International Journal of Forestry and Wood Science
Vol.4(1), pp. 036-040, July, 2017. © www.premierpublishers.org. **ISSN**: XXXX-XXXX

IJFWS

Research Article

Wood waste - carbon source for Polyhydroxyalkanoates (PHAs) production

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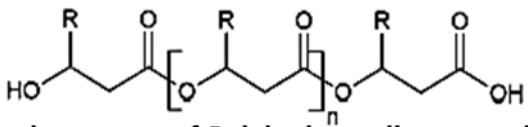
Polyhydroxyalkanoates (PHAs) are biodegradable and environmentally friendly bioplastics accumulate as storage materials by various bacteria. These PHAs serve as energy and carbon reserve materials for the bacteria. PHAs can be completely degraded within a span of 12-14 months by microbial consortia into CO_2 and water. Wood waste as a carbon source for the isolation and screening of polyhydroxyalkanoates (PHAs) accumulating bacteria was endeavored during our study. Bacterial strains collected from various environmental sources were subjected to culture studies for PHA synthesis. *Pseudomonas lignicola* was the single strain which was able to synthesize PHA using the carbon source derived from wood waste, when compared to other bacterial strains.

Keywords: Polyhydroxyalkanoates (PHA), bioplastics, carbon source, wood biomass, wood hydrolysate.

INTRODUCTION

Biological materials include chemically unrelated products that are synthesized by microorganisms or part of them under different environmental conditions (Alias and Tan, 2005). Bioplastics are an important class of biomaterials, which was demarcated as polyesters that are broadly dispersed in nature. These materials accumulate intracellularly in microbes in the form of storage granules. The physico-chemical properties of bioplastics look like that of synthetic plastics, *i.e.,* in terms of molecular weight, brittleness, stiffness, melting point and other physical properties PHA is comparable to some of the more common petrochemical-derived thermoplastics. Because PHAs are thermoplastics with biocompatible properties, they are being developed as new absorbable materials for implantable medical applications (Du and Yu, 2002). Other applications of PHAs include packaging material, osteosynthetic material in stimulation of bone growth, raw material for production of stereo regular compounds (PHAs being stereospecific), as mulch films in agricultural fields and as hot melt adhesives (Reddy *et al*., 2003).



General structure of Polyhydroxyalkanoates (PHAs)

Courtsey: Kootsrta *et al.*, 2017

Precisely, Polyhydroxyalkanoates (PHAs) belong to the polyoxoesters class of naturally occurring biopolymers (Steinbuchel and Hein, 2001). Polyhydroxyalkanoates are completely biodegradable synthesized by bacteria as intracellular storage materials under conditions of stress and act as carbon and energy reserve. Polyhydroxyalkanoates (PHAs) are accumulated as discrete granules to levels as high as 90% of cell dry weight and are generally believed to play a role as sink for carbon and reducing equivalents (Madison and Huisman, 1999).

Much effort has been devoted to reduce the price of PHAs by development of bacterial strains, more efficient

fermentation and more economical recovery processes (Godbole *et al*., 2003). High productivity is one of the major factors for economical production of biodegradable polymers (Chen *et al*., 2001). The carbon source should be inexpensive since it is the major contributor to the total substrate cost (Gao *et al*., 2002). Wood hydrolysate is a potentially inexpensive and renewable feedstock that can be produced through enzymatic or dilute acid hydrolysis of cellulose or hemicellulose to fermentable sugars, such as glucose, galactose, xylose, and mannose. Wood hydrolysate has already been utilized for the production of ethanol and xylitol. The isolation and development of bacterial strains that can utilize such wood hydrolysates as substrates is pursued intensively. In this regard the present research work was focused on isolation and screening of potential bacteria for synthesizing polyhydroxyalkanoates (PHAs) using cheap carbon source.

MATERIAL AND METHODS

Isolation of bacteria: Soil industrial effluent samples was taken from Bommasandra Industrial Area (Paper & pulp) – BIA and Kadugondana Halli (Tannery) – KH, Bangalore, Karnataka, India were used for isolation of the bacteria. Around 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates and incubated at 30˚C for 24 hours. Various colonies of different morphologies were individually picked and were screened for PHA production, potential isolates were sub cultured on nutrient agar plates as reported by Aarthi and Ramana, 2011. The original cultures were maintained as glycerol stock at –20°C for further use.

Rapid screening of native bacterial isolates for PHB production: All the bacterial isolates were qualitatively tested for PHB production by Nile blue (as a fluorescent) staining method (Ostle and Holt, 1982). For rapid screening of PHB producers, bacterial isolates were spread into nutrient agar plate and the plates were incubated at 30°C for 24 hours. Acetone solution of Nile blue (0.5µg/ml) was spread over the colonies and the plates kept undisturbed for 15 minutes.

