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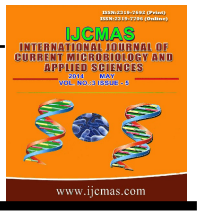

Original Research Article

Optimization and production of Polyhydroxybutarate(PHB) by *Bacillus subtilis* G1S1from soil

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Keywords

Bacillus sp. Biopolymer, optimization PHA, FTIR spectroscopy, Sudan black B

A B S T R A C T

Polyhydroxybutyrate (PHB), a lipid-like polymer of 3-hydroxybutyrate, is a representative member of polyhydroxyalkanoates (PHAs) formed in many bacteria.12 bacterial isolates were found to be the most promising PHA accumulating bacteria among 15 isolates screened from soil. Screening for PHA was done by Sudan black staining. *Bacillus subtilis* G1S1 shows PHA maximum production under the different carbon and nitrogen condition. PHA extraction was carried out by chloroform digestion method. Biochemical and 16s rRNA analysis showed that PHA producing bacteria belong to *Bacillus subtilis* G1S1 genera with Maximum production of PHA was analyzed by U.V spectrophotometer and finally it was characterized by FTIR spectroscopy.

Introduction

Plastics have been an integral part of our lives. From automobiles to medicine, plastics are utilized in almost every manufacturing industry in the world. Approximately 25 million tons of plastics are produced by the plastics industry every year (Wong *et al*., 2002).

The development of biodegradable plastics has become one of the major concerns in the present society because the disposal of the plastics has pointed out their major weaknesses. Plastics are produced from non-renewable resources such as petrochemical and are not compatible with natural carbon cycles because of their non-degradable characteristics.

Biodegradable plastics which also known as green plastics are plastics made from biodegradable polymer materials. Polyhydroxybutyrate (PHB) is a suitable source for biodegradable polymer material because their fully degradability and non-pollutant characteristics. Braunegg *et al*. (1998) defined biodegradability as the capability to be broken down, especially into innocuous products by the action of living things, known as microorganism. Bacteria and fungi are the natural occurring microorganisms that are capable to degrade PHA to carbon dioxide and water through secreting enzymes. It can also be degraded through non-enzymatic hydrolysis.

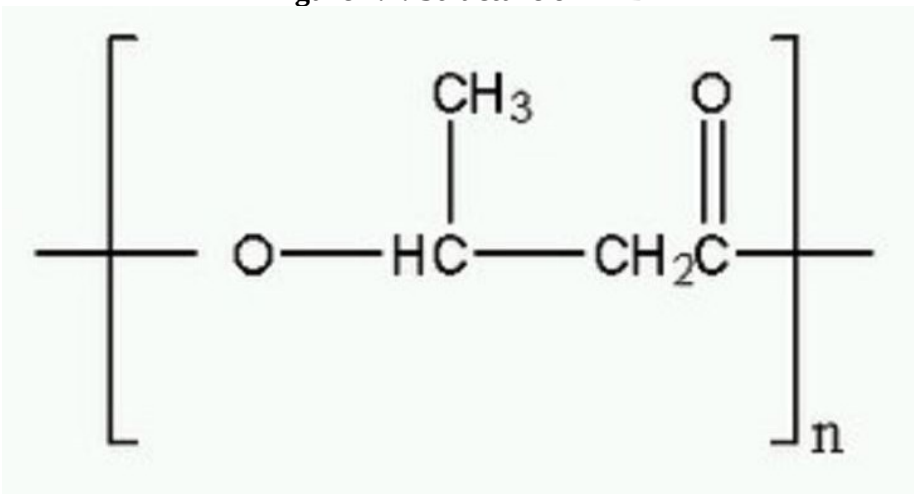
Recently, a large scale production of Poly-β-hydroxybutyrate (PHB) by bacteria has become a subject of increasing interest. PHB is a useful biodegradable polymer which can be used as a thermoplastic (Holmes, 1985). Biopolymers are possible alternatives to the traditional, non-biodegradable petrochemical derived polymers. In terms of molecular weight, brittleness, stiffness and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical-derived thermoplastics, such as polypropylene (Barham, 1990).

