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Production of Polyhydroxyalkanoates by *Rhodopseudomonas palustris*: Effect of Some Waste Materials and Toxic Substances

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Abstract: The ability of the phototrophic purple non sulfur bacterium, *Rhodopseudomonas palustris* SP5212 to grow and produce bioplastics, polyhydroxyalkanoates (PHAs) utilizing industrial and domestic wastes have been assessed during the present study. Growth and PHA accumulation by the isolate under one- and two-step cultivation in phototrophic, microaerophilic conditions have been determined. Municipal waste and pond sludge supplemented with acetate medium at 1:1 ratio favoured growth and synthesized PHAs accounting 16 and 19 % of cell dry weight (CDW) respectively. In two-step cultivation, the accumulated co-polymer, poly (3-hydroxybutyrate-co-3- hydroxyvalerate) [P(3HB-co-3HV)] increased up to 25 and 37 % CDW with 37 and 52 mol % of 3-hydroxyvaleric acid (3HV) monomers. The isolate tolerated heavy metals like Co(II) and Ni(II) and several aliphatic and aromatic compounds. While the aliphatic compounds favoured co-polymer synthesis, aromatic compounds led to the accumulation of lower amount of poly (3-hydroxybutyric acid) [P(3HB)] only. It was apparent that the bacterium, *R. palustris* could be effectively applied in utilizing domestic and industrial wastes simultaneously with the production of biodegradable polymers of commercial importance in a cost-effective manner. Moreover, successful implementation of the metabolic potential of the phototrophic bacterium will help in waste management, reduction of pollution in the environment along with production of eco-friendly PHA plastics.

Key words: Polyhydroxyalkanoates, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(3-hydroxybutyrate), waste materials, *Rhodopseudomonas palustris.*

Introduction

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoic acids accumulated as carbon and energy reserves and as sources of reducing power in a wide variety of micro-organisms when grown under nutrient limitating conditions. The nutrient limiting conditions are usually imposed by deficiencies in S, O or Mg in the presence of excess carbon in the growth medium [12]. Different bacteria are known to synthesize and intracellularly accumulate PHAs [12]. Since the discovery of poly (3-hydroxybutyric acid) [P(3HB)], the most common representative of PHAs [15], polymers possessing different number of carbon atoms and pendant groups have been reported [24]. Biologically produced PHAs are composed only of chirally pure (R) configuration monomers. These PHAs are the most important representatives of biodegradable and bio-compatible plastics and elastomers for wide range of applications [13].

In general, disposal of industrial wastes often

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causes considerable environmental problem due to its high biological and chemical oxygen demand [14]. The treatment of waste stream to purified effluent needs much efforts because the waste stream often contains various organic compounds. These organic compounds can be effectively utilized by the microbial systems for the production of PHAs. Production of PHAs from waste will provide double benefits; on one hand, it reduces the load of pollutants in the environment, while on the other hand it produces eco-friendly biodegradable plastics. Moreover, from commercial point of view, the process will significantly reduce the cost of PHA production [5].

Phototrophic purple non sulfur bacteria are ubiquitous in nature and have been reported to be utilized for treatment of different types of wastes like swine waste water [10,11], latex waste water [19], and agricultural wastes [9], soyabean waste water [8], and pharmaceutical waste water [16].

Keeping the above facts in view, the present study is an attempt to evaluate the potential of *Rhodopseudomonas palustris* SP5212, a purple non sulfur bacterium to produce biodegradable bioplastics, polyhydroxyalkanoates utilizing wastes of different sources and also to assess the influence of toxic compounds on such polymer production.

