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RESEARCH ARTICLE

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Utilization of sugarcane molasses for the production of polyhydroxyalkanoates using *Bacillus subtilis*

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ABSTRACT

Batch cultures of *Bacillus subtilis* AMN1 was screened for the production of polyhydroxyalkanoate (PHA) using sucrose as a carbon source. In subsequent experiments, sugarcane molasses was used as renewable carbon source since it was rich in sucrose (50%), leading to develop a low cost process of PHA production. The maximum PHA production (2.09 g/100 mL) and dry cell weight (DCW) production (1.5g/100 mL) was observed in the medium amended with 10% sugarcane molasses. The influence of pH on PHA production was also evaluated. It was found that pH 7.0 yielded a maximum DCW of 1.89 g/100 mL and PHA of 1.39 g/100 mL. The UV spectral analysis of PHA showed the presence of ester group (CH_3-COO) by recording a sharp peak between 230–240 nm. The Fourier transform infrared spectrum of the synthesized PHA showed a transmission band at wave number 1726 cm^{-1} which is a characteristic of C=O stretching of carbonyl group of the polymer. The major compounds of PHA was detected and identified as aliphatic polymer esters using Gas chromatography mass spectrometry. The prominent peaks of proton and carbon Nuclear magnetic resonance spectra of the polymer depicted that it can be hydroxyl hexanoate.

Keywords: *Bacillus subtilis*, biopolymer, fermentation, PHA, sugarcane molasses.

1. INTRODUCTION

The accumulation of petrochemical plastic wastes in the environment is a major problem as it takes several decades to decompose and also produces harmful toxins while degradation. In order to find alternative materials, researchers have identified fully biodegradable plastics, such as polyhydroxyalkanoate (PHA) from biotic communities. It has been established that material properties of PHA are similar to polypropylene [1]. The structure of PHA composed of 3-hydroxy fatty acids as shown in Figure.1. The value of n in the figure depends on the pendant group and the organisms in which the polymer is produced. The most common chemical structure of PHA is given in Table.1. Due to the similarity of chemical and physical properties with the conventional plastics, and full biodegradability, PHA constitutes one of the best alternatives for synthetic polymers replacement [2].

PHA is synthesized by both prokaryotic and eukaryotic organisms [3]. Plants and bacteria are mainly used for the large-scale production of PHA. However, plant cells can only cope <10% (w/w) of dry weight because >10% of dry weight retard

growth and development rate of plants [4]. In contrast, bacteria can accumulate PHA at levels as high as 90% (w/w) of the dry cell mass [5]. PHA accumulation in bacteria is a natural way to store carbon and energy, when nutrient levels are imbalanced or an excess amount of a nutrients are present in the environment [6]. The PHA is insoluble in water and thus the polymers are accumulated as intracellular granules inside the bacterial cells. It is beneficial for bacteria to store excess nutrients inside the cytoplasm, especially as bacteria physiological fitness is not affected [4].

Figure 1. Structure of Polyhydroxyalkanoate (PHA)

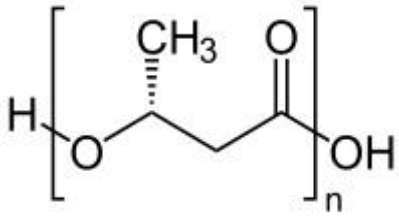


Table 1. Chemical structure of PHA

R-group	Full name	Short
CH_3	Poly(3-hydroxybutyrate)	PHB
CH_2CH_3	Poly(3-hydroxyvalerate)	PHV
$CH_2CH_2CH_3$	Poly(3-hydroxyhexanoate)	PHH

