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Polyhydroxyalkanoate (PHA) production from *Amphibacillus* sp.

Pushpa S.Murthy*, M.Madhumathi

Plantation Products, Spices and Flavour Technology Dept., Central Food Technological Research Institute, Council of Scientific and Industrial Research, Mysore-570020, Karnataka, (INDIA)

Tel : 91-821-2512352; Fax: 91-821-2517233

E-mail : Pushpamurthys@yahoo.com

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ABSTRACT

Many bacteria accumulate Polyhydroxyalkanoate (PHA) as an energy resource. The microbial thermoplastics are regarded as potentially useful polyester replacing petroleum-derived thermoplastics. Polyhydroxy butyrate (PHB) is the best-known member of the PHA series of polyesters. PHB producing strain of *Amphibacillus* sp was isolated from polymer industry soil and identified by comparison with keys given in Bergey's manual of determinative bacteriology. The initial yield of PHA was 39.90 ± 0.5% in the production medium. Different carbon and nitrogen sources were tested for PHB production by this bacterium Maximum yield of PHA was observed with glucose (82.20 ± 0.4 %) followed by fructose (47.09 ± 0.51%) and mannose (47.09± 0.4%), arabinose (41.42 ± 0.5 %). Sucrose, being a cheaper substrate, was used for further studies. Among the nitrogen sources beef extract and tryptone promoted PHB synthesis with 42.42 ± 0.3 % and 41.65 ± 0.02 % respectively. Growth conditions for production of PHA by the *Amphibacillus* sp were studied. The optimized conditions with 30 ± 0.5^{0}C of temperature and pH 7.0 ± 0.1 with 3 % inoculum at 250 rpm at 48 h of fermentation using sucrose as the carbon source yielded 67.03 ± 0.5% of PHB. The PHB produced was analyzed and confirmed by FTIR and NMR.

KEYWORDS

Polyhydroxyalkanoate (PHA);
Amphibacillus sp.;
Polymer industry soil,

INTRODUCTION

Many bacteria accumulate polyhydroxyalkanoate (PHA) as an energy resource[21]. PHAs have physical and chemical properties similar to those of synthetic polymers and are also biodegradable[11]. PHA accumulation usually occurs in the presence of excess carbon and with a limitation of one essential nutrient such as nitrogen, phosphorus, sulfur, magnesium, potassium or iron[14]. Efforts are being made to make this process economically feasible by further understanding the poly-β-hydroxybutyrate (PHB) accumulation process and improving the productivity. A number of *Bacillus* sp, have been reported to accumulate 9-44.5% dry cell weight (DCW) PHA[1,5,7,1113,22,19]. Biotechnological processes for the fermentative production of poly (3HB) and poly (3HB-co3HV) using strains of *Alkaligenes latus* and *Ralstonia eutropha* have been established since late 1970s for the manufacture of various products. To obtain a novel isolate that can grow on a cheaper

substrate, we have attempted to isolate bacteria that accumulate PHA from a soil. In this paper, we describe the isolation and characterization of gram-positive bacterium *Amphibacillus* sp which had 40-80 % PHA productivity and influence of nutritional and environmental conditions on the growth and PHB accumulation.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used in the studies were of analytical grade obtained from standard companies.

Isolation of bacterial strain by enrichment technique

Various bacterial species were isolated from the soil located near the polymer industry located near Mysore, India. The selected isolates were purified by dilution–streaking to obtain single colonies, and were grown in nutrient medium in order to produce large amount of cells. After 24 h, the cells were inoculated (10% v/v) to a sterilized liquid culture medium 100ml (gl^{-1}): $Na_2HPO_42H_2O$, 2.2, KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_47H_2O$, 0.2; Sucrose, 20; pH 7.The inoculated flasks were incubated at 30^0C at 250 rpm for 24h. Each of the purified isolates was screened for PHA accumulation after 24 hours of the growth and stained with Sudan black stain to detect the presence of intracellular PHA granules. Only the isolates, which could synthesize PHA, was selected and studied for PHA production.