Materials and methods

Bacterial strain and cultural condition

Rhodopseudomonas palustris SP5212, a PHA producing purple non sulfur bacterium isolated in this laboratory [18] was used through out the present study. The organism was maintained in phototrophic, microaerophilic condition using malate medium containing (g l^{-1}), malic acid, 2.0; K_2HPO_4, 0.9; KH_2PO_4, 0.6; $MgCl_2$, 0.2; nicotinic acid, 0.005, EDTA, 0.02; NH_4Cl, 1.0; and yeast extract, 1.0 (pH 6.8). For PHA production the medium was modified by replacing malate with acetate as the carbon source and ammonium chloride was omitted to impose nitrogen limiting condition.

Industrial and domestic wastes of semi-solid nature were collected in sterile containers from places in and around Kolkata, West Bengal, India and freshly collected samples were used for the experimental purpose. The waste samples at 10 % (w/v) level were centrifuged at 10,000xg for 10 min and filtered through Whatman No.1 filter paper to use as culture media. They were used in three different combinations: i) as crude waste (10 %, w/v), ii) waste (10 %, w/v) supplemented with 0.5 % (w/v) acetate and iii) 10% waste and acetate medium in the ratio of 1:1.

In single-step culture, the media in different combinations were distributed in 300 ml glass bottles and inoculated with freshly grown culture at 2.0 % (v/v) level. The bottles were incubated at 30°C with an illumination of 10,000 lux. In two-step cultivation, the bacterium was initially grown in malate medium for 4 days, cell mass were harvested aseptically by centrifugation at 12,500xg for 10 min in a Hitachi SCR 20B centrifuge and transferred to acetate medium supplemented with waste solution. Incubation conditions were same as in single-step cultivation.

Measurement of growth

Growth of the organism was determined by measuring the dry weight of the biomass. Cells were harvested by centrifugation at 12,500xg for 10 min in a Hitachi SCR 20B centrifuge, washed thoroughly, transferred to pre-weighed aluminium cups and dried to constant mass at 80°C for 24 h. Relative growth was calculated considering the growth in the control set as100.

Determination of polyesters

The PHA composition of lyophilized cell mass was determined by preparing methyl ester derivatives following the method of Brandl *et al*. [4]. The methyl esters was assayed by Gas Chromatography using Hewlett Packard Model 5890 Gas Chromatograph with SE-30 stainless steel column and a flame ionization detector. Nitrogen was used as a carrier gas. The temperatures of the injector and the detector were 200°C and 275°C respectively.

The intracellular polymer was extracted directly with boiling chloroform [21], concentrated and precipitated with chilled diethyl ether, separated by centrifugation (12,000 xg for 10 min) and dried under vacuum at room temperature. For proton nuclear magnetic resonance (^{1}H NMR) spectro-

scopic analysis the purified polymer was dissolved in analytical grade deuterochloroform ($CDCl_3$) and the chemical shifts were recorded using a Bruker AMX 300 NMR spectrophotometer with a multinucleate probe head.

Results

Growth and PHA production by *Rhodo-pseudomonas palustris* SP5212 was tested in six different waste materials under three different combinations. Results in Table 1 indicated that the waste materials with the exception of domestic waste were not suitable for growth of the organism but when they were supplemented with acetate medium in the ratio of 1:1 allowed growth as well as PHA accumulation particularly in municipal waste and pond sludge. However, the accumulated co-polymers, poly (3-hydroxy-butyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] from municipal waste and pond sludge contained 10.19 and 12.11 mol% of 3-hydroxy-valeric acid (3HV) respectively.

Considering the performance of the isolate in municipal waste and pond sludge, a two-step culture method was adopted with these two waste materials. Cell mass harvested after 4 days of growth in malate medium was transferred to media containing waste material and acetate medium at 1:1 ratio. The two-step culture in municipal waste and pond sludge samples resulted

Table 1. Effect of different waste materials on growth, PHA accumulation and composition of polyhydroxyalkanoates produced by *R. palustris* SP5212

