Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PRODUCTION OF EXOPOLYSACCHARIDE AND POLYHYDROXYBUTYRATE BY NEWLY ISOLATED *BACILLUS* AP03 FROM INDUSTRIAL EFFLUENTS

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ABSTRACT

An exopolysacharide (EPS) along with internal granules of polyhydroxybutyrate (PHB) producing bacterium *Bacillus* sp AP 03 *was* isolated and identified from the industrial effluents. It was motile,aerobic, Gram positive, rod shaped, endospore forming and catalase producing bacterium. The isolated organism produced exopolymer and PHB in E_2 medium consisting of glucose, nitrogenous substances. IR and GC spectra confirmed the presence of PHB and HPLC analysis confirmed the presence of EPS. Optimum conditions for polymer production were determined. Antimicrobial activities were also studied.16S rRNA analysis revealed that isolate AP03 had 99 % identity to *Bacillus cereus* and shared many similarities in terms of biochemical reactions and microscopic observations. The sequence of the 16S rDNA gene of strain AP 03 was determined (1341 bp) and deposited in the Gene bank sequence database under accession no. FN663621.

KEYWORDS: ***Bacillus*** **sp., Exopolysaccharide, PHB, EPS**

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INTRODUCTION

Polyhydroxyalkanoic acids (PHB) are polyesters synthesized from optically active thiol esters that accumulate as granular inclusions in the cytoplasm of various bacteria[1, 2]. *Bacillus* spp have adapted successfully in harsh environmental conditions, to the stress and nutrient imbalance etc. Hence isolation of *Bacillus* sp and its characterization for PHB synthesis was undertaken. There are advantages using *Bacillus* spp as PHB production hosts as they grow fast can produce copolymers from single carbon substrate and they can utilize cheaper carbon sources for polymer synthesis. They synthesize industrially important enzymes such as proteases, lipases and amylases. The genome data for some *Bacilli* are available so it is easier to manipulate them genetically. A vast number of *Bacillus* spp can be isolated from the environment, which may produce varied quantity and quality of the polymer. The synthesis of intracellular PHB is significantly increased by the addition of various complex nitrogen sources[3]. A variety of *Bacillus* spp are known to produce PHB[4, 5]. Generally *Bacilli* produce single chain length PHB, which comprises of PHB of C4-C6 and a few strains are reported to the ability of some members in the genera to produce medium chain length polymer also[6, 7].Bacteria produce a wide range of extracellular polymeric substances composed of polysaccharides, proteins, nucleic acids and lipids. EPS are often classified as capsules, sheaths or slimes dependent upon their proximity or attachment to the cell wall[8]. Cost-effective production process and high polymer yield is one of the main targets of many research groups worldwide[9, 10]. Therefore, this study was aimed to benefit from the unplanned exopolymer produced from the strainAP03 which also produces PHB. Bacteria can concomitantly produce different polymers (e.g., EPS and PHB) in different amounts[11].In fact the structure, composition, and viscosity of EPS depend on several factors, such as the composition of the culture medium, carbon and nitrogen sources and precursor molecules, mineral salts, trace elements, type of strain, and fermentation conditions such as pH, temperature, oxygen concentration, and agitation[12].Exopolysaccharides (EPS) have found multifarious applications in the food, pharmaceutical, and other industries. Both extremophilic microorganisms and their EPS suggest several biotechnological advantages, like short fermentation processes for thermophiles and easily formed and stable emulsions of EPS from psychrophiles[13, 14, 15].Many species of microorganisms such as Gram-positive and Gram-negative bacteria, archaea, fungi and some alga are known to produce extracellular polysaccharides. EPS is a term first used by Sutherland[16] to describe high-molecular weight carbohydrate polymers produced by marine bacteria.

