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FTIR analysis of polyhydroxyalkanoates by novel *Bacillus sp.* AS 3-2 from soil of Kadi region, North Gujarat, India

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Abstract

Soil sample collected from industrial area of Kadi and Mehshana, India. Twelve 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. PHA extraction was carried out by chloroform digestion method. Biochemical and 16s rRNA analysis showed that PHA producing bacteria belong to *Bacillus* genera with Maximum production of PHA was analyzed by U.V spectrophotometer and finally it was characterized by FTIR spectroscopy.

Keywords : *Bacillus sp.* Biopolymer, PHA, FTIR spectroscopy, Chloroform, Sudan black B

Introduction

Biopolymers may be defined as products which are based on renewable agricultural or biomass feedstock, capable of behaving like conventional plastics in production, utilization and biodegradable through microbial processes. It is progressive development of biopolymers which has led to a surging interest of a plastic and composite industry based on biological materials. Some of the biodegradable plastic materials under development include polyhydroxyl- alkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and copolymers and blends of starch and polypropylene (Lee et al, 1996).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. Biopolymers are possible alternatives to the traditional, non-biodegradable petrochemical derived polymers (Madison et al 1999). 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.

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Economic and technological barriers are the main concerns regarding large scale microbial production of PHAs and poly-β-hydroxybutyrate (PHB) until Byrom (1897) stated the large scale production of poly-β-hydroxybutyrate(PHB) by bacteria. 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. Applications focus in-particular on packaging such as containers and films (Bucci et al 2005).

Keeping these points in view, the following objectives were addressed in the present study.

1. Isolation of PHB producing bacteria from different environmental samples.
2. Screening for high PHB producers from the isolated bacteria.
3. Identification of Bacterial Strain by 16S rRNA Method
4. Characterization of PHA By FTIR Spectroscopy.

Materials and Methods

Screening and isolation of PHB producing bacteria

1 gm soil samples were inoculated into 250 ml flask containing 100 ml sterile Nutrient broth. Flasks were incubated at 37^0C 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.

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 and 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 they were 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 and 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 and 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 strains AS 3-2 were assigned to a genus based on the obtained 16S rRNA gene sequence similarities *Bacillus* Sp.

Results and Discussions

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. Among those 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. 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 sp* AS3-2 was screened for PHB production in nutrient agar medium. 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).

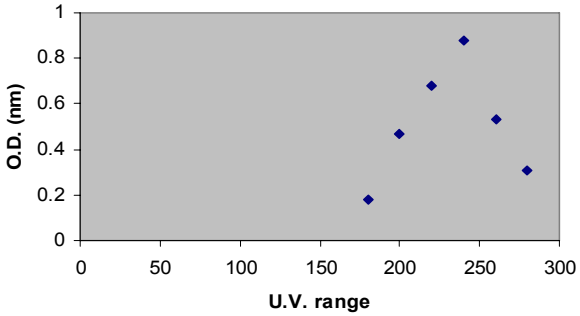

Figure 1: PHA analyses by U.V.

Dry cell weigh

Culture sample (10 ml) was centrifuged (15,000 rmp, 15 min, 4°C) and the cell pellets were washed with deionized water, recovered by centrifugation again and dried (105°C, 24 h) until it reaches constant weight. *Bacillus* sp. AS 3-2 gave the highest value for PHB concentration (1.44 ± 0.20 g/l) and PHB content (59.9% of DCW) 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.

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 2. 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 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 1639 cm−" was detected in AS3-2, as is expected for the C=O valence vibration of a thioester bond (Colthup et al. 1964) The intensity of this band was proportional to the sulfur content, which was determined by elemental analysis, as is shown in the inset in Fig.6. The IR spectroscopic the chemical structure without a previous hydrolysis of the polymer. PHAs with modified backbones have already been identified. These PHAs consisted of 2- methyl-3-hydroxybutyric acid (Satho et al. 1992; Fuchtenbusch et al. 1996) or 3-hydroxypivalic acid (Fuchtenbusch et al. 1998) and contained one or two methyl groups at the *a*-carbon atom

