PRODUCTION OF PHB (POLYHYDROXYBUTYRATE) BY *RHODOPSEUDOMONAS PALUSTRIS* KU003 AND *RHODOBACTER CAPSULATUS* KU002 UNDER PHOSPHATE LIMITATION

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ABSTRACT : Nine bacterial species of purple non sulphur bacteria were isolated from Warangal district of South India from leather industry effluents which included *Rhodopseudomonas palustris, R.rutila, R.acdiophila, Rhodopila globiformis, Rhodospirillum rubrum, Rsp.photometricum, Rhodobacter sphaeroides, Rb.capsulatus, Rhodobacter* sp and *Rhodocyclus gelatinosus* were isolated. Among these *Rhodopseudomonas palustris* KU003 and *Rhodobacter capsulatus* KU002 were selected for the production of Polyhydroxybutyrate (PHB). The extracted PHB was characterized by IR and NMR spectral analysis. Effect of nutrient limitation in the form of phosphate KH_2PO_4 was tested to enhance the production of the polymer. Maximum yield of PHB was recorded at a concentration of 280 mg/L of KH_2PO_4 in *Rps.palustris* while no significant increase in the production of the polymer was observed in *Rb.capsulatus*.

Keywords: *Rps.palustris*, *Rb.capsulatus,* Polyhydroxybutyrate, phosphate limitation.

INTRODUCTION: Plastic materials have become an integral part of contemporary life because they possess many desirable properties, including durability and resistance to degradation. Over the past 10-20 years, their widespread uses have been increasingly regarded as a source of environmental and waste management problems (Anderson and Dawes 1990). PHA-producing bacteria can be generally classified into two groups (Steinbuchel. and Valentin, 1995). The first class of bacteria, including *Ralstonia eutropha,* produces short chain length PHA with monomer units ranged from C_3 to C_5, while the other class, including *Pseudomonas oleovoran*s, produces medium chain length PHB with monomer units from C_6 to C_{14} (Anderson and Dawes 1990). Rohini *et al.* (2006) characterized PHB from *Bacillus thuringienesis* R1 strain isolated from soil sample. Bacteria such as *Ralstonia eutropha*, *Alcaligenes latus* and *Azotobacter vivelandii* may be induced to synthesize PHB by imposing a chemical stress. This is normally done by depriving the organism of a nutrient such as nitrogen or phosphorus or sulfur, which are required for cell growth (Babel *et al*.,2001, Khanna and Srivastava, 2005). Of these, nitrogen is the preferred stress-creator (Patwardhan and Srivastava, 2004 and Shahhosseini, 2004), but recent work (Koutinas *et al*., 2007) points to the possibility of limiting the supply of phosphorus to generate PHB.

Brandl *et al* .(1991) reported that *Rhodobacter sphaerodies* produced PHB as the major component (97%) and a small amount of PHV(3%) under anaerobic light conditions. PHA production from some waste material has been studied by Yigit *et al.* (1999) and Ali Hassan *et al.*(1996) from the waste waters of sugar refineries and palm oil waste respectively. PHA production from acetic acid was reported in *Rb.sphaerodies* S and *Rb.sphaerodies* IL 206 by Noparatnaraporn *et al.* (2001). Influence of cultural conditions on the synthesis and accumulation of PHB by *Rps.palustris* SP5212 was investigated by Mahuya *et al.*(2005). Combinations of various carbon and nitrogen substrates were used to study poly-β-hydroxybutyrate accumulation and H_2 evolution by *Rhodobacter sphaeroides* strain RV (Khatipov *et al.*,1998). In this investigation, an attempt was made to procure PHB from two phototrophic bacteria isolated from tannery effluent and to study the effect of phosphate limitation on its production.

MATERIAL AND METHODS

Phototrophic bacteria were isolated from the effluent samples by enrichment techniques by inoculating into the Biebl and Pfennig's medium and incubated anaerobically in the light. The cultures obtained by enrichment technique were streaked on to the solid medium repeatedly and colonies were picked up to inoculate into the liquid medium and maintained by subculturing. Bacteria thus isolated were identified by studying the cultural characteristics (colour, size and shape), utilization of carbon and nitrogen sources, vitamin requirements, absorption spectral analysis, bacteriochlorophyll and carotenoids with the help of Bergey's manual of Systematic Bacteriology (1989).

