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Research Article

PRODUCTION AND FTIR ANALYSIS OF BIO-POLYMER BY *BACILLUS SP* ISOLATED FROM VELLAR ESTUARY SEDIMENT

K. Muthazhagan and M. Thangaraj*

C A S in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608 502, Tamil Nadu, India.

ABSTRACT

Polyhydroxybutyrate (PHB) a lipid-like polymer of 3-hydroxybutyrate and the representative member of polyhydroxyalkanoates formed in many bacteria. In the present study, six different bacterial isolates were obtained from sediment sample and the bacterial isolates were screened on solid agar medium using Sudan black. On the agar medium, out of 6 isolates, one was produced PHB. The biochemical investigations of the isolated bacteria showed gram positive rod shape and confirmed as *Bacillus* sp. As the optimized conditions like salinity (2%), temperature (40°C), pH (4.0), carbon (maltose) and nitrogen (yeast extract) showed higher PHB production. Fourier Transform Infra Red (FTIR) spectrum of the PHB sample revealed 5 major peaks at 3430, 1651, 1645, 1636 and 649 cm^{-1}. And the structural analysis confirmed the presence of polyester group. On the basis of data obtained in this study it is concluded that the *Bacillus sp* is capable of producing PHB and can be used for manufacturing of biodegradable goods.

Keywords: *Baciilus sp*, estuary sediment, PHB, optimization, FT-IR

INTRODUCTION

Plastics have become an important part of our modern life and are used in different sectors of operations like packaging, building materials, consumer products and many more. Each year, about 100 million tons of plastics are produced world. Wide Demand for plastics in India reached about 4.3 million tons in 2001-2002and was expected to increase and the exact time for their degradation is unknown (Kalia *et al*., 2000). Presently plastic and synthetic polymers are mainly produced by using the petroc hemical material that cannot be decomposed easily. Thus they contribute to the environmental pollution and are dangerous to a number of animal species (Ojumuu and Solamon, 2004). The use of non biodegradable plastics causing the environment pollution, hence biodegradable plastics have emerged as a useful alternative to over come the environmental problems.

During the last decade, environmental pollution and exhaustion of non renewable resources have created much interest in natural materials like Poly β-hydroxybutyrate as a biodegradable. The physical properties of the PHB are similar to those of some conventional plastics (Howells, 1982). Because of their good biodegradability and biocompatibility, PHB have attracted interest in their use as an alternative to petroleum based plastic including fine chemicals,plastics, printing materials, bio fuel, agriculture, marine, medical and other fields (Doi, 1990).The first identified PHA, poly-β-hydroxybutyrate (PHB) from *Bacillus megaterium*, is drawing much attention due to having physical properties similar to petroplastic polypropylene with advantage of being completely biodegradable. The high production cost of PHB can be curtailed by strain development, improving fermentation and separation processes, and using inexpensive carbon source. In PHB production, about 40% of the total production cost is accounted for raw material, and thus, the use of in expensive carbon source or even waste organic materials could be highly significant (Braunegg *et al*., 1998).

PHB has wide spread occurrence in both Gram-positive and negative bacteria (Naranjo *et al*.,2013).C_4 to C_{18} hydroxyalkanoates can be the monomers of different polymers (Elsayed *et al*., 2013). PHB is found to accumulate in large number of microorganisms as reserve food material *e.g*. *Ralstoniaeutrophes*, *Azotobacter beijerinckia*, *Bacillus megaterium*, *Pseudomonas oleovorans* and various nitrogen fixing microorganisms (Haywood *et al*., 1988). A bacterial isolate *A. macrocytogenes* from the soil was selected and its PHB production reached 24% per dry weight after 48 h incubation (Elsayed *et al*., 2013).

However, only limited work has been carried out on the large-scale production of PHB from the Gram-positive genus *Bacillus*. The only report so far, (Wu *et al*., 2000) scaled up the production of PHB by *Bacillus* sp. Keeping this in mind, the present study was planned to isolate PHB producing microorganisms and optimize the culture condition such as incubation time, temperature, pH, carbon and nitrogen sources for maximum PHB production.In the present investigation we have carried out the isolation of various bacterial

strains from the estuary environment and optimization of culture condition for better production of PHB.