Poly-β-hydroxybutyrate (PHB) belongs to the class of biodegradable plastics PHAs. PHB was first among the family of PHAs to be detected by Lemoigne in 1926 as a constituent of bacterium *Bacillus megaterium* (Lemoigne, 1926). Approximately 150 different hydroxyalkanoic acids are at present known as constituents of these bacterial storage polyesters (Steinbüchel and Valentin 1995). Polyhydroxyalkanoates (PHAs), a family of bacterial polyesters, are formed and accumulated by various bacterial species under unbalanced growth conditions. PHAs have thermomechanical properties similar to synthetic polymers such as polypropylene, but are truly biodegradable in the environment (Lee *et al*, 1996). The molecular structure of PHB are describes in Figure 1.1. PHB act as an energy storage facility, and are developed when the bacteria's surroundings include excess carbon, and a deficiency of another nutrient.

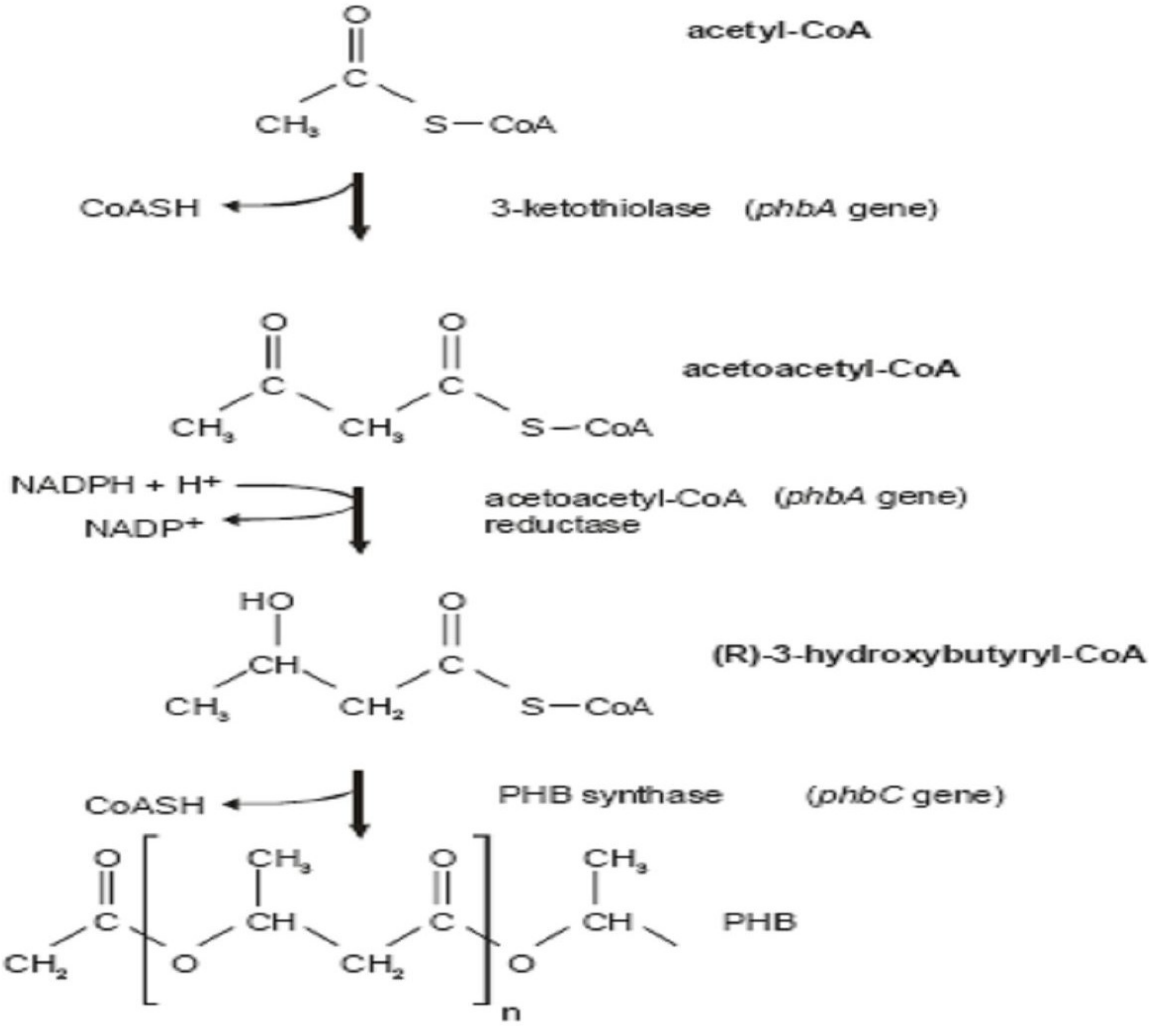
Synthesis route / Production of PHB

PHB are produced by many genera of bacteria as inclusion bodies to serve as carbon source and electron sink. PHB is synthesized from acetyl-CoA produce by the bacteria in sequential action of three enzymes. 3-ketothiolase (phbA gene) catalyses the formation of a carbon-carbon bond by condensation of two acetyl-CoA (Masamune *et al,* 1989) NADPH dependent acetoacetyl-CoA reductase (phbB gene) catalyses the stereoselective reduction of acetoacetyl-CoA formed in the first reaction to R-3- hydroxybutyryl CoA. The third reaction of this pathway is catalyzed by the enzyme PHB synthase (phbC gene) that catalyzes the polymerization of R-3- hydroxybutyryl-CoA to form PHB. The EC number is yet to be assigned to PHA synthase (Steinbüchel et al 1991). Figure 2.2 showed the biosynthetic pathway of PHB from acetyl-CoA PHB is a partially crystalline polymer which has material properties similar to polypropylene (PP) and polyethylene (PE) (Holmes *et al*., 1988; Lee, 1996).Therefore, PHB has been considered as one of the most promising biodegradable plastics and as an alternative to petrochemical plastics. This is due to their biocompatibility, biodegradability and versatile properties make it an eco-friendly