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PHA Production Using Low-Cost Agro-Industrial Wastes by *Bacillus* sp. Strain COL1/A6

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Abstract: Recycling of wastes generated from agro based industries for polyhydroxyalkanoate production is not only crucial for waste management but also in economizing and commercializing the polymer. In this study, the heterotrophic bacterium *Bacillus* sp. strain COL1/A6 isolated from humus was biologically characterized and explored for its potential to synthesize PHA using agroindustrial wastes. Qualitative analysis using Nile blue A staining revealed that starch, wafer residue, citrus pulp and cane molasses proved to be excellent carbon substrates for PHA accumulation. Growth and PHA producing ability of the isolate on cane bagasse and rice chaff improved after dilute acid hydrolysis. Highest cellular PHA content was obtained using wastes such as hydrolyzed wafer residue (62.41±1.04% of dry cell wt.) followed by cane molasses (54.68±1.36% of dry cell wt.) and hydrolyzed citrus pulp (47.5±1.01% of dry cell wt.). This is the first report wherein a *Bacillus* sp. has been reported to grow and utilize wastes such as wafer residue and citrus pulp as carbon feedstock for PHA production.

Key words: *Bacillus* sp., humus, low-cost, polyhydroxy alkanate, agroindustrial wastes

INTRODUCTION

The extensive usage of petrochemical plastics due to their versatile properties especially durability is causing severe problem in waste management affecting the aesthetic quality of cities, water bodies and natural areas (Full *et al.*, 2006). As a result, lot of research is now focused on the production of biodegradable plastics. Polyhydroxyalkanoates (PHAs) are the only naturally occurring polymers that are 100% biodegradable (Khanna and Srivastava, 2005). The wide spread use of this polymer is restricted only to areas where conventional plastics find limited applications such as the medical field due to its high production cost (Verlinden *et al.*, 2007; Valappil *et al.*, 2007a).

Process economics reveal that the use of inexpensive and renewable carbon substrates viz. agro industrial wastes and byproducts as PHA feedstock can contribute to as much as 40-50% reduction in the overall production cost (Choi and Lee, 1999; Kim, 2000). Other parameters which also influence the total production cost are bacterial strains, fermentation strategies and recovery processes. Currently, Gram negative microorganisms such as *Cupriavidus necator*, *Alcaligenes latus* and recombinant *Escherichia coli* are used for commercial polymer production (Valappil *et al.*, 2007a). They contain Lipopolysaccharide (LPS) endotoxins which co-purify with PHA. This limits the application of the polymer in medical field as LPS can elicit severe immunological reactions (Valappil *et al.*, 2007a; Chen and Wu, 2005). Synthesis of LPS free polymer requires additional purification step increasing the production cost. Gram positive bacteria such as *Bacillus* sp. are ideal candidates for industrial scale PHA production due to the lack of LPS layer. Members of this genus are known to grow rapidly, possess various hydrolytic enzymes and produce copolymers from structurally unrelated carbon sources (Valappil *et al.*, 2007b; Halami, 2007). These very characteristics of *Bacillus* sp. can be exploited for the production of PHA with desirable material properties from various low-cost agricultural feedstocks.

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Till date, very limited research is conducted on PHA production by Gram positive microorganisms such as *Bacillus* using agroindustrial wastes. This paper focuses on biological characterization of a PHA accumulating bacterium, COL1/A6 and production of the polymer using diverse agro industrial wastes.

MATERIALS AND METHODS

Bacterial Strain and Maintenance

The strain used in the present study, designated as COL1/A6 was isolated from humus sample. The isolate was maintained by streaking periodically on Nutrient Agar (NA) plates containing (w/v) peptone (1%), beef extract (1%), NaCl (0.5%) and agar (2%). The culture was incubated at 30°C for 24 h and stored at 4°C.