Waste material	Grothh condition[a]	Growth, g l^{-1}	PHA, % CDW	Composition of PHA, Mol %	
				3HB	3HV
Biscuit industry waste	A	0.11	7.50	100.00	0.00
	B	0.16	10.0	97.83	2.16
	C	0.48	12.91	94.04	5.95
Domestic waste	A	0.40	5.40	95.13	4.86
	B	0.44	8.91	89.89	10.10
	C	0.67	10.13	84.95	15.04
Municipal waste	A	0.06	7.80	100.00	0.00
	B	0.14	11.66	94.11	5.88
	C	0.43	16.70	89.80	10.19
Tannery waste	A	0.04	5.25	95.52	4.47
	B	0.05	6.31	89.46	10.53
	C	0.36	9.50	74.36	21.63
Pond sludge	A	0.07	5.85	93.85	6.14
	B	0.10	10.13	91.86	8.13
	C	0.43	18.94	87.88	12.11
Swine waste	A	0.00	0.00	0.00	0.00
	B	0.40	2.53	100.00	0.00
	C	0.51	3.65	100.00	0.00
Control (Half strength medium)	-	0.43	12.33	100.00	0.00

[a] Growth condition A, Crude waste solution,(10%,w/v)
B, Waste solution(10 %, w/v) + 0.5 % acetate
C, Waste solution (10 %, w/v) : acetate medium (1:1)
Cultures were incubated under microaerophilic condition at a light intensity of 10,000 lux, total PHA content was estimated according to the method of Brandl *et al*. (1988)

in high amount of co-polymer production (25.77 and 37.93 % CDW respectively) (Fig. 1A and 1C). The co-polymers were composed of 3-hydroxybutyric acid (3HB) and 3 hydroxyvaleric acid (3HV) (Fig. 1B and 1D). The ^{1}H NMR spectra of co-polymer from 8 days grown cells in municipal waste revealed 37 mol % 3HV incorporation (Fig. 2A), whereas, under identical conditions, the pond sludge yielded co-polymers with nearly 52 mol % 3HV (Fig. 2B).

In view of the ability of *R. palustris* SP5212 to grow in such waste environment, it's potential to tolerate some heavy metals and toxic compounds were also taken into consideration. Of the five heavy metals tested, *R. palustris* SP5212 was most resistant to Co(II) (MIC, 212.5 µg ml^{-1}) followed by Ni(II) (MIC, 180.0 µg ml^{-1}). The isolate was highly sensitive to Hg(II) followed by