PHA has been commercially produced by fermentation process using several bacterial species such as *Escherichia coli*, *Bacillus* sp., *Alcaligenes latus*, and *Ralstonia eutropha* [7-9]. Although high yield (>75%) content was obtained in fermentation process, the production costs of PHA is still high when compared to synthetic polymers production. The best way to reduce the production costs is the utilization of low cost raw materials, such as industrial wastes, agriculture wastes and by-products as carbon source. Several low cost raw materials such as milk whey, starchy wastewater, agro-industrial oil waste, etc have been used as a carbon source for the production of PHA by fermentation techniques [10-12]. However, limited studies have been focused on the application of sugarcane molasses for PHA production using *Bacillus subtilis*. Thus, the objective of the present study was to (i) assess the potential of sugarcane molasses for PHA production using *Bacillus subtilis* AMN1, (ii) characterization of synthesized PHA, and (iii) assess the experimental variables affecting PHA production.

2. MATERIALS AND METHODS

2.1. Bacterial strain

The *Bacillus subtilis* AMN1 was collected from the Department of Environmental Science, PSG College of Arts and Science, Coimbatore, India. The culture was maintained in Luria Bertani (LB) agar (Hi media, India) slants at 4°C.

2.2. Screening for PHA production

The *B. subtilis* AMN1 was cultured in LB broth (50 mL) supplement with 0.5% (w/v) sucrose as carbon source. The flasks were incubated at 30°C for 48 h on an orbital shaker at 150 rpm. After incubation, the cells were stained with Sudan Black B according to Wei et al. (13). Briefly, a thin smear was made on a clean glass slide and heat fixed. This slide was initially flooded with 0.3% (w/v) Sudan Black B (in ethylene glycol) for 15-20 minutes, immersed in xylene, and blot dried with absorbent paper. Later, the smear was counter-stained with safranin (0.5% w/v) for 10 sec, rinsed with tap water, blot dried, and examined under a microscope.

2.3. Extraction and quantitative assay of PHA

PHA was extracted from *B. subtilis* AMN1 by hypochlorite method according to Slepecky and Law [14]. Briefly, the cells were separated by centrifugation at 6000 rpm for 5 min. The cell pellet was re-suspended in a volume of sodium hypochlorite equal to the original volume of medium. After 1 h incubation at 37°C, the lipid granules (PHA) were centrifuged and washed with water, acetone and ethanol. The polymer was dissolved by extraction with three small portions of boiling chloroform and the filtrate was used. The excess of chloroform was evaporated, and 10 mL of concentrated H_2SO_4 was added and heated in a water bath (10 min at 100°C) to convert PHA into crotonic acid. PHA was determined as crotonic acid and quantified by spectrophotometry at 235 nm.

The LB broth was also used to calculate residual cell mass (RCM), dry cell weight (DCW) and PHA content. The cell pellet was dried to estimate the DCW in units of g/L. RCM was estimated by evaluating the difference between DCW and dry weight of extracted PHA. The RCM was calculated to determine the cellular weight and accumulation other than PHA. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight as follows:

Residual cell mass (g/L) = DCW (g/L) – Dry weight of extracted PHA (g/L)

PHA accumulation (%) = [Dry weight of extracted PHA (g/L) × 100%] / DCW (g/L)

2.4. Carbon substrate and shake-flask fermentation

Sugarcane molasses were procured from the sugar industry and pre-treated to remove colorants. The mid-log phase culture (10^8 cells/mL) of the isolate was aseptically inoculated in sugarcane molasses medium and flasks were incubated in a shaking incubator (150 rpm) at 30°C for 48 h. The PHA production was analyzed by monitoring the optimization parameters. Experimental variables considered were pH (3-11) and (ii) dosage of molasses (10-100%). The optimized condition of 10% molasses and pH 7.0 was used for mass production of PHA.

2.5. Characterization of PHA

The initial cell disruption was quantified with UV spectroscopy (14). The polyesters content of the cell and the composition of polyesters were determined by using Gas chromatography-mass spectrometry (GC/MS) analysis [15]. The Fourier transform infrared (FTIR) spectra was recorded on polymer films cast from chloroform solution on a spectrometer (Shimadzu, Model 8400S) in a diffuse reflectance mode at a resolution of 4 cm-1 in KBr pellets [9]. The structure and mole fractions of hydroxy alkanoates were investigated in 100MHz 1H nuclear magnetic resonance spectroscopy (NMR) (JEOL Ltd, Tokyo, Japan- spectrometer) and ^{13}C NMR spectra at 27°C on a $CDCl_3$ solution of polyester.