substitute for synthetic polymers (Brandl *et al*., 1998). In addition, PHB has more advantages because it is far less permeable than PE and PP, this known as a better material for food packaging needless to use antioxidant. However, industrial application of PHB has been hampered owing to its low thermal stability and excessive brittleness upon storage (Matsusaki *et al*., 2000). Due to the poor physical properties of PHB, the incorporation of a second monomer unit into PHB can significantly enhance its properties. This has led to an increased interest to produce hetero-polymers with improved qualities. The incorporation of 3-hydroxyvalerate (3HV) into the PHB has results in a poly-(3-hydroxybutyrate-co-3-

Figure 1.1: Structure of PHB



Biosynthetic pathway of PHB from acetyl-CoA (Taguchi et al.)



hydroxyvalerate) [P(3HB-co-3HV)] which is more flexible and tougher than PHB, and more easier to degrade when discarded into the natural environment.

Application of PHB

Economic and technological barriers are the main concerns regarding largescale microbial production of PHAs and poly-β-hydroxybutyrate (PHB). Byrom cited that large scale production of poly-β-hydroxybutyrate(PHB) by bacteria has become a subject of increasing interest (Byrom, 1897).Applications focus in particular on packaging such as containers and films (Bucci and Tavares, 2005). It is also processes into toners for printing applications and adhesives for coating applications (Madison and Huisman, 1999*)*. PHB could replace some of the more traditional, non biodegradable polymers. Polymer blends is expected to be more widely accepted. It is cited that such blends will greatly increase the spectrum of possible applications by expanding the range of available physical properties. PHB in combination with other biocompatible and nontoxic polymers would also have an enhanced scope in biomedical applications (Christi *et al*, 1999).

The success in the biodegradable plastic strategy largely depends on the isolation of potent PHA producing bacteria and optimizing culture parameters for maximum PHA biosynthesis. Keeping these points in view, the following objectives were addressed in the present study.