Tubes were inoculated with 1ml log phase cultures of two anoxygenic phototrophic bacteria and incubated at 30 ± 2º C under the light intensity of 2000 lux in fifteen ml screw cap tubes. Carbon source in the form of glucose for *Rps.palustris* and acetate for *Rb.capsulatus* were maintained at a concentration of 1.0%. After inoculation, growth and PHB was calculated at various concentrations of Potassium dihydrogen phosphate.

Bacterial pellet was suspended in 5ml of hypochlorite and incubated for 10 minutes. The suspension was centrifuged at 8000 rpm for 10 minutes. The pellet was washed with diethylether and was then assayed for PHB. PHB extracted by the above method was assayed by Law and Slepcky (1960) method. PHB sample was treated with 5 ml of concentrated H_2SO_4 and a placed in a boiling water bath for 20 min. On cooling absorbance was recorded at 236 nm on a UV-Vis spectrophotometer. Standard was run using poly hydroxy butyrate.

The ^{1}H NMR were recorded in the indicated solvent on a Varian 500 MHz and 200 MHz spectrometer with TMS as internal standard. All chemical shifts (δ) were reported in ppm from internal TMS. Infrared spectra were recorded in KBr on Brucher-IFS-66 FT-IR spectrophotometer. The homogeneity of the compounds was checked using precoated TLC plates (E.Merk Kieselgel 60 F_{254}).

RESULTS AND DISCUSSION

Structure:

[O - CH -(CH_3)- CH_2- (C= O)-] *n*

IR spectra : 1290 (C-O), 1680 (C=O aliphatic), 2670 (CH_2), 2910-2960 (C-H streching)

NMR : 1.08 (3H,d,-CH_3), 2.35 (2H,d,-CH2-), 5.15(1H,m,-CH-)

The infrared spectra and proton NMR data clearly suggest the molecule obtained is Polyhydroxybutyrate (PHB). Table 1 reveals the effect of phosphate limitation on the production of PHB. Maximum yield of PHB was recorded at a concentration of 280 mg/L of KH_2PO_4 by *Rps.palustris* while no significant increase in the production of the polymer was observed in *Rb.capsulatus*. No correlation could be observed between dry cell weight and polymer accumulation. Less than the limitation of phosphate mentioned above did not result in higher yields of PHB. Nutrient limitation is necessary to trigger PHB accumulation, and generally ammonia is used as the critical control factor for uncoupling the growth of cells and PHB production. Nitrogen limitation in the form of NH_4Cl for PHB production was reported in *Alcaligenes eutrophus* (Koutinas *et al*., 2007), *Methylobacterium* sp. (Kim *et al*., 2006), *Sinorhizobium fredii* (Liangqi *et al*., 2006) and *Rhodopsuedomonas palustris* (Ramchander *et al*., 2010). Production of Poly(3-hydroxybutyrate) by *Ralstonia eutrophus* with phosphate limitation was reported by Shang *et al*.(2003). Shamala *et al*. (2003) has shown that of all the three nutrient limitations nitrogen, phosphate and sulphate, nitrogen limitation proved to be the best for various bacterial species. In the present study, increase in the synthesis of PHB by *Rps.palustris* and no such increase in *Rb.capsulatus* indicates that phosphate limitation may or may not result in more production of the polymer.

Table 1: Effect of phosphate limitation on production of PHB in two anoxygenic phototrophic bacteria

Organism	KH2PO4	Growth	DCW	PHB
	(in g/L media)	(O.D)	(g/L)	(mg/L)
Rps.plaustris	70	0.768	1.3	123
	140	0.842	1.4	142
	210	0.924	1.4	143
	280	1.124	1.8	181
	350	0.985	1.6	120
Rb.capsulatus	70	0.662	1.1	66
	140	0.810	1.3	130
	210	0.962	1.5	153
	280	0.984	1.6	161
	350	1.186	1.8	206

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