MATERIALS AND METHODS

Isolation and identification of bacterial strain:

The estuary sediment sample collected from Vellar estuary (Lat.11˚29' N; Long.79˚46'E), Parangipettai, in Southeast coast of India. Samples were collected from 3 to 4 cm depth with the help of sterile spatula and transferred in to sterile plastic bags. They were brought to the laboratory inaseptic conditions. Collected samples from sediments of Vellar estuary cultured in a broth medium called marine broth, incubated in 30°C for three days and then transferred in a marine agar medium so that the colonies appeared. After sub culturing each of the colonies, isolation was achieved. The morphological and biochemical characteristics of the selected isolate were performed according to the standard method (Baron and Finegold, 1990; Delost, 1997).

Screening for PHB accumulation:

The isolates were further screened for PHB production by Sudan Black B test. Isolates were grown as single colony on a plate containing nutrient agar. The plates were incubated at 30°C for 24 hours. Sudan Black B solution (0.2% Sudan black B in 96% ethanol) was spread over the colonies and the plates kept as undisturbed for 30 minutes. They were washed with ethanol (96%) to remove the excess stain from the colonies. The dark yellowish color colonies were taken as positive for PHB production.

Quantitative analysis of PHB:

Determination of the amount of PHB was performed chemically. The samples were centrifuged for 45 min at 6000 rpm. Then the pellets were incubated at 60°C for 1 h with sodium hypochlorite to break the cell walls. Supernatant was obtained by centrifugation at 6000 rpm and transferred to a Soxhlet system. Cell lipids and other molecules (except PHB) were extracted by adding 5 ml of 96% (1:1 v/v) ethanol and acetone. PHB was extracted by chloroform. Chloroform extract was dried at 40ºC and 10 ml of concentrated sulfuric acid was added. They were heated at 100°C in a water bath for 20 min. After cooling, the amount of PHB was determined on a spectrophotometer, at wave length of 235nm (Thirumala *et al*.,2010).

Extraction of PHB from *Bacillus sp:*

PHB was extracted from the isolate by using the hypochlorite method (Rawte and Mavinkurve 2002). For this, the isolate was grown in 250 ml Erlenmeyer flasks containing 50 ml of E2 mineral medium with different carbon sources. These flasks were incubated at 28 ºC for 48 h at 150 rpm. Cell suspension (10 ml)

was centrifuged at 6,000 rpm for 10 min. The cell pellet was washed once with 10 ml saline and was recentrifuged to get the pellet. The cell pellet was then suspended in 5 ml of sodium hypochlorite (4% active chlorine) and incubated at 37 ºC for 10 min with stirring. This extract was centrifuged at 8,000 rpm for 20 min and the pellet of PHB was washed with 10 ml of cold diethyl ether. The pellet was again centrifuged at 8,000 rpm to get purified PHB.

Effect of salt concentration on PHB production:

For NaCl optimization, culture was incubated in carbon rich nutrient medium with different NaCl concentration *i.e*. 1%,2%,3%, and 4%. Each culture was taken from different flask and PHB production was determined.

Effect of temperature and pH on PHB production:

The inoculated culture in production medium was incubated at different temperature *viz.* 25, 30, 35 and 40°C for temperature optimization. After 72 h of incubation, growth and PHB production was determined and optimum temperature was selected.

For pH optimization, culture was incubated in carbon rich nutrient medium with different pH *i.e.* 4.0,5.0, 6.0 and 7.0. Each culture was taken from different flask and PHB production was determined.

Effect of carbon and nitrogen sources on PHB production:

Effect of media ingredient like carbon sourceson PHB production was determined by simply replacing the carbon sources *i.e* (maltose, glucose, sucrose, and lactose, all at 1.0 g/l concentration).

Effect of different concentration of best nitrogen source on PHB production was determined by simply replacing the different concentration of best nitrogen source*i.e* (ammonium chloride, gelatin, urea, Peptone and yeast extract (1.5 g/l concentration). Each culture was taken from different flask and PHB production was determined. (Soam *et al*., 2012)

FTIR spectrum analysis:

The lyophilized PHB (10mg) sample was mixed with 100mg of dried potassium bromide (kbr) and compressed to prepare as a salt disc. The disc was then read spectrophotometerically (Bio-Rad, FTIR-40-model, USA). The frequencies of different components present in each sample were analyzed.

RESULT

Isolation and identification of bacterial strain:

Different bacterial isolates were obtained from the sediment sample in Vellar estuary. The serial dilution factor 10^{-4}, 10^{-5} and 10^{-6} were used for isolation of bacteria (Fig.1). All the bacterial isolates were screened on solid agar medium using Sudan black (Fig.2). On ager medium, out of 6 isolates, one was selected as PHB producers by Sudan black.The biochemical investigations of isolated bacteria studied were indole test, vogesproskauer test, urea test and citrate utilization test. The result revealed the positive activity for oxidase, catalase and Voges-Proskauer in isolated bacteria (Tabl.1). Accordance of gram stain the isolated bacteria showed gram positive and rod shape like *Bacillus* sp (Fig.3).