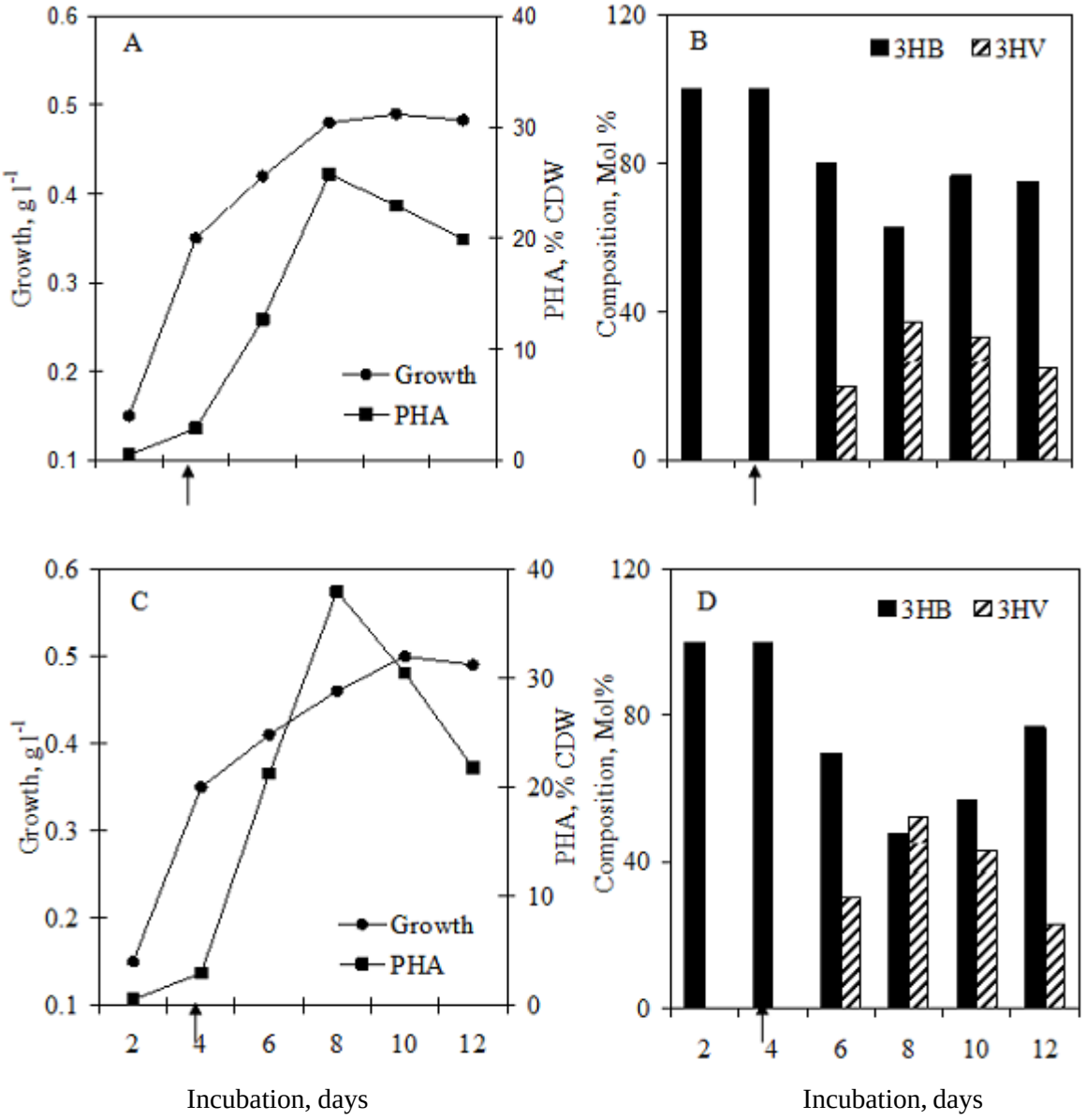

Figure 1. Growth, polyhydroxyalkanoates content and monomer composition of the polymer accumulated by *Rhodopsuedomonus palustris* utilizing municipal waste (A, B) and pond sludge (C, D) in two-step culture. Arrow indicates transfer of cell mass from malate medium to acetate media supplemented with waste materials.