PHA production

The PHA production was carried out in duplicate in 500ml Erlenmeyer flasks in a liquid medium (100ml) mentioned under inoculum preparation. Overnight grown cultures of the isolated bacteria were inoculated and incubated at 250 rpm and 30^0C for 72h. The cell biomass was collected by centrifugation. The cell pellet was washed with distilled water and dried. The PHA content was determined after sodium hypochlorite hydrolysis and chloroform extraction. PHA (%) was defined as the percentage of the PHB to dry cell weight (DCW). The isolate that produced maximum yield of PHA was considered for further investigation. The stock culture was maintained at 4^0C.

Characterization of the bacterium

The morphological and physiological properties of the isolate were investigated according to Bergeys manual of determinative bacteriology[6].

Structural analysis

The polymer analysis of PHA were carried by FTIR and NMR. Along with the standard PHB (Sigma,Germany). The FTIR was performed using perkinn Elmer, FTIR spectrometer, Spectrum-2000. The IR source with 400-4000cm^{-1} intensity was used. The NMR spectra of PHA were obtained in deuterated chloroform ($CDCl_3$) for protons using BRUKER AVANCE 500 ultra shield spectrometer. The ^{1}H NMR spectra were recorded using standard PHB with a 30 pulse, 10.50μs, 10,000Hz spectral width and a 3 sec repetition rate.

Production of PHA from various carbon and nitrogen sources

Different sugars like sucrose, glucose, fructose, galactose, lactose, xylose, maltose, arabinose, some of the sugar alcohols such as dulcitol, mannitol, were investigated for the PHA production by the isolated strain. Likewise organic and inorganic nitrogen sources such as beef extract, yeast extract, peptone and tryptone, potassium nitrate, ammonium nitrate, ammonium phosphate were also studied with sucrose (2.0%) along with mineral salts and trace elements were incubated for 48h of fermentation at 30^0C at 250 rpm. The cell mass and PHB content was determined.

Optimization of PHA production

The influence of pH, temperature, duration, aeration and inoculum was determined to obtain maximum yield of PHA production from the bacterial strain. Various pH levels i.e. 6.0,6.5,7.0 and 7.5 was studied. Fermentation temperature at 30, 37 and 42^0C and aeration for bacterial growth in terms of shaking (250rpm) and static conditions along with different fermentation time at 24, 48 and 72h was investigated. Also the inoculum concentration at 1, 2, 3 % were studied with sucrose as the carbon source to obtain the best yield of PHA. The PHA production was estimated as mentioned earlier.

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TABLE 1 : Morphological and taxonomical characteristics of amphibacillus sp.

Characterization morphology	Observations
Shape, Size (μm), Motility	Medium Rods, 1.2×2.0 – 3.0, motile
Gram staining, Sudan Staining, Spore formation	+, +, +
Features of colonies: (Plate) Features of the colony	Small, circular, whitish cream
Pigmentation	No pigmentation
Margin, Elevation	Irregular, Flat
Growth in Nutrient broth	Uniform with less turbidity
Flocculent	Evenly dispersed in medium
Pellicle / sediment	Sediment
Growth in Nacl:3%,5%,7%,10%	Faint growth only in 3%
Growth on Macconkey	-
Optimum pH and temperature	7.0, 30°C
Physiological Characteristics /Biochemical properties	-
Catalase	-
Indole	+
MR-VP	++ --
Starch, Citrate production, Oxidase	-
Casein, Nitrate reduction	-
TSI, Litmus reduction, Gelatin, H_2S	+
Aerobic Conditions	+
Utiltisation of sugars: Arabinose, Sucrose, Starch, Glucose, Fructose, Galactose, Inulin, Maltose, Arabinose, Dulcit, Rhamnose ,Mannose, Xylose, Lactose, Mannitol, Nanonoic acid, Hexanoic acid, Heptanoic acid	+