Identification of Bacterial Strain

Genomic DNA was isolated according to Sambrook *et al.* (1989) and 16S rRNA gene sequence amplification was carried out using the isolated chromosomal DNA as template. The following oligonucleotide sequences S-D-Bact-0011-a-S-17 5'-GTTTGATCCTGGCTCAG-3' were used as forward and S*-Univ-1392-b-A-15 5'-ACGGGCGGTGTGTTTC-3' reverse primers (Alm *et al.*, 1996). The amplified gene was gel purified and sent to Macrogen Inc., Korea for sequencing. The sequence of the strain was compared with similar sequences of reference organisms by BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>). The 16S rRNA gene sequence of the bacterial strain was aligned and clustered against those of the family Bacillaceae, which are available in the GenBank using ClustalW analysis. The 16S rRNA gene sequence is deposited in GenBank with accession number EU702754. ClustalX version 2.0.7 was used to generate multiple alignments between the selected sequences using IUB matrix (Larkin *et al.*, 2007). Neighbor-joining tree was obtained with 100 seeds and 3000 bootstraps. Final tree obtained was rooted and drawn using MEGA 4 (Tamura *et al.*, 2007).

Procurement of Agroindustrial Wastes

Citrus waste was procured from a local commercial food joint Sanyog (Goa, India). Cane molasses and bagasse were obtained from Sanjeevani sugar mill (Goa, India). Rice chaff, coconut oil cake and cotton seed cake were procured from a local market in Margao (Goa, India) and starch based wastes from a Fast Moving Consumer Goods (FMCG) industry at Ponda (Goa, India). Bakery wastes were collected from a local bakery in Bardez (Goa, India).

PHA Production by Plate Assay Method

Qualitative analysis was performed by spot inoculating 24 h old culture on E2 mineral medium (Lageveen *et al.*, 1988) agar plates containing pure carbon substrates (2% w/v). Further, the ability of the isolate to utilize carbon rich wastes as sole carbon source was also tested. All the plates were incubated at 30°C for three days. PHA accumulation was visualized after every 24 h using Nile blue A stain (0.05% w/v) dissolved in ethanol (Kitamura and Doi, 1994).

Dilute Acid Hydrolysis of Solid Wastes

PHA accumulation was also tested using hydrolyzed wastes. Except molasses, all the carbon wastes were dried to a constant weight and powdered to a particle size of 500 µm using electrical grinder. Wafer residue and citrus pulp waste (7% w/v) were hydrolyzed using 0.75% (v/v) sulphuric acid (Buhner and Agblevor, 2004) and refluxed at 100°C for 60 min. Cane bagasse and rice chaff were hydrolyzed at 121°C and 15 Psi for 30 min. The hydrolyzed samples were centrifuged and clarified supernatants were neutralized using sodium hydroxide (6N). Reducing sugar content was quantified

using DNSA method (Miller, 1959). The hydrolyzed wastes were added in the medium at a final concentration of reducing sugar equivalent to 20 g L⁻¹ of glucose.

Quantitative Analysis

PHA accumulation by the isolate grown in modified E2 mineral medium containing respective carbon wastes was monitored by individually growing the culture in a 250 mL Erlenmeyer flask containing sterile 100 mL production medium [ammonium chloride (0.895 g L⁻¹), potassium phosphate buffer (0.07 M, pH 7), yeast extract (0.5 g L⁻¹), magnesium sulphate (100 mM) and mineral trace element solution (1 ml L⁻¹)]. Hydrolyzed carbon wastes were autoclaved separately and added to the medium prior to inoculation. Flasks were incubated at 30°C for 48 h at 170 rpm on Orbitek environmental shaker.

Analytical Procedures

For the determination of biomass content, 25 mL culture broth was centrifuged at 10,000 g for 15 min and washed thrice with double distilled water. The cell pellet obtained was transferred to pre-weighed aluminum foil cup and dried at 60°C till constant weight was obtained. The polymer was extracted from bacterial cells using sodium hypochlorite method (Rawte and Mavinkurve, 2002) and PHA content determined gravimetrically.