EPS are high-molecular-weight polymers that are composed of sugar residues and are secreted by microorganisms into the surrounding environment. The *Bacillus* have developed various adaptation strategies, enabling them to compensate for the deleterious effects of extreme conditions, high temperatures and salt concentrations, low pH or temperature, and high radiation. Among these strategies, the EPS production is one of the most common protective mechanisms. In their natural atmosphere, most bacteria occur in microbial aggregates whose structural and functional integrity is based on the presence of a matrix of extracellular polymeric substances and the EPS production seems to be essential for their survival[17]. EPS possess a protective nature and form a layer surrounding cells provide an effective protection against high or low temperature and salinity or against possible predators.

MATERIALS AND METHODS

(i) Bacterial strain

Different samples of industrial effluents were collected, mucoid colonies were selected and screened for PHB-producing bacteria and have

been described elsewhere[18] .Bacterial strain AP03 was isolated on Luria Bertani (LB) medium (Hi media, India).

(ii) Medium and cultivation

The selected isolate was grown in 250 ml Erlenmeyer flask containing 50 ml E2 mineral medium[19], modified by adding 2% beef extract, 1% Nacl and 2 % (w/v) glucose as carbon source. The flasks were incubated at 28° C for 48 h on scientific environmental shaker at 150 rpm. For PHB and exopolymer production, 24 h grown bacterial cultures in LB broth was used as the starter culture adjusted to a cell concentration (optical density, OD660) of 0.5 was transferred into the fresh medium. The culture was taken every 4 hrs for growth and exopolymer measurements. Growth was determined in terms of turbidity by using a spectrophotometer (Hitachi U-2000) at a wavelength of 600 nm. Both cell dry weight (CDW) and PHB quantification were determined gravimetrically. The PHB content was defined as the ratio of PHB concentration to cell concentration given as percentage.

(iii)Biochemical characteristics

Growth and biochemical characteristics, carbon assimilation and sensitivity of cultures to different antibiotics were determined by previously described methods[20]. Biochemical characteristics were also checked with the Hi25 Enterobacteriaceae identification kit (KB003) and HiCarbohydrate kit parts A, B and C (KB009) (both from HiMedia) according to the manufacturer’s protocol. The isolate was tested for antibiotic sensitivity, using the antibiotic assay medium. Neomycin, Penicillin, Novobiocin, Ampicillin, Nalidixicacid, Nitrofurazone, Kanamycin, Oxytetracyclin and polymixin B were the antibiotics used for the assay.

(iv) Molecular phylogeny of Bacillus spp AP 03

The DNA amplification involved the primer set 8 forward and 1492 reverse, (5’AGAGTTTGATCCTGGCTCAG3’) (5’ACGGCTACCTTGTTACGACTT3’) (Numbers corresponding to the positions in *E. coli*) [21] flanking a region of 16S ribosomal DNA. Primers were purchased from Bioserve India and were suspended in Tris buffer (10mM, pH 8.5). All amplifications were run on a DNA Engine gradient (BioRad).

(v) Phylogenetic Analysis

The sequences of 16S rDNA genes were compared with those from the GeneBank database using the NCBI Blast program. Sequences were aligned using the ClustalW program[22].Genetic relationships were determined by the neighbor-joining method with the MEGA4 program[23] using nucleotide sequences of the 16S rDNA gene. To determine the degree of statistical support for branches in the phylogeny, 1,000 bootstrap replicates of data were analyzed. The gene sequences that were >97.0% identical to sequences of species in the NCBI database were assigned genus names[24].

(vi) Estimation of biomass

The microorganism was grown aerobically in 250 and 500 ml Erlenmeyer flasks with 50 ml of the culture medium and incubated in a rotary shaker at 150 rev/min and 30 °C during 48 h.