1. Isolation of PHB producing bacteria from soil samples.
2. Screening for high PHB producers from the isolated bacteria.
3. Identification of Bacterial Strain by 16S rRNA Method
4.Optimization of PHB production under defferent carbon and Nitrogen source
5. Characterization of PHA by FTIR Spectroscopy

Materials and Methods

Screening of PHB producing bacteria.

Screening and Isolation of bacterial strains: 1 gm soil samples were inoculated into 250 ml flask containing 100 ml sterile Nutrient broth. Flasks were incubated at 37°C for 24 hrs on rotary shaker at 120 rpm. Isolation was carried out on Nutrient agar plate, MRS plate and Ashby's Mannitol Agar.

Fifteen bacterial colonies were isolated and preserved using nutrient agar medium. All isolates were screened for PHB by staining with Sudan black B stain (0.3 in 70% alcohol and observed under microscope X100x). Hartman (1940) was the first to suggest the use of Sudan black B, as a bacterial fat stain. Subsequently, Burdon et al, (1942a) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan black B solution and counterstained with safranin. The selected isolates were then identified on the basis of their morphological, cultural and biochemical characteristics.

Optimization of culture medium and conditions

To observe the effects of culture conditions for maximum bacterial polymer production, cultures were incubated at different incubation period (4, 8, 16, 24, 30, 36, 40 and 48 h) at 37°C.The production of bacterial polymer under different carbon and nitrogen were also studied using liquid

complete medium Glucose, Fructose, sucrose ,maltose and lactose were used as carbon source, whereas Ammonium sulfate, Malt extract, Peptone and Yeast extract were tested for their ability to utilize nitrogen source.

Cell cultivation

For large-scale growth, Inoculums was prepared in nutrient broth medium at 37^0C and transferred to 500 mL of nutrient broth in a wide-necked 1 L culture flask, incubated at 37°C for 48h with continuous gentle shaking.

Harvesting

After incubation, cells were harvested by centrifugation at 8000 rpm for 12 min, washed in sterile water and recentrifuged similarly. Pellets were collected aseptically, dried at 60°C

PHA Extraction

The PHA was directly extracted using the solvent chloroform. First, the bacterial cultures were harvested and then lipids were removed from the cell pellet-using methanol (40 times the volume of cell pellets) after cells were incubated at 95°C for 1 h. Then it was filtered to remove the methanol completely and the sediment granules were incubated in an oven at 65°C till dry. Chloroform was added to the dried granules and was incubated at 95°C for 10 min. after cooling; the mixture was gently mixed overnight. The solution was then filtered to get the debris. Finally, the PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water. The precipitated PHA was then washed with acetone and dried. It was analyze by U.V. spectrophotometer by using crotonic acid as a standard (Law , Slepecky et al 1961)

Analytical methods

PHA production was estimated as previously described by Law and Slepecky (1961). Briefly, 3 ml of bacterial culture grown in N-free medium was transferred to glass centrifuge tubes (tubes were washed with acetone and methanol to remove plasticizers if plastic tubes were used) and centrifuged at 5000 rpm for 10 min. The cell pellet was suspended in 1 ml of standard alkaline hypochlorite solution and incubated at 37°C for 1 - 2 h for complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHA granules in the sediment were washed twice with three portions of acetone, methanol and diethyl ether, respectively. The polymer granule was dissolved with boiling chloroform and the chloroform was allowed to evaporate. Finally, the granules were mixed with 10 ml of concentrated H_2SO_4 and the tube was capped and heated for 10 min at 100°C in a water bath. The concentration of PHA was determined from an established standard graph in which the absorbance was plotted against the concentration of crotonic acid. The presence of PHA was confirmed by the presence of a peak obtained between 230 - 240 nm (Figure 2).

Fourier transform-infrared spectroscopy (FT-IR analysis)

The PHA extracted from the organism was analyzed by FT-IR spectroscopy (JASCO FT/IR). It was used under the following conditions: spectral range, 4000-400 cm-1 to confirm the functional groups of the extracted polymer.