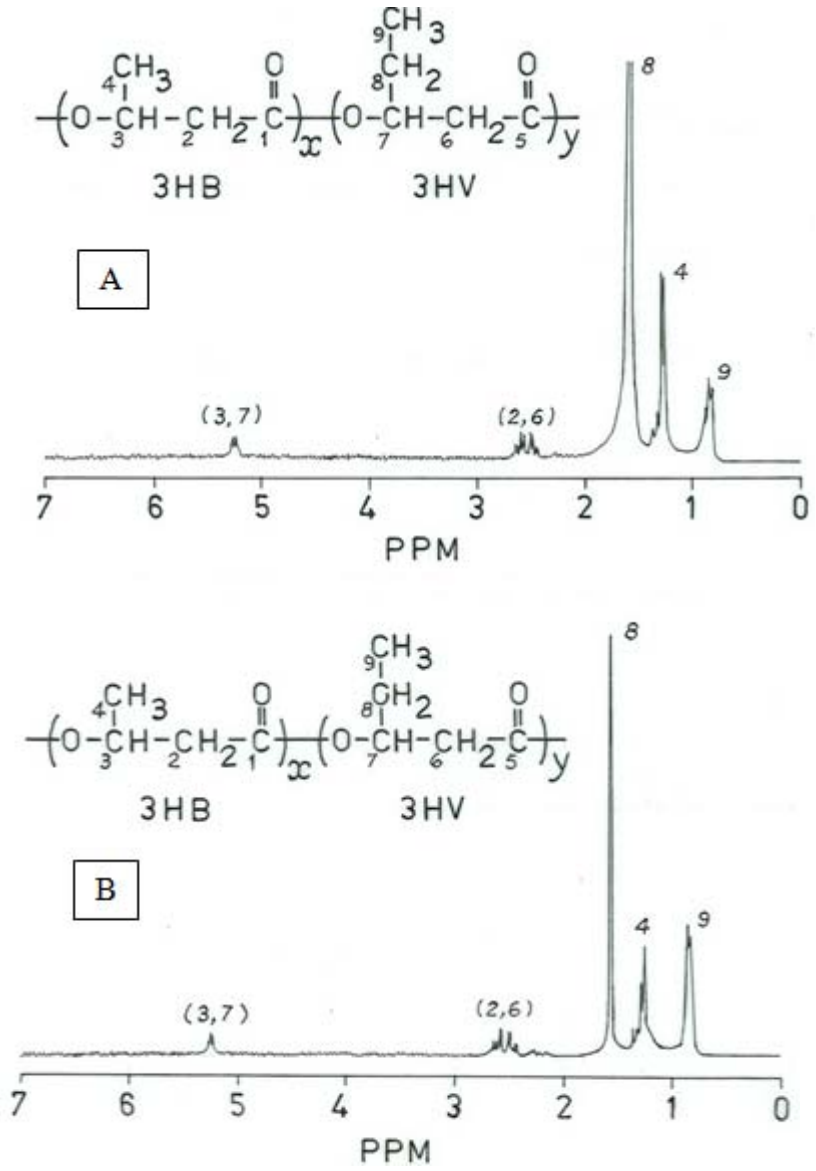

Figure 2. ^{1}H NMR spectra of polyhydroxyalkanoates extracted from by *R. palustris* SP5212 cells grown in municipal waste (A) and pond sludge (B) in two-step culture

Cr(VI) and Cu(II). Growth and polymer synthesis by the isolate at LD_{50} level of these metals was severely affected (Table 2). Cobalt, the relatively non toxic one at LD_{50} (105.0 µg ml^{-1}) yielded PHA accounting only 5.12 % of CDW. Other metals were highly inhibitory to PHA biosynthesis by *R. palustris* SP5212.

Effect of different aliphatic compounds, namely cresol, phenol, formaldehyde, hydroxybutyrate and valerate were tested on the growth and PHA accumulation by *R. palustris* SP5212 (Table 3). The isolate grew well in hydroxybutyrate and valerate as evident from 91.66 and 88.33 % relative growth compared to control. The accumulated PHA represented by P(3HB-co-3HV) accounted for about 33 and 21% of CDW in hydroxybutyrate and valerate grown cells respectively. Phenol and formaldehyde were toxic to growth and PHA synthesis by the isolate.

On the contrary, aromatic compounds, with the exception of nitrobenzene, were well tolerated by *R. palustris* SP5212 for its growth (44-93 % relative growth) but not for polymer synthesis which ranged between 5.3 to 12.6 % of CDW

Table 2. Effect of different heavy metals on growth and polyhydroxyalkanoates synthesis by *R. palustris* SP5212

Heavy metal	MIC, μg ml^{-1}	LD50, μg ml^{-1}	Growth, g l^{-1}	PHA, % CDW	Composition of PHA, mol %	
					3HB	3HV
CO(II)	212.5	105.0	0.32	5.12	100	00
Ni(II)	180.0	88.0	0.36	3.02	100	00
Cr(VI)	15.6	7.6	0.29	2.19	100	00
Cu(II)	7.0	3.4	0.32	1.83	100	00
Hg(II)	0.325	0.16	0.31	1.51	100	00
Control	-	-	0.61	15.0	100	00