(vii) Preparation of exopolymer

After removing the cell pellet by centrifugation, 5 mL of the supernatant was precipitated with 3 volumes of chilled ethanol. The precipitated exopolymer was then re-dissolved in distilled water and dialysed against distilled water overnight using the membrane with 10,000 daltons molecular cut-off. Suspensions were centrifuged, and supernatants were decanted and mixed with previous supernatants and pressure filtered through cellulose nitrate filters. EPS was precipitated by adding three volumes of chilled ethanol to filtrates and incubating overnight at 4°C. EPSs were recovered by centrifugation, and to remove impurities, it was dialysed for 48 h using 8 kDa MW cut-off dialysis bags against distilled water at 4 °C for 24 h. An aliquot (1 mL) was transferred to preweighed tubes, dried overnight, and

weighted. EPSs were lyophilised and stored at –20 °C until further analyses[25]

(viii) Accumulation of PHB (microscopic method)

The presence of cytoplasmic PHB inclusions in strainAP03 was evidenced by Nile blue staining and observed the cells under the fluorescence microscope[26].The stained smears were observed under Olympus microscope with fluorescence attachment using a green filter. The PHB granules appeared bright orange against a dark back ground.

(ix) PHB extraction and purification

For the extraction of PHB, 300mL of the cells were harvested by centrifugation at 5000×*g* and then lyophilized. The following methods were then employed. The PHB extracted from the cell pellet by the hypochlorite method[27], was washed with methanol and acetone consecutively and centrifuged at 8000 rpm for 20 min. The polymers were then dissolved in hot chloroform (60°C) and the solution poured into glass trays. The chloroform was allowed to evaporate slowly by placing the trays in the cold room at 4°C. The film of PHB so obtained was used for further analysis.

(x) Analytical methods

Microbial growth was monitored by measuring the cell density of the culture at 600 nm after suitable dilution with distilled water. Organic nitrogen in the samples was estimated following its mineralization with hot sulphuric acid. PHB quantification was quantitated according to the method of Law and Slepecky (1961), whereby the dried pellets containing intracellular PHB were hydrolysed using concentrated sulfuric acid for 1 h to obtain crotonic acid, which was quantified by measuring absorbance at 235 nm. Analysis was performed in triplicates for shake flask samples. Cell dry weight (cdw) was measured by lyophilizing harvested cells from 3 ml culture broth. PHB content and its composition were determined by gas chromatography using PHB standards. Cell concentration was defined as cell dry weight per litre of the culture medium[28].

(xi) Gas chromatography (GC)

PHB was quantified using a slight modification of the gas chromatographic method of Riis and Mai (1988).Instead of whole cells, pure, extracted PHB was used. The precipitated polymer was weighed in tightly sealable vials (volume 10 ml). Two ml of 1,2-Dichloroethane (DCE), 2 ml n-Propanol containing hydrochloric acid (HCl) (1 volume concentrated HCl + 4 volume n-Propanol) and 200 µl internal standard (2.0 g benzoic acid in 50 ml n-Propanol) were added. The mix was incubated for 4 h in a water bath at 850C. The mixture was shaken intermittently. After cooling to room temperature, 4 ml water were added and the mixture shaken for 20 – 30 s. The heavier DCE-Propanol phase was collected and injected directly into the gas chromatograph. Quantitative evaluation was affected by means of the peak areas of standard polymer and benzoic acid[29].

(xii) Infrared spectroscopy

For FT-IR analysis, the PHB was precipitated from the chloroform using cold ethanol.The Precipitated polymer was used to prepare KBr discs (sample: KBr, 1:100). An FT-IR spectrum 1720X (spectrometer) (Perkin Elmer, USA) was used under the following conditions: spectral range, 4,000–400 cm–1; window material, CsI; 16 scans; resolution 4 cm–1; the detector was a temperature- stabilized, coated FR-DTGS detector.

RESULTS

1. Morphological and biochemical characterization

Morphological, growth and biochemical studies were performed using standard methods[30,31]. Nutrient agar was used for growth, maintenance of the strain and the determination of the phenotypic characteristics. Gram-positive rods, 1.0 x2.0 - 3.0µm, peritrichously flagellated, occurring singly. Motile, ellipsoidal endospores are located subterminally.Cells grown on media containing 2% glucose have polyhydroxyalkanoate granules in the

cytoplasm. Circular colonies may be cream or white, mucoid with entire edges, about 1±2.5 mm diameter. Colonies capable of accumulating PHB were identified by examining the fluorescence was considered as potential PHB producer (figure.1). Microscopic investigation of the strain, stained with Nile blue A was conducted to confirm the presence of PHB. PHB granules fluoresced as bright orange, with individual granules often visible within a cell. The fluorescent micrographs showed these fluorescing orange granules as an intense bright image within the cells. The strains were found to accumulate granule inclusions by phase contrast microscopy.