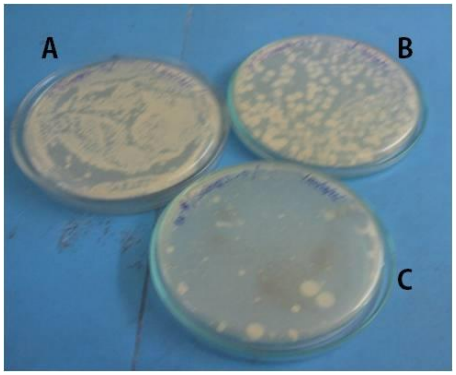

Figure 1: PHB producing bacteria at different serial dilution (A, B & C-10^{-4}, 10^{-5} and 10^{-6})

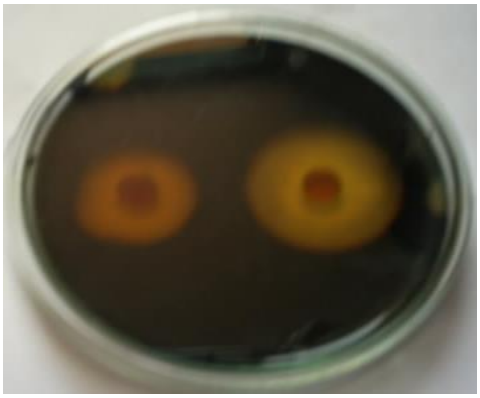

Figure 2: The plate showing the PHB activity by *Bacillus* sp

Biochemical test	**Results**
Gram staining	+
Morphology	Rod
Motility	+
Oxidase	+
Catalase	+
Methyl rad	+
Indole	-
Voges-Proskauer	+
Urea	-
Citrate utilization	-

Table 1: Biochemical test result for isolated bacteria

+ (positive) – (negative)

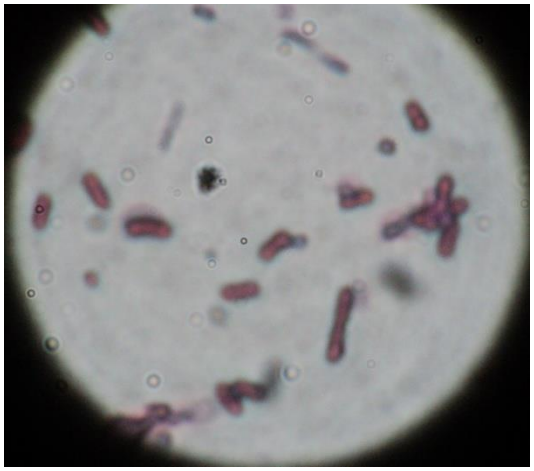

Figure 3: Gram positive*Bacillussp*

Effect of salt concentration on PHB production:

The marine bacteria were isolated from the sediment and the effect of NaCl on PHB production was also tested. The effect of salinity (1%, 2%, 3%, and 4%) on PHB production is given in Fig. 4. Among the five different salinity conditions, 2% NaCl shows higher PHB production (9.7mg/100ml).

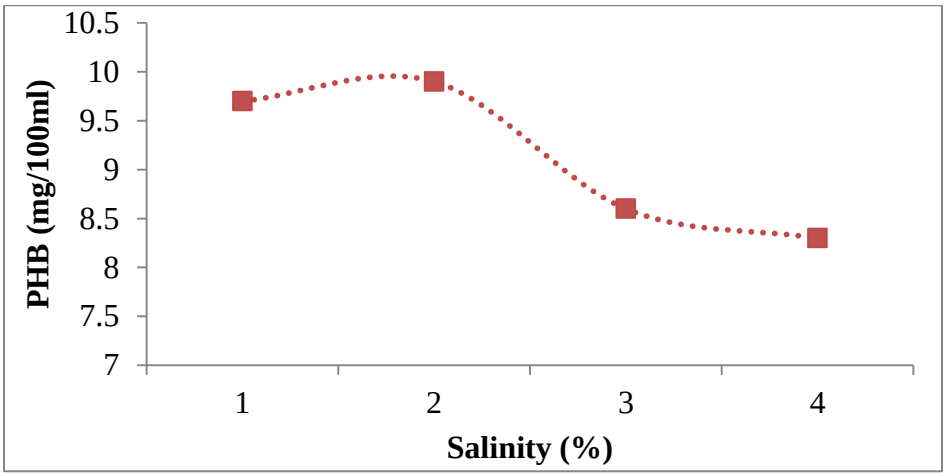

Figure 4: Production of PHB at different NaCl concentration

Effect of different temperature and pH for the production of PHB:

The effect of temperature (25, 30, 35 and 40°C) on PHB production is given in Fig. 5. Among the five different temperature conditions, 40°C shows higher PHB production (10.7mg/100ml) in the liquid media.