3. RESULTS

3.1. Sudan Black B staining, PHA extraction and quantification

The PHA granules (dark colored) were observed inside the *B. subtilis* AMN1 and it indicates that bacteria were capable of PHA production. The bacterial DCW of 2.96 g/100mL produced a maximum biopolymer of 1.5 g/100mL at neutral pH range (7.0), with 0.5% sucrose as carbon source and 0.04% ammonium sulphate as nitrogen source.

3.2. Effect of molasses concentration and pH on DCW and PHA production

The effect substrate concentration on PHA production is shown in Table.2. It is evident from results that maximum DCW and PHA production were observed only when 10% of molasses was amended in the medium. The DCW was 2.09 g/100 mL and PHA concentration was 1.5 g/100 ml. The percentage of PHA produced by using 10% sugarcane molasses was 75.5%. Similarly the effect of medium (molasses amended) pH on DCW and PHA production was recorded and the PHA production was observed according to increasing concentration of sugarcane molasses. The changes in pH from optimum value of 7.0 also showed a decrease in the production of PHA (Table.3).

3.2. Characterization of PHA

PHA granules extracted with the boiling chloroform method were dissolved in concentrated sulfuric acid and UV spectrum was recorded between 200 and 800 nm (Figure.2). The presence of PHA was confirmed by the presence of a peak obtained between 230 - 240 nm, which indicated the presence of ester group. The FTIR spectrum of the polymer sample was evaluated and the results are shown in Figure.3. The intense band at 1726.13 cm^{-1} represented the presence of aliphatic carbonyl (C=O) group of R-CO-A in PHA polymer and the band at about 1058.12 cm^{-1} characterizes the valence vibration of the carboxylic group (COOH) [16]. The peak at 1177.12 cm^{-1} confirmed the carbonyl group (C-O) stretching of the esters present in PHA [17]. Apart from these, the polymer also showed a sharp peak at 1400.49 cm^{-1} corresponding to alkanes (CH_2) groups and the band at 1636.8cm^{-1} is the characteristic feature of the (C=O) of amide group, which absorbs even at lower frequency [18].

The structure and mole fractions of methanolysis product of polymer sample were analyzed using GC/MS and results are shown in Figure 4 and 5. By investigating the potential fragmentation patterns and the molecular mass of the fragments, the identities of specific peaks in mass spectra were correlated to that of carbonyl and hydroxyl ends of the corresponding alkanoates. In GC (Figure.4), the methyl ester derivative of PHA showed three characteristic peaks with retention time of 5.28, 7.616, and 9.873 min respectively. The results indicate that the compound is a hetero polymer of PHA. The peak obtained in mass spectrum at m/z 133 corresponds to that of polyhydroxyl hexanoate, and the peak at m/z 119 and 103 were rationalized to that of polyhydroxyl valarate and butyrate, respectively (Figure.5).

The carbon NMR spectra of the polymer were recorded in the range of 0-220 ppm and the results are shown in the Figure.6. Six prominent peaks appeared at 24.91, 29.08, 34.49, 63.93, 67.06, and

Table 2. Effect of molasses concentration on Dry cell weight (DCW), Residual cell mass (RCM) and polyhydroxyalkanoate (PHA) production