Table 1
Taxonomic characteristics of strain Bacillus sp. AP 03

Characteristics	*Bacillus* sp. AP 03	Metabolic activity	*Bacillus* sp. AP03
Shape	Rod	Nitrate	+
Size (μ m)	1.0 x2.0 - 3.0	VP	+
Motility	+	Citrate	-
Gram's character	+	Indole formation	-
Endospore	+	H_2S formation	+
Catalase	+	Methyle red	-
Oxidase	-	Arabinose	+
Lipase	+	Xylose	+
Cellulase	-	Adonitol	-
Amylase	+	Cellobiose	+
Urease	+	Saccharose	+
Protease	+	Glucose	+

FLOURESCENCE MICROSCOPY

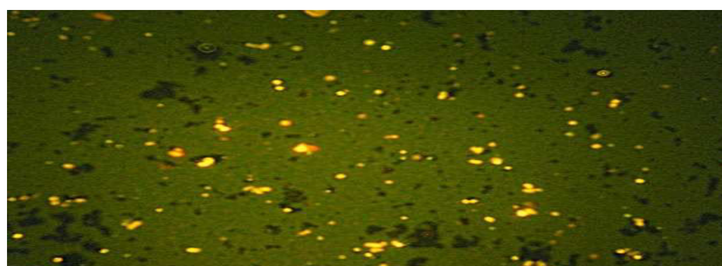


Figure1
Flourescence micrograph of Bacillus sp. AP 03

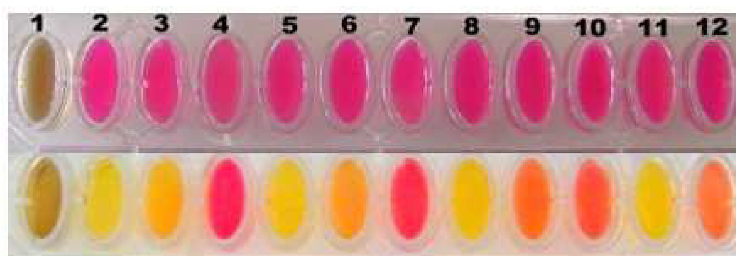
BIOCHEMICAL TESTS

Figure 2
Biochemical tests of Bacillus sp. AP 03

1. ONPG; β galactosidase; 2. Lysine decarboxylase; 3. Ornithine decarboxylase; 4. Urease; 5.Phenylalanine deamination; 6. Nitrate reduction 7. H_2S production; 8. Citrate utilization; 9.Voges Proskauer's; 10. Methyl red; 11.Indole and 12.Malonate.

CARBOHYDRATE UTILIZATION TESTS

Figure 3
Carbohydrate utilization tests of Bacillus sp. AP 03



Positive-Yellow; Negative-Pink 1.Esculine; 2.Arabinose; 3.Xylose; 4.Adonitol; 5. Rhamnose; 6.Cellobiose; 7.Melibiose; 8.Saccharose; 9.Raffinose; 10.Trehalose; 11.Glucose and 12.Lactose

Cells are strictly aerobic, catalase- positive, oxidase- negative, and urease positive. Positive for Ornithine and H_2S production. Does not produce indole (table.1). Egg-yolk amylase, urease,protease and lipase reactions are positive. Voges±Proskauer test are positive, methyl -red, and ONGP are negative (figure.2). Produces acid from arabinose, d-glucose, *meso*-inositol, d-mannitol, dmannose, d-rhamnose, trehalose, raffinose and xylose, but not from esculine, melibiose, adonitol. Phenylalanine deaminase reaction is negative (figure.3). Growth occurs at 10±45 °C. Optimal growth temperature is about 30 °C. Growth occurs in the presence of 10%NaCl (table.1). Cells are resistant to penicillin G (10 IU ml^{-1}), ampicillin (10 µg ml^{-1}), and oxytetracycline (50 µg ml^{-1}) but sensitive to neomycin (50 µg ml^{-1}), novobiocin (5 µg ml^{-1}), nalidixicacid (50 µg ml^{-1}), nitrofurazone (30µg ml^{-1}), and kanamycin (30 µg ml^{-1}) (figure.4).