Preparation of wood hydrolysate: Wood extracts processing was carried out, for wood chips (at 160°C for 120 min), and hydrolysed with 2% sulphuric acid at 95°C for 20 min., followed by concentration, neutralization, and centrifugation for removal of precipitates (Chen *et al*., 1984).

Analysis of wood hydrolysate for its contents: The analysis of wood hydrolysate was performed with GC-MS (JEOL GCMATE II GC-MS with Data system is a high resolution, double focusing instrument, Maximum resolution: 6000 Maximum calibrated mass: 1500 Daltons. Source options: Electron impact {EI}, Chemical ionization {CI}) for its knowing the actual sugar content of the source material used for PHA bacteria.

Substrates for PHB production: Wood waste samples was collected and dried in an oven at 60°C to reduce moisture content and was milled into fine particles. The following parameters such as reducing sugar, starch and cellulose content in the raw seed were characterized (GC-MS).

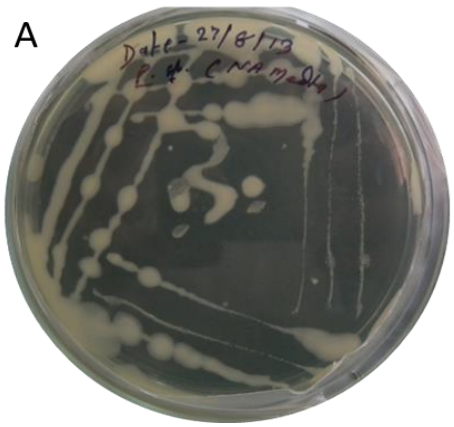
Bacterial Growth in specific media: Pure culture of selected bacterial strain was revived in nutrient broth initially and then grown in a defined Mineral Salt Media (MSM) containing: 10g Hydrolyzed Seed, 5g Glucose, 5g Sodium Chloride, 5g Di-Potassium Hydrogen Phosphate, 1g Potassium Chloride, 1g Magnesium Sulphate, and 1g Ammonium Sulphate in 1L of distilled water. The pH of the media was maintained to be 7.5±0.5. The culture flask was kept in shaker at 150 rpm at 35 °C for two days (Amirul *et al*., 2008, Du *et al*., 2001 and Yamanka *et al*., 2010).

Identification of PHA granules: The bacterial cells were stained with Nile blue stain and visualized under UV transilluminator (Geneflash, Syngene Bioimaging, Cambridge, UK) and that gives a bright orange fluorescence at a wavelength of 460nm. The accumulation of PHA in the form of granules would be identified from the fluorescing cells (Amirul *et al*., 2008).

RESULTS

Isolation of PHA producing bacteria

Polyhydroxyalkanoates (PHAs) are storage materials that accumulate by various bacteria as energy and carbon reserve materials. They are biodegradable, environmentally friendly, and also biocompatible bioplastics. Unlike petrochemical-based plastics that take several decades to fully degrade, PHAs can be completely degraded within a year by variety of microorganisms into CO_2 and water. Polyhydroxyalkanoic acids (PHAs) are common intracellular compounds found in bacteria, archaea, and in few eukaryotes such as yeasts and fungi. PHAs are carbon and energy reserve polymers produced in some microorganisms (e.g. *Alcaligenes latus (*FULAI WANG AND SANG YUP LEE (1997)), *Ralstonia eutropha (*Markus Potter, Mohamed H. Madkour, Frank Mayer and Alexander Steinbuchel (2002)), *Azotobacter beijerincki (*Soma PalA. MannaA.K. Paul (1999)), *Bacillus megaterium*(GABRIEL J. MCCOOL AND MAURA C. CANNON (1999)), when carbon source is in plentiful and other nutrients such as nitrogen, phosphorus, oxygen or sulfur are limited. Among the members of PHA family, polyhydroxybutyrate (PHB) is the most common biodegradable polymer and promising alternative to synthetic non-degradable plastics. These polymers are accumulated intracellular membrane enclosed inclusion



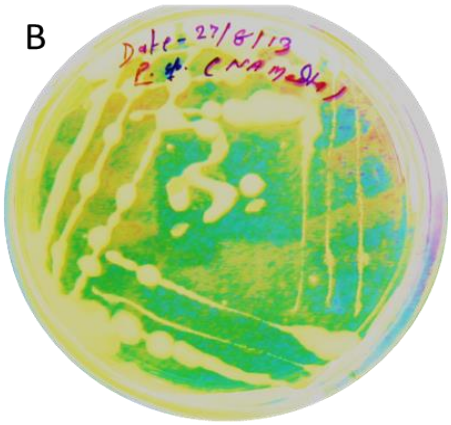


Figure 1: (A) Isolate BIA 3 (refer Tab. No.1) from industrial effluents soil and (B) Isolate BIA 3 showing Fluorescence of PHA using Nile blue staining