	Sugarcane molasses (%)									
	10	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
DCW (g/100 mL)	2.09 ± 0.61	0.34 ± 0.04	0.39 ± 0.06	0.77 ± 0.05	0.82 ± 0.06	0.83 ± 0.05	0.83 ± 0.05	0.85 ± 0.05	0.89 ± 0.04	1.07 ± 0.09
RCM (g/100 mL)	0.79 ± 0.2	0.25 ± 0.24	0.34 ± 0.15	0.68 ± 0.22	0.53 ± 0.25	0.35 ± 0.2	0.34 ± 0.15	0.16 ± 0.34	0.52 ± 0.2	0.68 ± 0.2
PHA (g/100 mL)	1.5 ± 0.4	0.12 ± 0.02	0.068 ± 0.01	0.1 ± 0.08	0.34 ± 0.12	0.5 ± 0.2	0.51 ± 0.20	0.75 ± 0.31	0.4 ± 0.16	0.4 ± 0.16
PHA%	75.5	26.4	12.82	11.69	35.36	57.83	59.03	81.17	41.57	36.44

Values shown are means (± SD). Data presented are representative of those obtained in independent experiments done in triplicates

Table 3. Effect of pH on Dry cell weight (DCW), Residual cell mass (RCM) and polyhydroxyalkanoate (PHA) production in a medium amended with sugarcane molasses (10%) as carbon source.

	pH				
	3	**5**	**7**	**9**	**11**
DCW (g/100 mL)	0.42 ± 0.2	0.25 ± 0.05	1.89 ± 0.66	0.5 ± 0.56	0.15 ± 0.05
PHA (g/100 mL)	0.06 ± 0.01	0.07 ± 0.01	1.49 ± 0.08	0.08 ± 0.16	0.03 ± 0.16
RCM (g/100 mL)	0.41 ± 0.3	0.17 ± 0.18	1.39 ± 1.19	0.44 ± 0.22	0.13 ± 0.16
PHA %	2.38	32	83.59	12	13.33

Values shown are means (± SD). Data presented are representative of those obtained in independent experiments done in triplicates

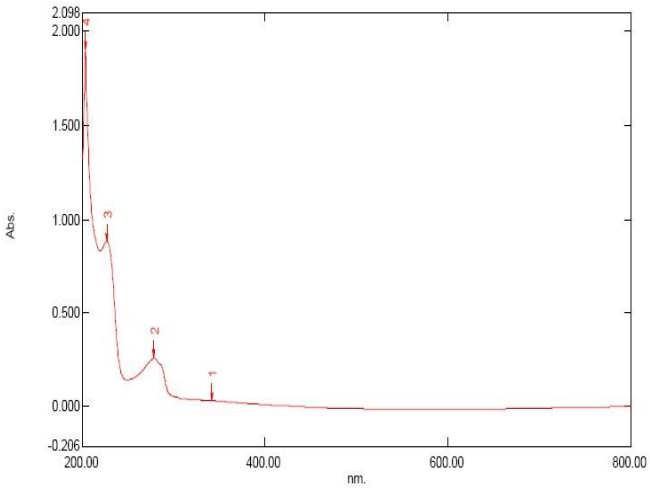


Figure 2. UV visible spectrum of PHA produced from *B. subtilis* AMN1. The peak at 230-240 nm confirms the presence of ester group in the polymer

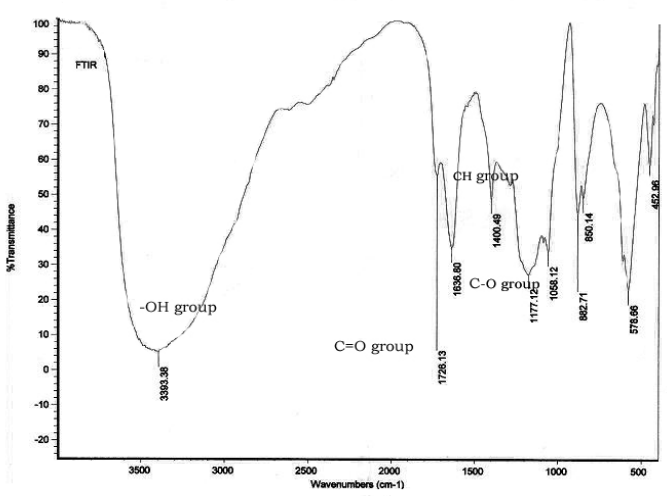


Figure 3. FTIR spectra of PHA produced from *B. subtilis* AMN1 indicates the presence of several functional groups in the polymer.