RESULTS AND DISCUSSION

During an exhaustive screening for microorganisms capable of producing PHA, a Gram positive, rod shaped, sporulating bacterial strain was obtained on Nutrient Agar (NA). This strain was isolated from humic soil found below the leaf litter of various plants at a sampling site approximately 1 km away from the coastline. The culture was designated as COL1/A6 and identified to be *Bacillus* sp. based on 16S rRNA gene sequencing. A BLAST (NCBI) search using the 16S rRNA gene sequence showed 90% and above homology with 30 known taxa of Bacillaceae and maximum homology of 96% to *B. megaterium* and *B. flexus*. The evolutionary relationship of the isolate with selected species of the Bacillaceae family was analyzed using the Neighbor-joining method (Saitou and Nei, 1987) (Fig. 1). It is evident from the phylogenetic tree that strain COL1/A6 forms a separate group with the closest relatives being *B. megaterium*.

Quantitative estimation of the polymer content revealed that *Bacillus* sp. strain COL1/A6 could accumulate unequivocally about 65.25±0.91% of dry cell weight as PHA within 24 h using the simplest sugar *viz.* glucose as carbon substrate. Generally, members of the genus *Bacillus* are known to accumulate PHA content ranging from 6.53 to 48.2% (Shamala *et al.*, 2003; Aslim *et al.*, 2002; Chen *et al.*, 1991). Recently few researchers have reported higher amounts of accumulated PHA in different *Bacillus* sp. such as *Bacillus thuringiensis* R1 (64.1%), *Bacillus mycoides* RLJ B-017 (69.4%) and *Bacillus* sp. CL1 (90%) (Rohini *et al.*, 2006; Borah *et al.*, 2002; Full *et al.*, 2006). On further incubation till 72 h, the intracellular PHA content accumulated by strain COL1/A6 was found to be stable indicating that PHA depolymerase enzyme of this organism remains inactive over a longer period of time. *Bacillus cereus* SPV also exhibits similar pattern for PHA production (Valappil *et al.*, 2007c). It is advantageous for industrial PHA production to use an organism that is able to synthesize as well as stably maintain the accumulated polymer. This will prevent wasteful degradation of PHA after it has been synthesized in the bacterial cells (Valappil *et al.*, 2007c). *Bacillus* sp. strain COL1/A6 possesses this important characteristic making it an ideal candidate for industrial scale PHA production.

The production cost of commercially available PHA, approximately 4 to 6 \$ per kg is comparatively higher than that of conventional synthetic plastics (Van-Thuoc *et al.*, 2008). This high