ANTIBIOTIC SENSITIVITY TESTS

Figure 4
Antibiotic sensitivity tests of Bacillus sp. AP 03

Key: 1.Neomycin; 2. Penicillin; 3.Novobiocin; 4.Ampicillin; 5.Nalidixic acid 6. Nitrofurazone; 7.Kanamycin and 8.Oxytetracyclin

Time-course analysis revealed that PHB was a growth-associated product and its accumulation significantly increased when all cultures reached the exponential phase (after 18 hrs) till stationary phase (about 40-50 hrs). The maximum values were achieved at 48 hrs cultivation. After 50 hrs, a slight decrease in the level of dry cell weight coincided with a small decrease in PHB content during decline stage. This indicated that the presence of an intracellular PHB depolymerase and PHB concentration decreased significantly after 50 hrs cultivation due to nutrient depletion and cells consumption of PHB as a carbon source. Over all, production of PHB was increased with the increase of EPS.

2. Phylogenetic analysis

The sequence of the 16S rDNA gene of strain AP 03 was determined (1341 bp) and deposited

in the Gene bank sequence database under accession no. FN663621. A phylogenetic tree (figure.5) demonstrated that the isolated strain was a member of the genus *Bacillus*, and it formed a monophyletic lineage. 16S rRNA analysis for bacteria identity revealed that bacteria AP03 had 99 % identity to *Bacillus cereus* and shared many similarities and difference in terms of biochemical reactions and microscopic observation.

PHYLOGENETIC TREE

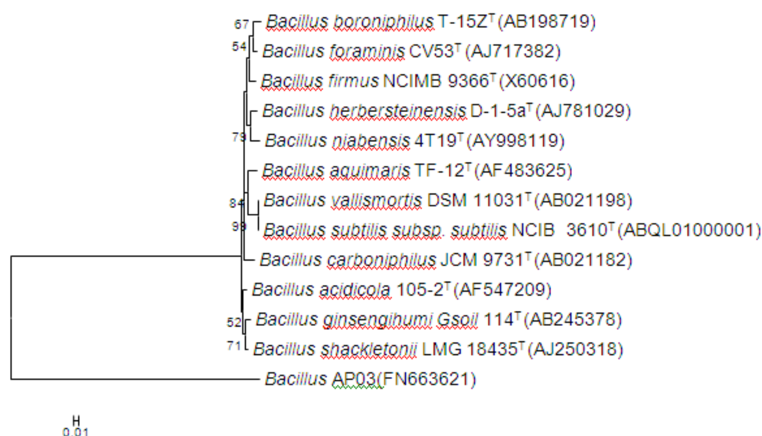


Figure 5

Phylogenetic tree based on 16S r DNA sequences of members of the genus Bacillus, showing the location of strain Bacillus sp. AP 03.Gene bank accession numbers are provided in parenthesis. Scale bar represents 0.01 substitutions in nucleotide sequence