Identification by 16S rRNA

DNA extraction

The bacteria were grown in (LB) medium at 25°C for 18 h. After growing, they were suspended by mixing with a vortex mixer in suspension buffer, (TE) buffer (10 mM Tris, 1 mM EDTA)in pH in 8.0. Genomic DNA was obtained from pure cultures by lysozyme-proteinase K-sodium dodecyl sulfate (SDS) treatmentfollowed by phenol-chloroform extraction and subsequent ethanol precipitation (Braker et al., 2003). The purity and concentration of the DNA preparations were determined spectrometrically. Electrophoresis of the extracted DNA was carried out on 0.8 % agarose gels at 3.0 Vcm- out on 0.8 % agarose gels at 3.0 Vcm-1 in TAE buffer.

16S rRNA amplification

The DNA amplifications were performed with general methods (Sambrook et al., 2001). Total bacterial 16S rRNA genes were amplified by PCR using the universal primer. The 16S rRNA sequences were compared with all accessible sequences in databases using the BLAST server at NCBI (National Centre of Biotechnology Information). The sequences were aligned with those belonging to representative organisms of the L-subclass of Furmicutes. The strain GISI was assigned to a genus based on the obtained 16S rRNA gene sequence similarities *Bacillus* Sp.

Results and Discussion

Fifteen isolates were isolated from soil of KADI region. With the reference to gram reaction eight isolates shows gram positive and nine isolates shows gram negative (Table 1). Gram staining showed that among 15 isolates, 2 were Gram positive cocci, 8 were Gram positive bacilli and 5 were Gram negative bacilli. 15 bacterial isolates were found to be positive for Sudan black staining which indicated PHA accumulation in bacterial cells. Gram reaction and Morphological analysis revealed that the PHA producing strains belong to *Staphylococcus, Bacillus, Pseudomonas and Escherichia* genera. From These fifteen isolates, AS3-2 gave good biopolymer production which is Bacillus genes with respect of morphological, Biochemical, colony character and 16s rRNA sequencing technique.

The strain of *Bacillus subtilis* GISI was screened for PHB production in Nutrient medium where glucose as a carbon source. Time-course analysis (Figure 1) indicated that PHB was a growth-associated product and its accumulation significantly increased when the culture reached stationary phase (about 48-60 hrs). The maximum values were achieved at 48 h cultivation. After 52 hrs, a slight decrease in the level PHB content. This indicated that the presence of an intracellular PHB depolymerase. Concentration of PHB decreased significantly after 60 hrs cultivation due to nutrient depletion and cells consumption of PHB as a carbon source. The lower total dry cell weight of the bacteria corresponded with high amount of PHA production within 48 hrs cultivation these was similar *Ralstonia eutropha* (recent name *Cupriavidus eutropha*), which accumulated PHB at the stationary phase (Madison et al 1999).

Dry cell weigh

Culture sample (10 ml) was centrifuged (15,000 rmp, 15 min, 4^{o}C) and the cell pellet was washed with deionized water,

recovered by centrifugation again, and dried (105^{o}C, 24 h) to constant weight. *Bacillus* sp.G1S1 gave the highest values for PHB concentration(1.41 ± 0.20 g/l and 55.0% of DCW, respectively) The PHA content was within the range of 50-90% of DCW produced by commercial PHA producing bacteria such as *Cupriavidus eutropha* or recombinant *E. coli* (Steinbüchel, 2001). Chen et al 1991 studied PHA in 11 different *Bacillus* spp. and found PHB consisting 50% (w/v) of dry cell weight of the bacteria.

Optimization of Carbon and Nitrogen

Effect of carbon sources on production of PHB, G1S1 isolates have shown maximum production of PHB in media with fructose and Glucose. However with maltose yield was not significant. Glucose and fructose, being monosaccharides were readily utilizes by bacteria, hence their growth and subsequent production of PHbwas higher. In case of sucrose and maltose, complexity of the carbon increased and hence PHA yields were low. Similar conclusion was made by Chandrashekharaiah et al. However, for the PHB production by *Rhizobium* bacteria, sucrose was found to be better source of carbon