Figure 4. Gas chromatograph of PHA isolated from *B. subtilis* AMN1 shown the presence of methyl esters derivatives in the polymer.

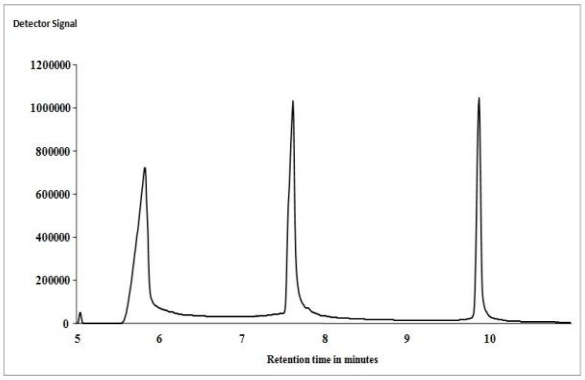


Figure 5. Mass Spectrum of PHA produced from *B. subtilis* AMN1. The peaks correspond to poly hydroxyl hexanoate, poly hydroxyl valarate and poly hydroxyl butyrate.

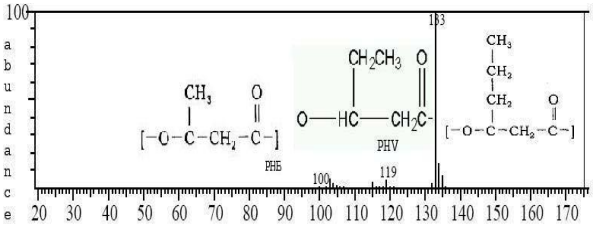
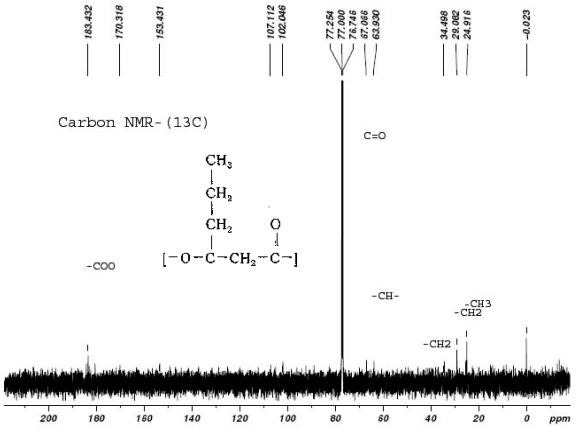
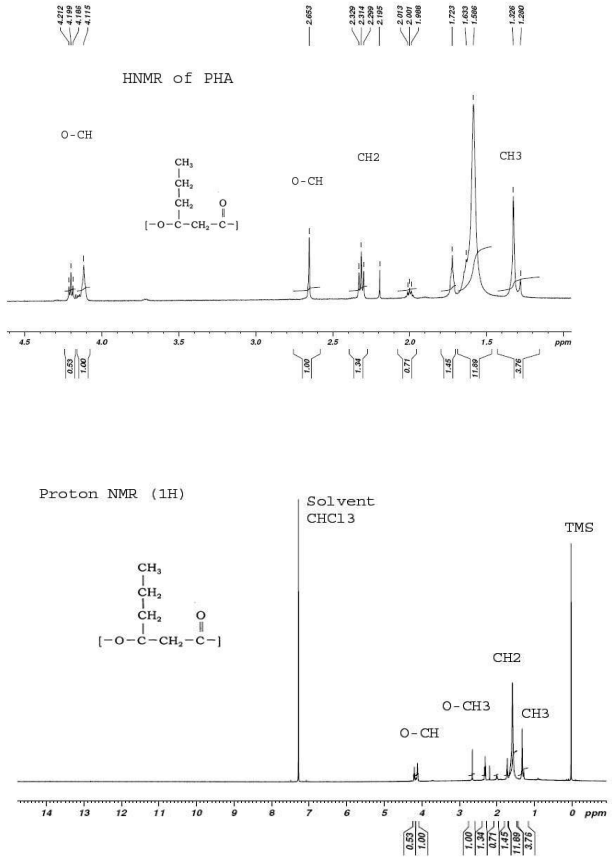


