicted in Table 2. From these results strain 11 showing high PHB production whereas the strain 4 showing less PHB production compared with other strains. Similar results have been showed by with glucose as a carbon source in the medium[16].

**Table 2: PHB accumulation for PHB isolates**

| Name of the Organism | Cell dry weight (g/ml) | Dry weight of PHB (g/ml) | Residual biomass (g/ml) | % PHB Accumulation |
|---|---|---|---|---|
| Strain 2 | 0.128 | 0.048 | 0.080 | 37.5 |
| Strain 4 | 0.152 | 0.038 | 0.114 | 25.0 |
| Strain 5 | 0.110 | 0.044 | 0.066 | 40.0 |
| Strain 9 | 0.116 | 0.046 | 0.070 | 39.6 |
| Strain 11 | 0.122 | 0.056 | 0.066 | 45.9 |
| Strain 16 | 0.122 | 0.050 | 0.072 | 40.9 |

## CONCLUSIONS

The main aim of this present study was to isolate the PHB producing bacteria from sewage sample. Now days researchers are focusing on biopolymer producing microorganisms for developing biodegradable plastics. The medium used for the PHB isolates was simple medium and less cost effective and the PHB yield from these isolates was high compared with the earlier reports. Among 6 isolates the strain 11 is showing more production of biopolymer. The PHB produced from this strain will further be characterized by analytical techniques like Infra Red spectra and Gas Chromatography analysis.

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