It has been reported that the complex nitrogen sources increased the yield of PHB by *Bacillus megaterium.* Growth conditions including pH, temperature, carbon and nitrogen sources play an important role in the production rate of PHA. G1S1 was gave good PHA production on yeast extract (Table 1) . Similar results were obtained from cultivation of *Bacillus licheniformis, Anaerobiospirillum succiniproducens* and *Phaffia rhodozyma* in the presence of yeast extract, and a combination of yeast extract and peptone (Lee et al. 1999.shah 2012)

16s rRNA sequence

G1S1 was identify by 16s rRNA technique and it is confirmed that it is *Bacillus* Sp. The sequence of this species is approximately 98 % similarity with *B. subtilis* . GenBank: HE577170.1

Similar sequence(s) found in database. < Family of BLAST hit with highest sequence identity: Bacillaceae

Bacillus subtilis subsp. Spizizenii

Family = Bacillaceae; Sequence identity = 98.1%; Alignment length = 964 (86.7%); E-Value = 0.0; Accession = AF074970

Bacillus vallismortis

Family = Bacillaceae; Sequence identity = 98.1%; Alignment length = 997 (89.7%); E-Value = 0.0; Accession = AB021198

Bacillus subtilis subsp. subtilis

Family = Bacillaceae; Sequence identity = 98.1%; Alignment length = 997 (89.7%); E-Value = 0.0; Accession = AJ276351

Brevibacterium halotolerans

Family = Brevibacteriaceae; Sequence identity = 98.1%; Alignment length = 985 (88.6%);
E-Value = 0.0; Accession = AM747812

FTIR Analysis for Functional group identification

The functional groups of the extracted PHA granules were identified as C=O group by FT-IR spectroscopy the results of FT-IR spectroscopy are shown in Figures 3. The IR spectroscopic analysis gave further insights into the chemical structure of the polymer and reflects the monomeric units. In this study, the functional groups of the polymer PHA was confirmed as

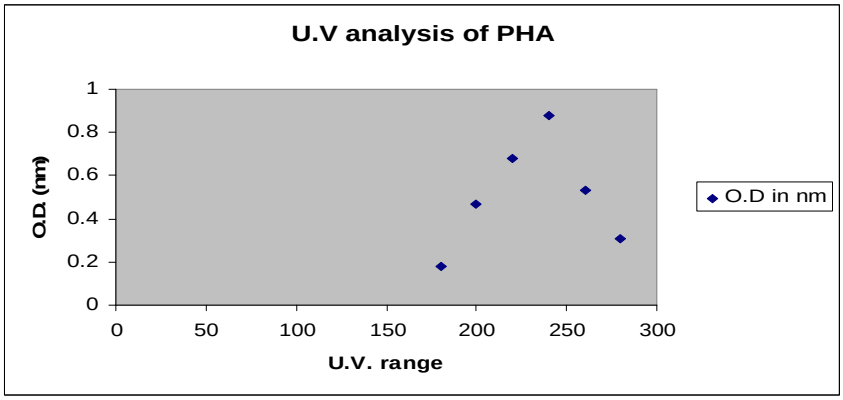
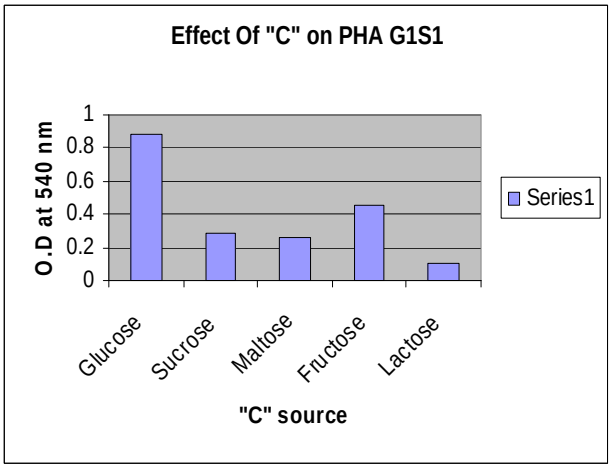
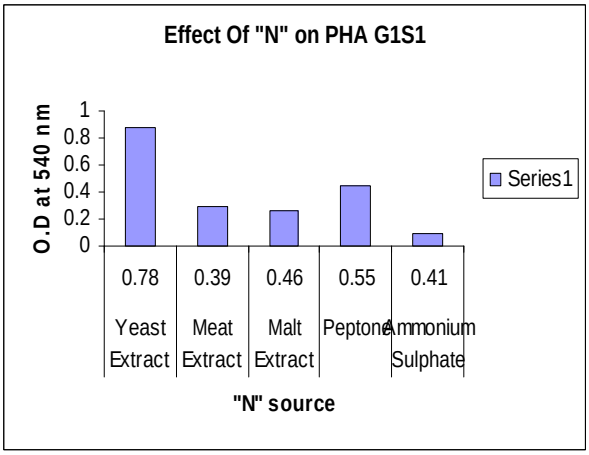