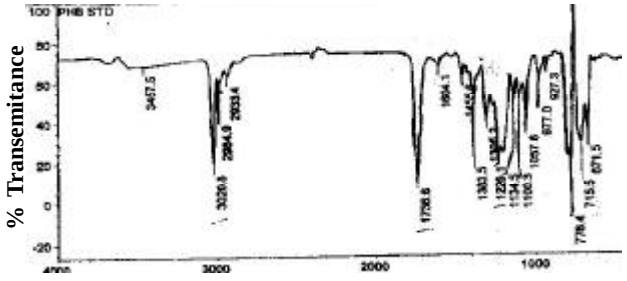
Positive: + and Negative: -

RESULTS AND DISCUSSION

Screening of bacteria accumulating PHA

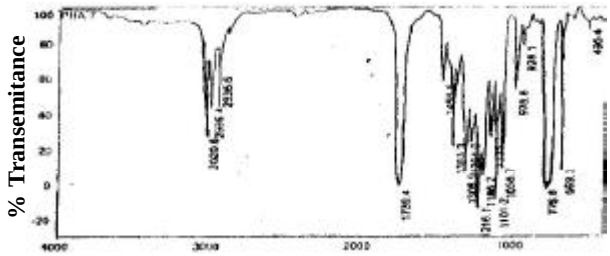
We attempted to isolate bacteria that accumulate PHA from polymer industry soil. Forty-six strains were isolated by enrichment culture method and were classified according to their morphology and their ability to synthesis PHA was determined. The isolate with the best yield was further studied for nutritional and biochemical characterization.

The isolated strain was gram positive, rod shaped cell (1.22μm-2.0μm) and motile. The bacterium could grow and accumulate PHA with optimal growth temperature at 37°C. Based on the results obtained from various biochemical test performed (TABLE 1) and Bergeys manual of determinative bacteriology, the strain was identified and designated as *Amphibacillus* sp.



Wavenumbers(cm^{-1})

Figure 1: FTIR of standard PHB



Wavenumbers(cm^{-1})

Figure 2: FTIR of PHB from Amphibacillus sp

Production of PHA

Amphibacillus sp produced 39.90 ± 0.3% dry cell weight (DCW) of PHA in the production media with respect to dry cell mass. Sucrose was rapidly utilized during the growth and PHB accumulation was observed. *Amphibacillus xylanus* has been reported to produce PHA[12]. No other species apart from *A.xylanus* is reported. In the recent past, species of the genus *Amphibacillus* such as *A. sediminis, A.fermentum, A.tropicus* are reported[23] as a new isolates isolated from sea and sludge. Our isolate with genus *Amphibacillus*, could be a new species and is potential producer of PHA and is of industrial interest.

Structural analysis

The FTIR analysis of the polymer suggests very prominent peak at the 1724-1760 cm^{-1}. The major peaks obtained from our sample corresponds to 1739.4 cm^{-1}, which is very close to peak 1740cm^{-1}ester carbonyl groups when compared with the standard PHB spectrum (Figures 1 and 2). This indicates that the sample contains PHB ester groups. The results of the NMR

indicate that the peaks corresponding to methyl proton appear as a doublet at δ 1.28 (d, 3H, J = 6.0 H z), one methine proton appears as a multiplet at 5.27 and two methylene protons appear at 2.62 (dd, 1H,J=7Hz & 15.5Hz) and 2.49 (dd, 1H,J=5.5 Hz and 15.5 Hz) as double doublets in the spectrum of the PHB that was synthesized from sucrose (Figure 3) .

Influence of carbon source and nitrogen source on cell growth and poly-β- hydroxybutyrate accumulation

Carbon sources serve three different functions such as biomass synthesis, cell maintenance and for PHB polymerization. Among the carbon sources tested glucose, fructose, mannose galactose and sucrose were found to be most suitable for growth and PHB accumulation (Figuer 4). Xylose, lactose, starch, rhamnose, dulcitol, inulin, hexanoic acid supported growth but were only moderately utilized for PHB accumulation. Maximum PHA accumulation was 82.20 ± 0.4 % dry cell weight in case of glucose. The pentose sugar (xylose and arabinose) also supported PHB accumulation. However the PHA accumulation of PHB was observed to be growth associated.