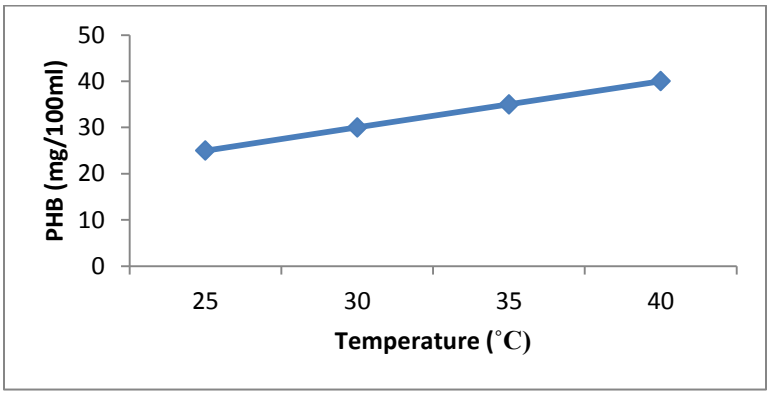

Figure 5: Production of PHB at different temperature

It was evident that pH also significantly influenced the production of PHB. The bacteria were able to release maximum PHB (7.10 mg/100ml) at pH 4.0 after 30 h (Fig.6).

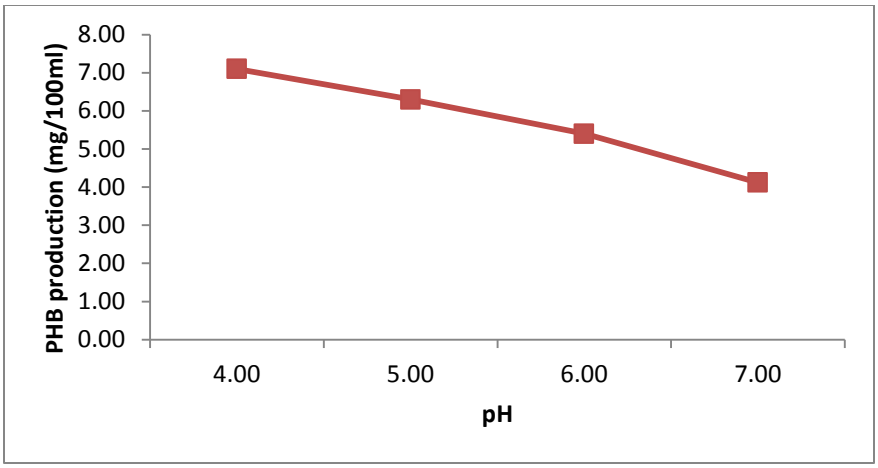

Figure 6: Production of PHB at different pH

Effect different carbon and nitrogen source for the production of PHB:

Five different carbon sources such as,sucrose, maltose, lactose and glucose were tested at 2%for PHB production. The highest level of PHB accumulation (12.8mg/100ml)was observed in the medium with maltoseas carbon source (Fig.7).

Figure 7: Production of PHB at different carbon source

Five different nitrogen sources such as ammonium chloride, urea, gelatin, yeast extract, and peptone were tested for PHB production.The highest level of PHB accumulation (6.3mg/100ml) was observed in the media with yeast extractas nitrogen sources.(Fig.8).