Figure 2: Culture plate of PHA producing microorganism using Nile blue staining

Table 1: Characterization & Screening of PHA producing bacteria

S. No.	Designation of Isolate	Gram Reaction	Shape	Plate Accumulation	
				Plate Count	Nile Blue Staining
1	BIA 1	Gram Negative	Cocci	+++	+++
2	BIA 2	Gram Positive	Cocci	+++	++
3	**BIA 3**	**Gram Negative**	**Small rod**	**++++**	**++++**
4	BIA 4	Gram Positive	Cocci	+++	+++
5	BIA 5	Gram Positive	Dispersed rod	+++	+++
6	KH 1	Gram Negative	Small rod	+++	+++
7	KH 2	Gram Negative	Small rod	++	++
8	KH 3	Gram Negative	Dispersed rod	+	++
9	KH 4	Gram Positive	Cocci	+++	+++
10	KH 5	Gram Positive	Small rod	++++	+++

up to 90% of the cell dry weight under conditions of nutrient stress and act as energy reserve material. These bacteria have been reported from various environments, but only a few from paper pulp and tannery effluents. For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Nile blue A staining viable colony method was used. The isolation of PHA producing bacteria was done from paper pulp and tannery effluent water samples. Most of the isolates showed positive result with Nile blue A staining (Fig. No. 1). Both gram-positive and gram-negative bacteria showed PHA production, but gram-negative bacteria dominated the waste material microflora of paper pulp and tannery industry.

The Nile Blue Staining method as experimented by Ostle and Holt, 1982, was adopted visualizing PHA producing microorganisms. The Nile blue was dissolved in acetone, and was added to the agar medium for viable colony staining. PHA producing microorganisms were visualized as bright yellowish orange colonies under digital microscope (Fig. No. 1B and 2).

Several bacteria were isolated from paper pulp and tannery effluents by serial dilution method (Kumari Bhuwal *et al*., 2013). From this, ten isolates were selected for PHA production as shown in Table no. 1. Based on the intensity of the fluorescence were observed in the Nile blue staining method (Ostle and Holt, 1982), one potential PHA producer (BIA 3) was screened out of ten isolates. The granules showed Orange fluorescence under UV trans-illuminator at a wavelength of 460nm as shown in Figures 1 - 2 and the results obtained was similar to Cortes *et al*., 2008.

Characterization of wood hydrolysate

Hemicellulosic wood hydrolysate was analyzed by HPLC and GC-MS to identify major carbon sources and inhibitory compounds. The chromatographic profile of membrane treated wood hydrolysate obtained by GC-MS is shown in Fig. 3. Most of the pertinent peaks eluted within a retention time of 20–30 min. Based on standards, the most abundant peaks in the chromatogram were sugars, including xylose, rhamnose, mannose, glucose, and some minor sugar derivatives. Among the sugars, xylose was the most abundant monosaccharide component, and accounted for more than 85% of the total sugar content in the hemicellulosic hydrolysate, followed by mannose, rhamnose, and glucose (Table No. 2).

Chromatographic profile of membrane treated wood hydrolysate obtained by GC-MS. Most of the pertinent peaks eluted within a retention time of 18–35 min. Based on standards, the most abundant peaks in the

Table 2: Composition of wood hydrolysate before and after membrane filtration

Substrates	Before Membrane Treatment	After Membrane Treatment
Xylose	170.7 g/L	70.7 g/L
Furfural	0.59 g/L	N.D.
HMF	0.15 g/L	0.005 g/L
Acetate	0.78 m/L	0.05 m/L
Total phenolics (g/L GAE)	0.66 g/L	0.60 g/L

GAE – Gallic Acid Equivalent, N.D – Not Detected Molecular Weight cut-offs of 200 and 130 DA