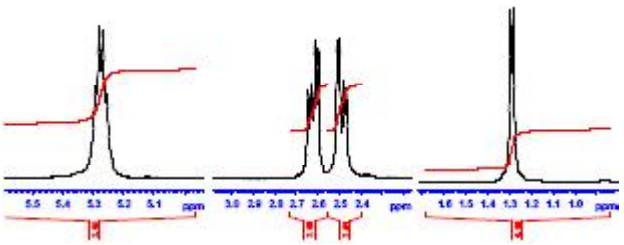


Figure 3: 1H NMR of the PHB produced by Amphibacillus sp

As sucrose is a cheap substrate it was used as carbon source for PHA production. It was reported earlier that *Alcaligenes latus* produced 50% of PHA when sucrose was used as a carbon source[4]. Maximum PHB production was obtained with glucose, sucrose and fructose as carbon source in case of *Bacillus mycoides*[3].

The efficiency of PHA synthesis obviously depends on the type of nitrogen source used[2]. Nitrogen is a component of protein, enzymes and nucleic acid and increased utilization would benefit overall formation of the cell. The addition of an organic nitrogen source increased PHB accumulation. The maximum PHB yield and productivity was obtained when beef extract was added to a medium containing sucrose (Figure 5). Also tryptone and ammonium carbonate supported good PHB yield. The enhancement of PHB accumulation may be due to the presence of amino acids and peptides in beef extract. Nitrogen sources have found to have impact on biosynthesis and PHB accumulation. Similar enhancement of PHB accumulation by *A.vinelandii*, *A.chroococcum* and *E.coli* has been reported when the organisms were grown in media con-