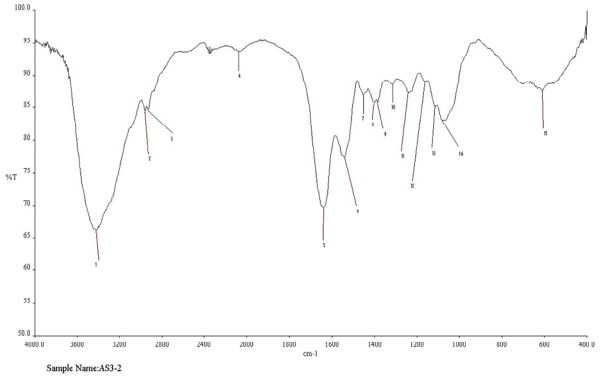

Figure 2: FTIR Analysis FT-IR Spectrum of PHB produced by *B.licheniformis AS3-2.* Accumulation =16; Resolution = 4cm-1 Zerofilling = Off;Apodization = Cosine Gain = 2; Scanning speed = 2 mm/s; 1). 3425.89 , 2). 2977.4, 3). 2934.42,4). 2073.77, 5). 1639.94, 6). 1541.44, 7). 1452, 65, 8). 1402.04. 9) 1384 10). 1315 11). 1240 12). 1162 13). 1113 14). 1082 15). 611

Identification 16S RRNA

To describe the isolated bacterial diversity, a molecular approach based on the sequence variability of the 16S rRNA gene was used. The results of the above biochemical tests along with the phylogenic identification of the bacterium using 16S rRNA sequence analysis, proved that the genus of the bacterium was *Bacillus* and close to the *licheniformis* sp (Table 1). In 16S rRNA sequence analysis, more than 99% similarity was observed between the sequences of the isolate strain and the sequences of *Bacillus lichenifomis* AS 3-2 in EMBL Data Center and Accession No is HE576762 (figure 3)

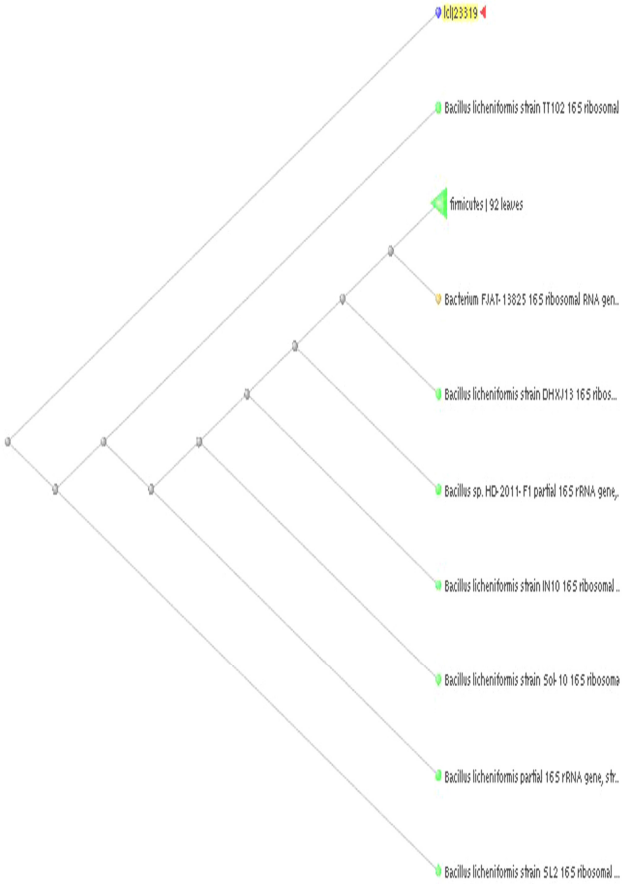

Figure 3: Phylogenetic tree constructed using BLAST output (AS3-2 (Sequence under study); ACWC01000034.1, NC_006270.3, NC_006322.1, NC_014639.1,AEFY01000003.1, AEFX01000001.1, AEFW01000019.1, AEFV01000003.1, AEFU01000002.1, AEFT01000022.1, AEFS01000001.1, AEFR01000025.1, AEFP01000019.1, AEFO01000022.1, AEFM01000008.1, NC_016047.1, NC_014551.1)

Conclusion

B. licheniformis AS3-2 may be an attractive candidate for production of biodegradable plastics. PHA was characterized by FTIR which gave thioester containing group which might be contain 2- methyl-3-hydroxybutyric acid. *Bacillus* species isolated from the soil samples can be employed in the industrial production of PHA. When compared to the values reported in the literature, the amount of PHB accumulated in lactic acid bacteria was generally lower than that accumulated by the soil bacteria *Ralstonia eutropha* (Bertrand et al., 1990), *Bacillus* species (Yilmaz et al. 2005), *Pseudomonas cepacia* (Celik et al. 2005) and *Rhizobium meliloti* (Tavernier et al. 1997)**.**

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