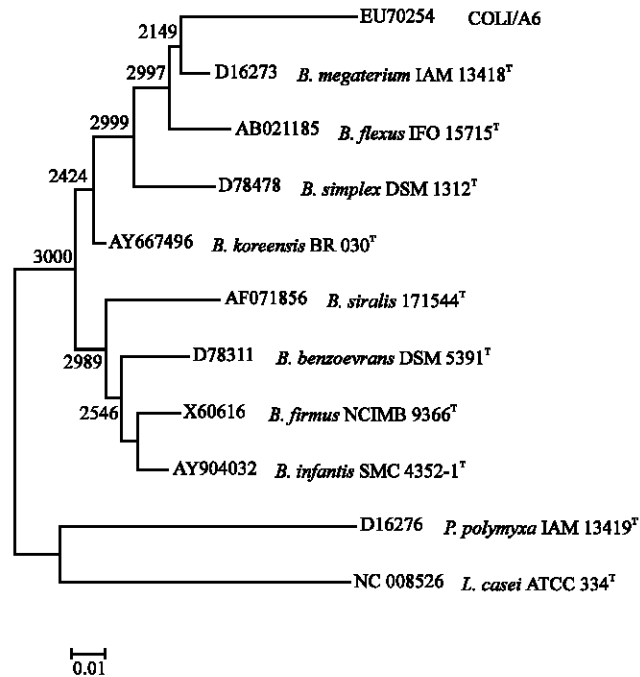


Fig. 1: Phylogenetic relationship of isolate COLI/A6 with closely related members of *Bacillus* based on 16S rRNA gene sequence. The rooted tree was generated using Neighbor-joining method with *Paenibacillus polymyxa* and *Lactobacillus casei* as outgroup. Nodes having 70% or greater support are shown with bootstrap value out of 3000. Bar = 0.01 nucleotide substitution per site

cost of polymer production is the major bottleneck in the commercialization of biodegradable plastics. For economical PHA production, cheaper renewable sources used as carbon feedstock and bacterial strains able to produce large quantities of intracellular PHA using such low-cost substrates are required. Wastes generated in the agricultural sector are abundantly available. These wastes are mostly used as cattle feed since they have little economic value. These renewable agricultural residues are rich in carbohydrates (Thomsen, 2005). Members of the genus *Bacillus* have innate ability to utilize such diverse and cheap carbon wastes as they possess heterologous enzymes capable of metabolizing these complex residues. Therefore native *Bacillus* strains are now being explored industrially using agroindustrial wastes for economic PHA production (Gouda *et al.*, 2001; Full *et al.*, 2006).

Bacillus sp. strain COLI/A6 was isolated from humus, an econiche known to harbor microorganisms able to degrade complex plant materials rich in carbohydrates (Ulehlova, 1998). Therefore its ability to utilize different carbon substrates which are generally found as major components in agro industrial wastes was tested. It was observed that the isolate could utilize a wide range of carbohydrates, polyols as well as low molecular weight organic acids for growth and PHA production (Table 1). This capability was exploited further by testing the efficiency of the isolate to accumulate PHA on different carbon rich wastes. Starch, wafer residue, citrus pulp waste and cane molasses supported excellent growth and PHA production (Table 2). Wastes such as cane bagasse and rice chaff were unable to support PHA accumulation. This could be attributed to the fact that the isolate is unable to utilize cellulose as a carbon source and these wastes are cellulosic in nature. Hence all the wastes except molasses were subjected to hydrolysis. Currently, the method used for

Table 1: PHA accumulation using diverse carbon substrates by *Bacillus* sp. strain COL1/A6

Substrate	Fluorescence intensity
Carbohydrates	
Glucose (control)	+++
Arabinose	+++
Xylose	+++
Fructose	+++
Galactose	++
Mannose	+++
Lactose	++
Sucrose	+++
Maltose	+++
Cellobiose	++
Melibiose	+++
Trehalose	++
Polyols	
Glycerol	+++
Mannitol	+++
Sorbitol	+
Organic acids	
Acetic	+++
Pyruvic	++
Lactic	++
Tartaric	+++
3-hydroxybutric	++
Succinic	++
Citric	++
Malonic	++

+, ++, +++ degree of PHA accumulation in comparison to glucose (positive control)

Table 2: PHA accumulation using diverse carbon wastes by *Bacillus* sp. strain COL1/A6

Substrate	Fluorescence intensity	
	Unhydrolyzed	Hydrolyzed
Starch	+++	+++
Pectin	+	++
Cellulose	-	-
Wafer residue	+++	+++
Citrus pulp waste	+++	+++
Cane bagasse	-	+
Rice chaff	-	+
Molasses	+++	ND
Coconut oil cake	++	++
Cotton seed cake	+	ND
Whey	++	ND

+, ++, +++ degree of PHA accumulation in comparison to glucose (positive control); ND: Not determined

hydrolysis of carbon wastes for industrial scale PHA production employs dilute acid which releases 75-90% of the substrate as metabolizable sugars (Yu, 2007). Dilute acid hydrolysis of the wastes not only improved the ability of the isolate to assimilate the released fermentable sugars as PHA but also avoided the interference caused by the insoluble present in the wastes during downstream processing. Strain COL1/A6 could produce as much as 47.5 ± 1.01 and $62.12 \pm 3.09\%$ PHA using hydrolyzed citrus pulp waste and hydrolyzed commercial pectin respectively (Fig. 2). Till date, there are no reports on PHA production by microorganisms using pectin as a carbon source.