Figure.2 PHA analyses by U.V.

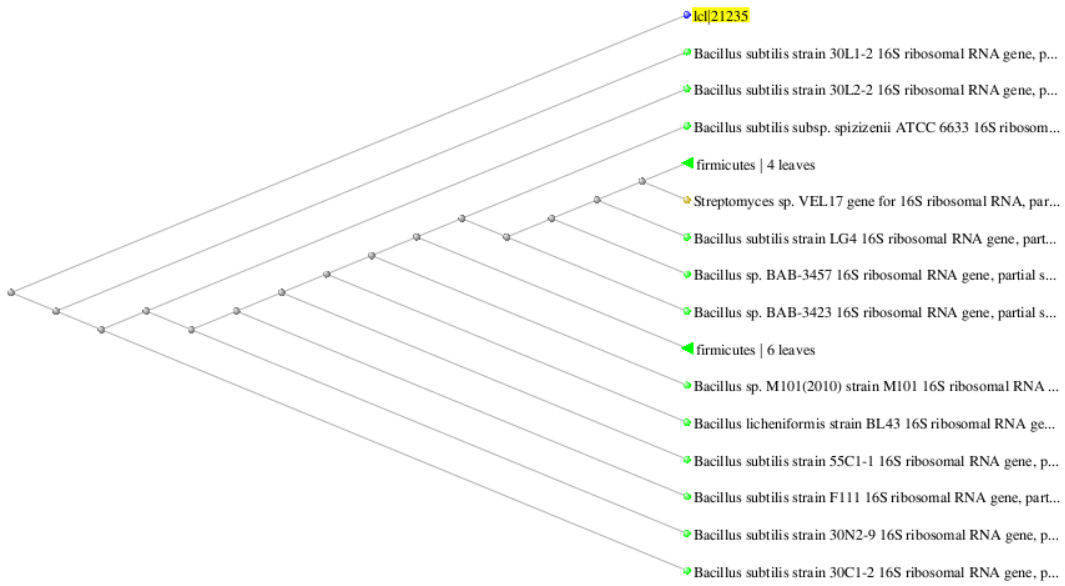
Table.1 Production of PHA of the G1S1 strain in media with different carbon and nitrogen sources

G1S1		
Carbon Sources	**DCW (g/L)**	**PHB (g/L)**
Glucose	0.765	0.020
Sucrose	0.30	0.016
Maltose	0.38	0.014
fructose	0.60	0.018
Lactose	0.10	0.007
Nitrogen sources		
Yeast extract	0.654	0.016
Meat extract	0.30	0.012
malt extract	0.38	0.014
peptone	0.49	0.015
$(NH_4)_2SO_4$	0.14	0.010





Family of BLAST hit with highest sequence identity: Bacillaceae
Bacillus subtilis subsp. spizizenii