3. Polymer analysis

IR spectra were recorded for the PHB dissolved in chloroform. Spectra showed two intense absorption bands at 1,734 and 1,279 cm^{-} 1, corresponding to C = O and C–O stretching groups, respectively. Other absorption bands at 1,445, 2,923 and 3,445 cm-1 corresponding to -CH2, -CH and O–H groups are shown in figure 6. In GC spectra, two major ester peaks were found for the PHB isolated from strain OUAP03, with retention times of 6.5, 9.5 min (figure 7).These were responsible for the presence of Benzoic acid which was used as internal standard and 3-hydroxy butyrate. The EPS was isolated, subsequently, was subject for further identification. EPS production was observed at all stages of culture growth. Partial purification of EPS was performed and characterized as glycoprotein. HPLC analysis (data not shown) of the polymer revealed the presence of a glucose chain. The EPS was soluble in water and insoluble in tested organic solvents and could able to flocculate over a wide range of pH (pH 3 to 9) and temperature (5 to 50°C) tested in the presence of CaCl2. Thus, it is possible that this glycoprotein along with PHB could be substituted for a commercial polymer with respect to flocculation.

DISCUSSION

Morphological and phylogenetic analyses have clearly confirmed that strain AP 03 is a member of the genus *Bacillus.* The optimal temperature for growth was between 28 and 30°C, and the optimal pH was 7.2. Phylogenetic analysis of 16S rDNA demonstrated that this bacterium is grouped with *B.subtilis*, a well-defined taxon.

IR SPECTRA

Figure 6
IR spectra of polymer from o Bacillus sp. AP 03, grown on Glucose

GC SPECTRA

Figure 7
GC spectra of polymer from Bacillus sp. AP 03, grown on Glucose

Among the carbon sources tested with the strain AP03, glucose and sucrose were found to be the choice of substrates for the polymer accumulation. The EPS production by any bacterial culture is known to be influenced by various factors such as nature and availability of carbon, nitrogen, aeration, and genetic potential. Monosaccharide, disaccharides, polysaccharides etc. are known to influence the growth of EPS producing capacity of different bacterial genera. Further, the composition of EPS produced by a large number of microorganism is well understood. Read and Costerton[32] have reported that the composition of EPS produced is influenced by the carbon sources employed in the growth. According to Rodrigues and Bhosle[33] among various carbon sources such as glucose, sucrose, fructose, mannitol, galactose, glycerol employed in the medium, glucose supported maximum EPS production. EPS were extracted and purified from bacteria AP03 as they produced mucous colonies. The utilization of different carbon sources by different microorganisms suggests the ability of these organisms to produce various polymeric substances that are soluble or insoluble.The results indicate that at pH 3, the EPS produced is minimal followed by pH 5. pH 9 showed the best EPS production. *Bacillus* sp. AP 03 showed the maximum production of EPS at pH9. This clearly indicates that the bacteria prefer alkaline conditions more than acidic. Therefore, *Bacillus* sp. AP 03 could be an interesting candidate for industrial production of biopolymers. Coproduction of EPS and PHB requires a regulation pathways and cell growth stage. Wang and Yu[34] studied EPS and PHB coproduction in R. eutropha and showed that in the early exponential phase, the PHB in the biomass was very high, while the concentration of EPS present in the culture broth was very low. In the stationary phase, the EPS

concentration increased while the initially stored PHB decreased. On the other hand, A chroococcum grown with different carbon and nitrogen sources showed high amounts of both polymers, principally in the presence of sucrose and ammonium nitrate[35].Our results show that in strain *Bacillus* sp. AP 03, the production of large quantities of EPS did not negatively affect the accumulation of PHB, thereby leading to a rise in PHB accumulation reaching 52% in glucose-supplemented medium. However, beside PHB production, it was able to synthesize extracellular polymer in elevated amount.

CONCLUSIONS

This study concludes that *Bacillus* sp. AP 03 could be an interesting candidate for industrial production of biopolymers. The strain *Bacillus* sp. AP 03 showed large variation in regard to their ability to produce the polymers evaluated. Furthermore, the isolate *Bacillus* sp. AP 03 presents the capacity of produce both polymer in high amounts, which suggests a very interesting biochemical regulatory mechanism, which must be investigated. Therefore further study is needed to explore the newly identified strain *Bacillus* sp. AP 03 for commercial production of these polymers.

ACKNOWLEDGEMENTS

Authors thanks to Osmania University and JNTU,Hyderabad, Govt of India, for facilities provided.

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