When hydrolyzed wafer residue was used as the substrate, strain COL1/A6 accumulated $62.41 \pm 1.04\%$ of PHA which was found to be much higher than the PHA content obtained using commercially available soluble starch ($43.44 \pm 0.25\%$). *B. cereus* CFR06 has been reported to accumulate 48% PHA using soluble starch as a carbon source (Halami, 2007). However this is the first report on PHA production by *Bacillus* sp. using starch based wafer residue. The highest reported

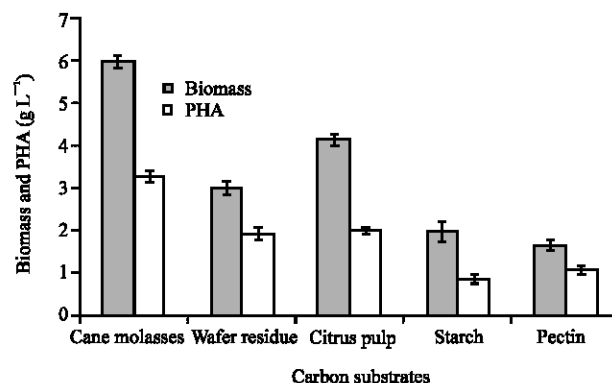


Fig. 2: Growth and PHA production by *Bacillus* sp. strain COL1/A6 grown in modified E2 mineral medium containing different carbon substrates

production of PHA with hydrolyzed potato processing waste is 76.9% using the Gram negative bacterium, *A. eutrophus* DSM 545 (Rusendi and Sheppard, 1995).

Cane molasses served as an excellent carbon substrate as maximum biomass and PHA was accumulated by strain COL1/A6. This could be attributed to the presence of rich content of sugars like sucrose, fructose, glucose and vitamins in molasses. The isolate could accumulate 54.68±1.36% PHA of dry cell weight using this waste. So far, *Bacillus* sp. strain JMa5 has been reported to synthesize PHA to a maximum of 35-40% of dry cell weight with molasses as carbon source (Wu, 2001). Production of PHB in *Bacillus megaterium* strain using molasses and corn steep liquor has also been studied by Gouda *et al.* (2001). They observed that a maximum yield of 46.2% PHA was obtained with 2% molasses. *A. eutrophus* DSM 545 can accumulate 44% of dry cell weight as PHA after 75 h of fermentation in medium containing 0.5 g L⁻¹ molasses (Beaulieu *et al.*, 1995). The optimum concentration of molasses and corn steep liquor for PHA production has been reported to be 65.4 and 13.2 g L⁻¹ respectively using *Azotobacter beijerinckii* DSM 1041 (Purushothaman *et al.*, 2001). The organism could accumulate about 25% of intracellular PHA under these optimized conditions.

Bacillus sp. strain COL1/A6 can efficiently produce higher quantities of PHA with less fermentation time and less substrate concentration as compared to the other reported organisms and therefore a potential strain to be explored for commercial PHA production.

CONCLUSION

Isolate *Bacillus* sp. strain COL1/A6 can utilize a wide array of renewable feedstocks for production of PHA. This ability is an added advantage for economical production and commercialization of the polymer as the production will not be dependent on the seasonal availability of the carbon feedstock. Utilization of agroindustrial wastes for synthesis of value added products such as biodegradable plastics will not only ensure the reduction in manufacturing costs of the polymer, but also solve the problems associated with waste disposal.

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