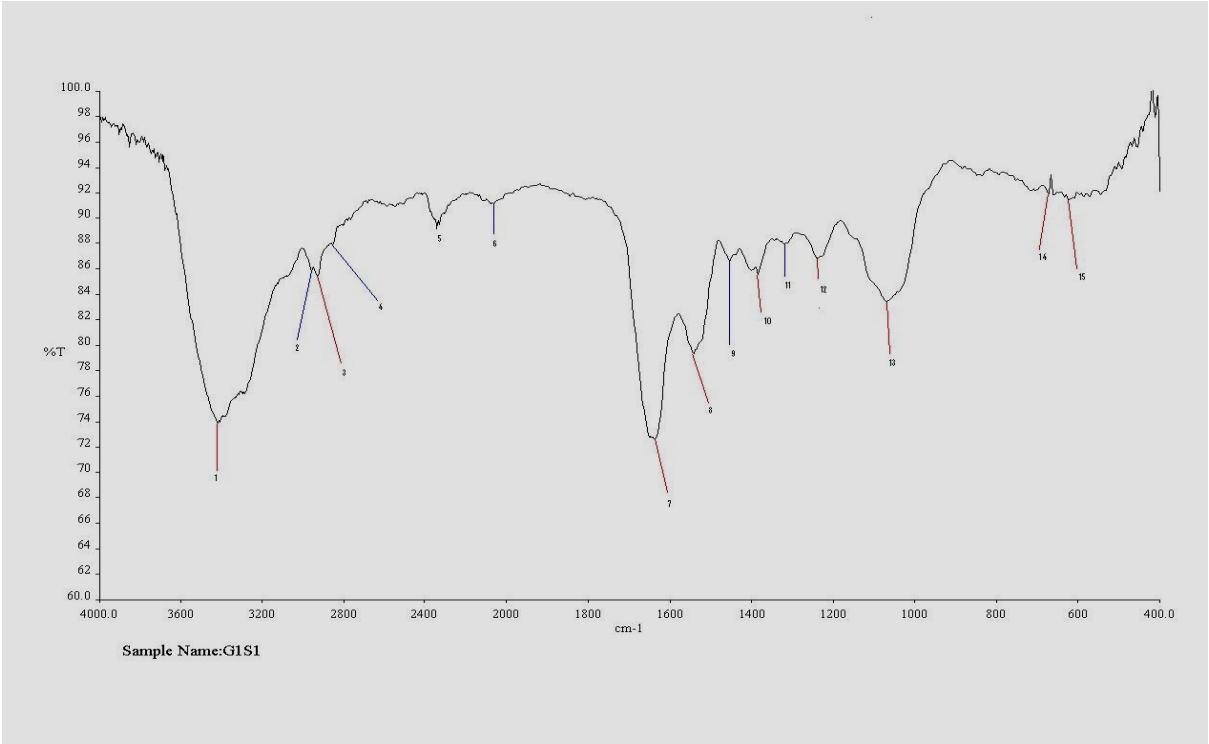


Figure.4 FT-IR spectrum of PHA produced by *B.Subtilis*. Accumulation = 16; Zerofilling = Off; Gain = 2; Resolution
= 4cm-1; Apodization = Cosine; Scanning speed = 2 mm/sec; **1.** 3422.81, 2 2959.01 **3.** 2932.70, **4.** 2852.45,
5. 2465.48, 6 2065. 7 1714.63, **8.** 1419.35, **9** 1384.73, **10.** 1317, 4. 11 1239.3**. 12 1070.46 13 673.3**

C=O groups by FT-IR spectroscopy. The result obtained by this is exactly similar to that of other researchers (De Smet et al., 1983; Castillo et al., 1986) 2983 m (CH,CH_2,CH_3) ; 2933 m (CH, CH_2,CH_3); 1720 (ester C=O valence); 1639 (thioester C=O valence); 1380 m; 1302 m; 1260 m (CH_2-S) ; 1162 s (ester C-O); The IR spectrum reflects both monomeric units in addition a strong absorption band at 1714 cm−" was detected in G1S1, as is expected for the C=O . 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 (ShahKR., 2012).

Among 12 strains of the bacteria , G1S1 identified with 16s rRNA sequence. This bacteria gave the highest value of specific growth with PHA production. PHA was characterized by FTIR which gave ester containing group which consisted of Polyhydroxybutyric acid (G1S1). Bacillus species isolated 1from the soil samples can be employed in the industrial production of PHA.

Acknowledgement

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