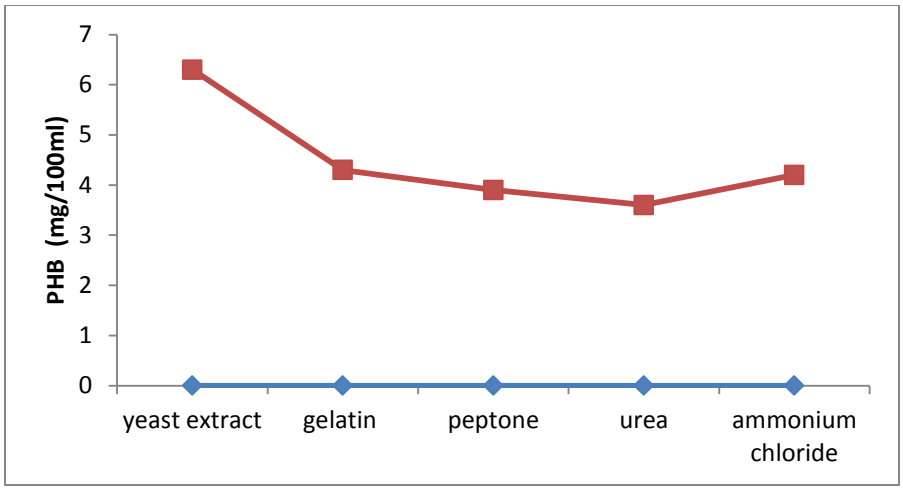

Figure 8: Production of PHB at different nitrogen source

FTIR spectrum analysis:

Fourier Transform Infra Red (FTIR) spectrum of the PHBsample revealed 5 major peaks at 3430, 1651, 1645, 1636 and 649 cm^{-1}, whereas the remaining peaks are closely lying between 3430 cm^{-1} and 649 cm^{-1}.

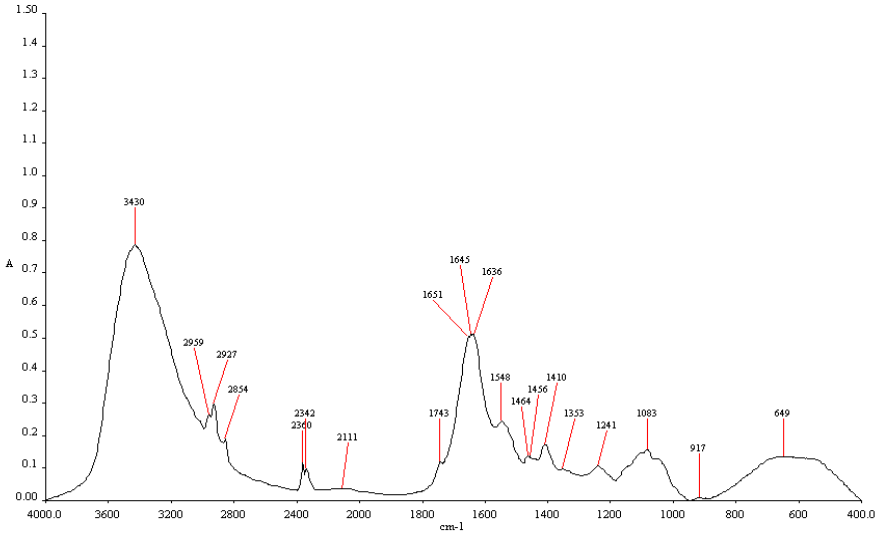

Figure 11: FTIR spectrum of PHB

DISCUSSION

Petroleum derivative plastics are widely used in our daily lives, but they cause environmental pollution because they are persistent for hundreds of years. Because of this, biodegradable polymer production (microbial termoplastics) has gained importance. Further more the continuous depletion of petroleum sources has placed more emphasis on the need for biodegradable microbial plastics. Poly-β-hydroxybutirate (PHB) is an important raw material for microbial plastics. Today, most research efforts in this field concentrate on the isolation of PHB producing microorganisms from different sources and improvement of PHB production abilities of microorganisms (Braunegg *et al*., 1998).

Natural polymers are synthesized and accumulated bya diverse group of microorganisms. hydroalkanoicacids have been detected in some microorganisms andif they are exploited for optimized PHB production this may reduce production cost and help in large scale uses. In the present study, different bacterial isolates were obtained from the sediment of Vellar estuary. On ager medium, out of 6 isolates, one was PHB producers as detected by Sudan black.The biochemical investigations of isolated bacteria studied were indole test, vogesproskauer test, urea test and citrate utilization test. Accordance of gram stain the isolated bacteria showed gram positive and rod shape like *Bacillus* sp. Previously, Benoit (1990) studied that the spores were produced during the stationary phase of *Bacillus* cultures and at a time when PHB was being produced and consumed.