Figure 6. Carbon NMR (C^{13} NMR) spectra of PHA produced from *B. subtilis* AMN1. The peaks indicate the presence of methyl, methylene, and carbonyl groups in the polymer.



183.43 ppm due to the presence of (CH_3) methyl group, (CH_2) methylene group, and (CO) carbonyl group, respectively. The results suggested that polymer could be an impure of polyhydroxy hexanoate. The H^1 NMR scans were studied in the ∂ range of 0-14 ppm and the results are shown in Figure.7. On the ∂ scale of H^1 NMR, simple hydrocarbons protons tend to absorb in the region 0.53-1.5 ppm. This could be due to methyne group. The singlet for O-CH_3 group is barely visible. The sharp singlet at 1.5 ppm corresponds to that of methylene group (CH_2) and the signal at 3.76 ppm corresponds to CH_3 methyl group.

Figure 7. H1 NMR spectra of PHA produced from *B. subtilis*



4. DISCUSSION

PHA are synthesized and intracellularly accumulated as granules in many bacteria. Results of Sudan Black B staining revealed that isolate *B. subtilis* AMN1 were capable of producing PHA polymer. The results are consistent with previous studies reporting PHA production [9, 19]. To further validate the PHA production, chloroform extracted PHA was subjected to UV spectra analysis. The prominent peak for ester group in UV spectra confirmed the presence of PHA [20]. FTIR analysis permits spectrophotometric observation of PHA in the range of 450-4000 cm^{-1} and possibly provides the information regarding the functional groups present in the PHA polymer. Expectedly, the polymer contains different functional groups including C=O, CO and CH2. The results are in agreement with previous study reporting the presence of similar functional groups in PHA [14]. Similarly, the results of GC-MS were correlated to

that of carbonyl and hydroxyl ends of the corresponding alkanoates as described by Matias et al. [21]. The peaks of NMR were consistent with poly-3-alkanoates produced by *Cupriavidus necator* [4].

Numerous studies reported that growth conditions highly influence the PHA production [22]. The growth conditions for PHA production in *B. subtilis* AMN1 were similar to several bacilli that can synthesize and accumulate PHA as carbon and energy storage materials under limitation in nitrogen source [9]. The results of the optimization studies indicate that 10% molasses concentration and pH 7 was the optimum conditions for PHA production. Although, poor medium content and low cost substrate (sugarcane molasses) used in the study, the yield (83.59%) of PHA was superior to those in the previous published works. *Bacillus megaterium* produced 29.7% of polyhydroxyalkonates in cassava starch amended medium [23]. The recombinant *Bacillus subtilis* cultivated in hydrolyzed malt waste produced 15.3% (w/w dry cell) of polymers [24]. Several reasons may explain the high yield (83.59%) of PHA as compared with other studies; the constituents in sugarcane molasses may increase the synthesis rate of polymers. Alternatively, the environmental conditions and growth rate of the bacteria may increase the PHA yield. The high yield of PHA indicated the potential of the isolate and sugarcane molasses for mass production of PHA polymer.

5. CONCLUSION

The results of this study indicate that *B. subtilis* AMN1 strain cabable of producing known PHA using sugarcane molases with reasonable yeild. The use of sugarcane molasses can substantially reduce the substrate cost and in turn even provide value to the wastes generated by these industries, and can downsize the production costs. Moreover, the fermentation conditions reported in this study prevent the degredation of the PHA and pave a way for the use of *B. subtilis* AMN1 in commercial PHA production.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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