Heavy metals were supplemented in the acetate medium at LD_{50} level, other conditions are same as under table 1

Table 3. Effect of different aliphatic and aromatic compounds on growth and polyhydroxyalkanoates accumulation by *R. palustris* SP5212

Compound	Concentration, % (w/v)	Relative growth, %	PHA, % CDW	Composition of PHA, Mol %	
				3HB	3HV
Aliphatic compound					
Cresol	0.05	71.06	11.01	100	0
Phenol	0.01	0	0	-	-
Formaldeyde	0.01	0	0	-	-
Hydroxybutyrate	0.1	91.66	33.71	62.52	37.47
Valerate	0.1	88.33	21.75	67.31	23.65
Aromatic compound					
Benzoic acid	0.1	93.33	12.6	100	0
p-Amino benzoic acid	0.025	44.16	6.13	100	0
Cytosine	0.1	90.83	10.13	100	0
Cinnamic acid	0.025	69.83	7.19	100	0
Salicylic acid	0.025	66.66	5.33	100	0
Nitrobenzene	0.01	0	0	-	-
Control	-	100	15	100	0

Aliphatic and aromatic compounds were supplemented in acetate medium at a specific concentration, other conditions were same as under table 1

(Table 3). Moreover, the accumulated polymer was represented only by P(3HB), unlike the co-polymers [P(3HB-co-3HV)] synthesized with aliphatic compounds.

Discussion

Phototrophic purple non sulfur bacteria have long been known to grow on waste material and explored for industrial and domestic waste management [3,6]. *R. palustris* in particular has been reported to grow in swine waste water [10], sago waste water [7] and synthetic sewage water [19]. Again production of PHA by this group of microorganisms utilizing waste materials of

different types is not uncommon [1,23] and reported to be greatly influenced by the composition of the wastes used [1,2]. The present experimental findings clearly revealed utilization of waste materials of different types for growth and synthesis of PHA by *R. palustris* SP5212 (Table 1). The accumulated co-polymers were primarily composed of short chain length (C4 and C5) hydroxyalkanoic acids similar to those of PHA co-polymers synthesized by *Rhodobacter sphaeroides* from palm oil mill effluent (POME) [1].

Two-step cultivation of *R. palustris* SP5212 with waste material was advantageous for polymer accumulation from qualitative as well as quantitative view points (Figure 1and 2). Sawayama *et al*. [23] have also adopted two-step cultivation of phototrophic bacteria in lighted up flow anaerobic sludge blanket (LUSAB) which increased the polymer content from 9.6 % to 32 % of CDW as observed in our investigation.

Rhodopseudomonas species showing multiple metal resistance have been effectively utilized in the reductive removal of Cr(VI) [17]. Likewise, removal of heavy metals by extracellular polymeric substances produced by resistant purple non sulfur bacteria isolated from contaminated shrimp ponds was also investigated and reported by Panwichian *et al*. [20]. The present study clearly revealed that *R. palustris* SP5212 could tolerate Co(II) and Ni(II) (Table 2) but its efficiency for detoxification or removal of these metals during growth needs to be confirmed.

The ability of purple non sulfur bacteria to utilize aliphatic and aromatic compounds of different types has been well documented [22,25]. It is interesting to note that like many purple non sulfur bacteria, *R. palustris* SP5212 was able of utilize aliphatic and aromatic compounds for growth and also produced P(3HB-co-3HV) (Table 3) which is preferred over P(3HB) for its physical and mechanical properties.

Conclusion

It may, therefore, be concluded that under phototrophic, microaerophilic growth conditions, *R. palustris* could be effectively utilized as an ideal candidate for the production of PHA co-polymers, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] from domestic and industrial wastes. Such biosynthetic potential of the organism will be of help in waste management, reduction of environmental pollution as well as production of bioplastic in a sustainable manner.

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