In the present study among the four different salinity conditions, 2% NaCl shows higher PHB production. Previously, Soam *et al*. (2012) showedthat the effect of NaCl concentration on PHB production by the *Bacillus mycoides* (WSS2). The highest PHB yield of 12.3mg/100ml was obtained after 72h of growth. Among the four different temperature conditions, 40°C shows higher PHB production in the liquid media. Previously, the results of PHB yields atdifferent temperature conditions are in accordance with Grothe *et al*. (1999). A lot of studies confirmed maximum PHB production at range of 33-37°C (Tabandeh and Vasheghani, 2003). It was pointed out that PHB production decreased at temperature extremes due to low PHB activity at such temperatures (Tamdogan and Sidal, 2011). Optimization of culture conditions for bio-polymer producing *Bacillus mycoides* bacteria from sewage and temperature optimum PHB production conditions were 45°C(Soam *et al*., 2012). Aly *et al*. (2013) showed that the 37°C was the optimum temperature to produce high amount of polymer in *B.cerrus* MM7. .Elsayed *et al*. (2013) reported that the maximum PHB production was achieved at temperature 37°C.

This study also showing the significance of the pH for production of PHB in *Bacillus sp.*The bacteria were able to release a maximum PHB at pH 4.0 after 30h. Earlier, the effect of pH during cultivation of the strain in liquid state on PHB production was investigated and compared to the range of pH 5-9 (Luengo *et al*., 2003).Grothe *et al*. (1999). reported that pH value ranging from 6.0-7.5was optimum for PHB production by

Alcaligenslatus, but in our study. pH 4.0 is best for PHB production.Nakata (1963) reported that PHB production occurs at pH 6.4 and that the lack of polymer accumulation at higher pH values.

It is well known that any bacteria capable of producing PHB needs excess carbon source in addition to a limited other source such as nitrogen or phosphate (Naranjo *et al*., 2013).In this study the highest level of PHB accumulation was observed in the medium with maltose as carbon sources. Mizuno *et al.,*(2005), in *B. megaterium,* the PHB content in the cell was reached a maximum level when it grown with glucose. Batch-fermentation experiments showed that, *Herbaspirillum seropedicae* Z69 was able to accumulate up to 36% of its biomass as PHB when grown on glucose as a carbon source (Ana Ines *et al*., 2007).The highest level of PHB accumulation was observed in the medium with maltose as carbon sources in *Bacillus mycoides* (Soam *et al*., 2012)

In PHB production, a variety of commercially available nitrogen sources such as ammonium chloride, urea, gelatin, yeast extract and peptone were tested by Page (1989). In this study, the highest level of PHB accumulation was observed in the media with yeast extract as nitrogen sources. Previously, the highest level of PHB accumulation was observed in the media with yeastextract as nitrogen sources in *Azotobacter vinelandii* UWD strain (Page, 1989). Prashad *et al*., (2001) utilized peptone for growth and PHB production by *Azotobacte rchroococcum.* Elsayed *et al*. (2013) estimated that the maximum PHB production percentage per dry weight was achieved using ammonium chloride followed by potassium nitrate.

In the present study the Fourier Transform Infra Red (FTIR) spectrum of the PHBsample revealed 5 major peaks at 3430, 1651, 1645, 1636 and 649 cm^{-1}, whereas the remaining peaks are closely lying between 3430 cm^{-1} and 649 cm^{-1}. The IR spectrum reflects both monomeric units in addition a strong absorption band at 1714cm " was detected in G1S1, as is expected for the C=O.(Shah.K,R.2014). All absorptions due to the PHB moiety appeared in the spectrum, and in addition a strong absorption band at 1639 cm " was detected a thioester bond (Shah,K.R*.,* 2012).

PHA was characterized by FTIR which gave ester containing group which consisted of Polyhydroxybutyric acid (G1S1). *Bacillus* species isolated from the soil samples can be employed in the industrial production of PHA.

The bacteria PHBextracts from the different sourcesshowed significant activity when compared with previous report. They represent potential pharmacological leads perhaps possessing novel and uncharacterized mechanisms of action that might ultimately benefit the ongoing global search for clinically useful biodegradable agents.

It is concluded that the biochemical investigations of isolated bacteria studied were positive activity showed gram positive bacteria rod shape like *Bacillus* sp.Among the different sources optimization conditions salinity (2%) temperature (40˚C) pH (4.0) carbon (maltose) and nitrogen (yeast extract) shows higher PHB

(bio-polymer) production. The Fourier Transform Infra Red (FTIR) spectrum of the PHB sample revealed 5 major peaks at 3430, 1651, 1645, 1636 and 649 cm·On the basis of data obtained in the present work it was concluded that the *Bacillus sp* is capable of producing PHB and that can be used for industrial purpose for the manufacture of biodegradable plastics.

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