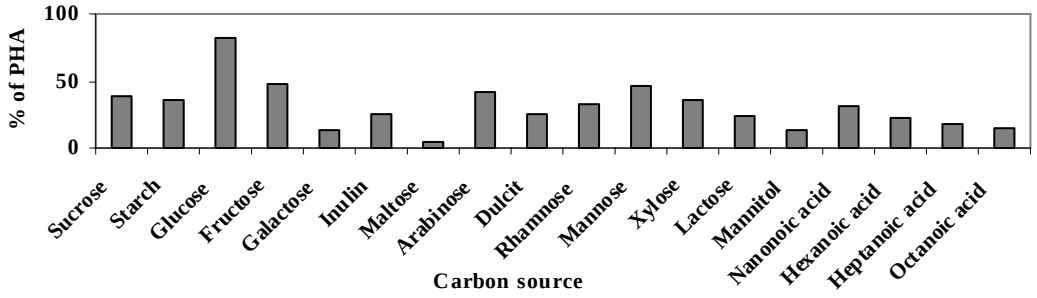


Figure 4: Effect of carbon source on PHA production

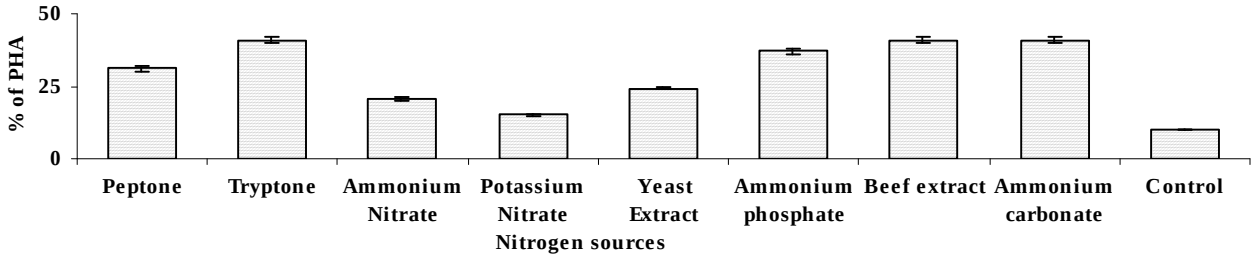


Figure 5: Effect of nitrogen source on PHA production

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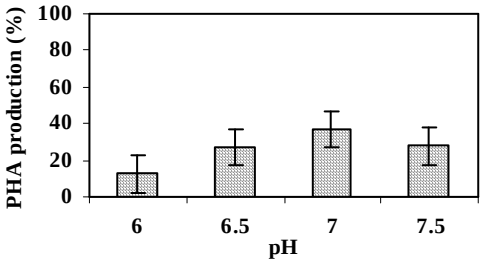

Figure 6 : Effect of pH on PHA production

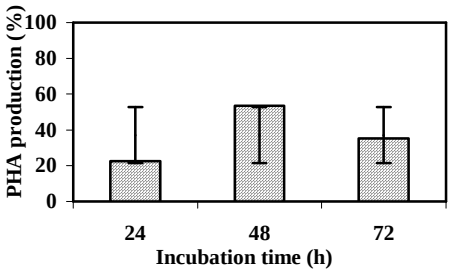

Figure 7: Effect of fermentation time on PHA production

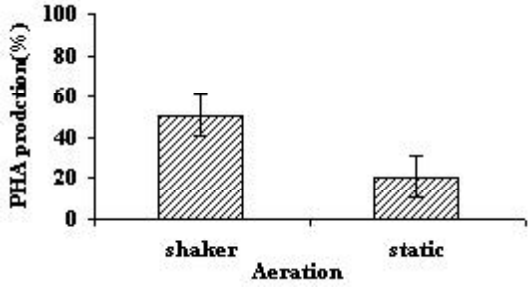

Figure 8: Effect of aeration on PHA production

taining organic nitrogen sources[10,15,16,17,18,20]. The addition of ammonium nitrate and yeast extract promoted cell growth, but PHB synthesis was suppressed. Maximum cell growth and PHB accumulation of 42.42% were obtained when supplemented with beef extract. However, the addition of inorganic nitrogen sources tested suppressed PHB accumulation. Influence of nitrogen source on PHB production will be useful for industrial applications of the strain, since it can synthesis polymers rich with ammonium containing wastes by products.

Optimization of PHA production

The influence of pH was effective on the growth of the cells and PHB yield. Maximum PHB was obtained at pH 7.0 ± 0.1. (Figure 6). The temperature required for the maximal PHB yield and productivity was found to be 30^{0}C ±0.5. With respect to incubation time, good yield was obtained after 48h of incubation period in shaking flasks at 30^{0}C (Figure 7). Prolonged incubation to 72h showed decrease in PHB yield. Though the biomass was more at 72 hrs, the PHA content was less. Our strain accumulates PHA during the stationary phase and requires limitation of N, P, Mg and oxygen and excess carbon sources (data not shown). Similarly earlier reports of Lee et al.,[11] indicates that maximum PHB accumulation at 48 h by fed batch culture.

With regard to aeration, the fermentation flasks on a shaker at 250 rpm resulted in twice the yield of PHB than in static condition (Figure 8). Earlier report also states that good percentage of PHA is obtained in shake flasks[3]. The PHA production was comparatively high with 3% inoculum with 69.7± 0.3% than that of 1 and 2 % with 7.2 and 16.0 % respectively. High inoculum concentration (250mg/l) using the mutant strain of the *Rhizobia* has reported to yield high PHA[8].

CONCLUSION

Polyhydroxyalkanoates have been attracting considerable attention as biodegradable substitutes for conventional polymers. Results from this study contributed significantly to our objective of seeking new isolate on the production of PHA from *Amphibacillus* sp isolated from soil with 66.03% of PHA in the medium containing sucrose with inoculum concentration of 3% at pH 7.0 and 30^{0}C temperature at 250 rpm. Maximum yield of 82.20% of PHA was obtained when glucose was used as a carbon source. This strain has been considered as potent organisms for industrial interest.

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