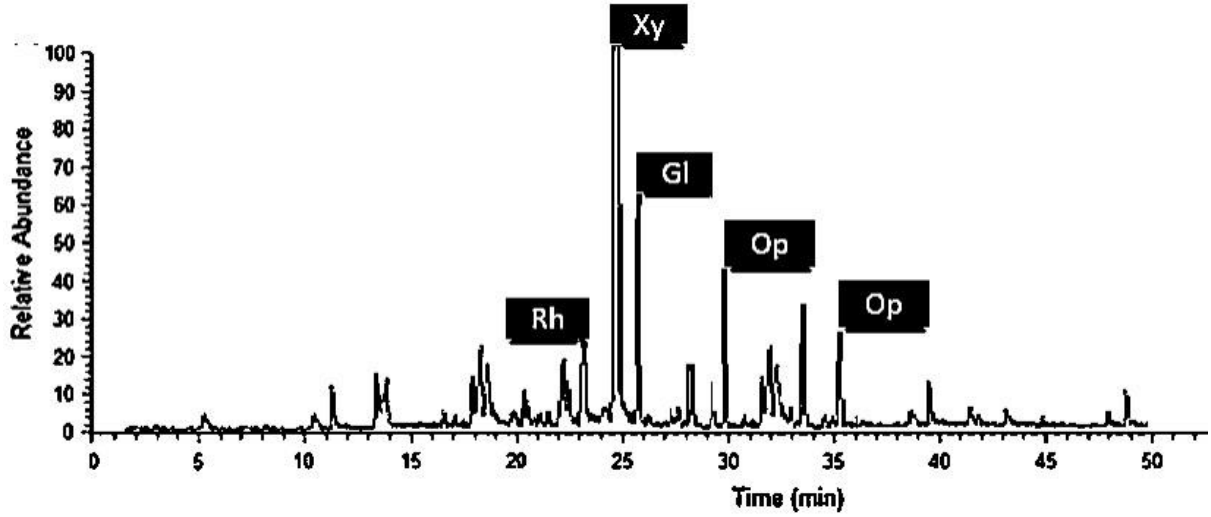


Figure 3: Chromatogram of membrane-treated wood hydrolysate

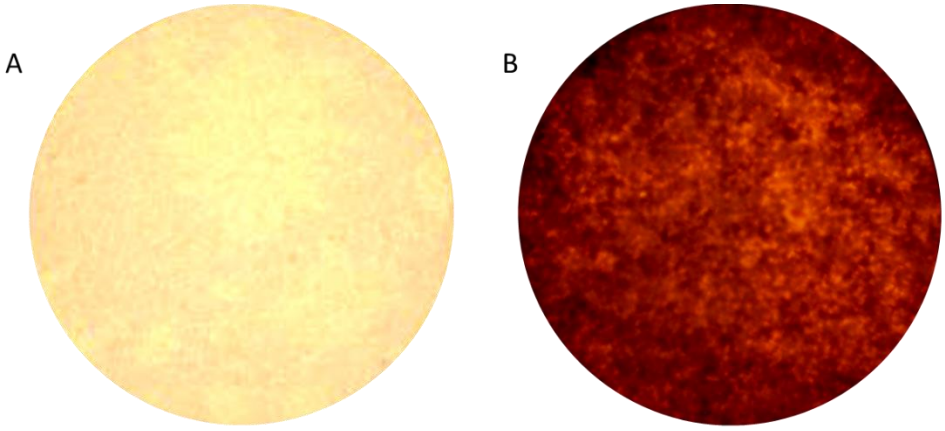


Figure 4: Detection of PHA(B) produced by *Pseudomonas lignicola* under light microscope. (A) Bacterial culture under light back ground (B) PHA bacteria under fluorescence back ground

chromatogram were sugars, including xylose, glucose and other pentoses. Among the sugars, xylose was the most abundant monosaccharide component, and accounted for more than 80% of the total sugar content in the wood hydrolysate, followed by glucose and rhamnose.

Identification of PHA granules

The accumulation of PHA in the form of granules would be identified from the fluorescing cells as shown in figure 4. Similar kind of studies was done earlier by Amirul *et al*., (2008) using fluorescence microscopy to visualize regions of intracellular PHA accumulation.

DISCUSSION AND CONCLUSION

The isolation of PHA producing bacteria was done from paper pulp and tannery effluent water samples. A large proportion (%) of isolated bacteria produced PHA as energy reserve material, proved by positive results with Nile blue A staining. Both gram-positive and gram-negative bacteria showed PHA production, but gram-negative bacteria dominated the waste material microflora of paper pulp and tannery industry. Hemicellulosic wood hydrolysate was analyzed by HPLC and GC-MS to identify major carbon sources and inhibitory compounds. The chromatographic profile of membrane treated wood

hydrolysate obtained by GC-MS, among the sugars, xylose was the most abundant monosaccharide component, and accounted for more than 85% of the total sugar content in the hemicellulosic hydrolysate, followed by mannose, rhamnose, and glucose. The accumulation of PHA in the form of granules was identified from the fluorescing cells (Amirul *et al*., 2008), using fluorescence microscopy to visualize regions of intracellular PHA accumulation. Detection of PHAs (B) produced by *Pseudomonas lignicola* was visualized under light microscope.

ACKNOWLEDGEMENTS

I sincerely thank The Director IWST for providing facilities and moral support for completing the project, similarly I would be grateful to ICFRE for sponsoring the project.

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Accepted 3 August 2017

Citation: Kumar M (2017). Wood waste - carbon source for Polyhydroxyalkanoates (PHAs) production. International Journal of Forestry and Wood Science 4(1): 036-040.