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Photoproduction of poly(3-hydroxybutyrate) by purple non sulfur bacteria under microaerophilic conditions

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Numerous prokaryotes belonging to physiologically and taxonomically diverse groups are able to produce poly(3-hydroxybutyrate) as reserve material. Phototrophic purple non sulfur bacteria (PNSB) are not exceptions. They produce the polymer from organic substrates when some nutrients are limiting in the growth medium. Screening of 32 purple non sulfur bacterial isolates led to the selection of isolates KT2105 and KD2108 having the ability to accumulate 6.2% and 5.12% of P(3HB) of cell dry weight (CDW). Succinate and acetate in nitrogen free condition favoured the polymer accumulation in KT 2105 and KD2108 respectively. Growth and polymer accumulation phases in both the organisms could not be segregated with time and polymer accumulation attained 9.8% and 7.7% of CDW in KT2105 and KD2108 after 8 days of incubation respectively. The extracted and purified polymers from both the isolates were characterized following UV-, IR- and ^{13}C NMR spectroscopic analysis. Finally the isolates KT2105 and KD2108 were tentatively identified as *Rhodobacter* sp. and *Rhodopseudomonas* sp. respectively on the basis of their morphological, physiological and biochemical characteristics.

Key words : Phototrophic bacteria, poly(3-hydroxybutyric acid), purple non sulfur bacteria, reserve material, *Rhodopseudomonas*, *Rhodobacter*

INTRODUCTION

The phototrophic, purple non sulfur bacteria (PNSB) are known to produce intracellular energy and carbon storage products, generally described as poly(3-hydroxybutyrate) [P(3HB)] (Dawes and Senior, 1973 ; Brandl *et al.*, 1988). P(3HB) is the most common member of polyester, polyhydroxyalkanoates (PHAs) and is accumulated as intracellular inclusions under unbalanced growth conditions, which are created by a limiting nutrient or growth factor in presence of sufficient carbon or energy (Nickles *et al.*, 1979). The quantity and quality of the accumulated polyesters are governed by the physiological abilities of the organism concerned and environmental conditions (Brandl *et al.*, 1989).

Studies on the accumulation of P(3HB) by phototrophic bacteria was initiated as early as 1962 (Schlegel, 1962) but the subject received momentum in early nineties (Vincenzini 1991). It was Brandl *et al.* (1989; 1991) who optimized the conditions for polymer accumulation in *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* using a range of linear and branched hydroxy carboxylic acids. Libergesell *et al.* (1991) have analyzed the composition of the polymer accumulated by 15 strains of PNSB and the polymer was found to contain 3 hydroxyvalerate (3HV) beside 3 hydroxybutyrate (3HB), when the cells were grown in presence of different n-alkanoic acids. Hashimoto *et al.* (1993) and Suzuki *et al.* (1995) described the effect of pH on the accumulation of the polymer by *R. sphaeroides* and *R. rubrum* respectively.

The present study reports the ability of purple non sulfur bacteria isolated from aquatic environment of West Bengal for P(3HB) accumulation under

microaerophillic conditions. Attempt has also been made to characterize and identify two selected strains. The identity of the accumulated polymer has been confirmed by UV-, IR- and ^{13}C NMR-spectroscopic analysis.

MATERIALS AND METHODS

Water and sludge samples collected from different places of West Bengal were enriched under microaerobic conditions in malate medium at a light intensity of 8,000 lux and pure cultures were obtained by agar dilution method of van Niel (1931). All the isolates were maintained in same medium by subculturing at an interval of 15 days.

The strains were primarily screened for the growth and P(3HB) accumulation in malate medium. In further experiments ammonium chloride was omitted and malate was replaced by acetate. The modified medium contained in 70 ml screw cap bottles was inoculated with a freshly grown culture at 2.0% (v/v) level and incubated under microaerophillic conditions with continuous illumination (10,000 lux) for 8 days at 30°C.

Growth of the isolates was measured by determining the dry weight of the biomass. Cells were harvested by centrifugation at 10,000 rpm in a Hitachi SCR 20B centrifuge, washed thoroughly, transferred to pre-weighed aluminium cups and dried to a constant mass at 80°C.

For estimation of P(3HB) the dried cell mass was extracted directly with boiling chloroform and quantified following the method of Sleepecky and Law (1960) using a Jenway UV- Vis Spectrophotometer model 6505 and expressed as the percentage of cellular dry mass.

Ultraviolate spectroscopic analysis involves conversion of the polymer to crotonic acid following digestion in concentrated H_2SO_4 for 10 minutes in boiliing water bath. Absorbance of the digested samples was scanned between 200-300 nm.

The infrared spectra of the whole cells, pigment-free cells and purified P(3HB) preparations were recorded with a Perkin - Elmer, Model 297 IR spectrophotometer following the method of Wakisaka *et al.* (1982).

The purified polymer dissolved in analytical grade deuterochloroform ($CDCl_3$) was used for ^{13}C NMR spectral analysis. A Bruker AMX 400 NMR spectrophotometer was used and chemical shifts were recorded in ppm scale.

RESULTS AND DISCUSSIONS

Systematic screening of 32 purple non sulfur bacterial (PNSB) cultures isolated from 10 water and sludge samples have indicated that majority (78.1%) of the isolates was able to accumulate P(3HB) but in limited amount. The PNSB isolates KT2105 and KD2108 with P(3HB) content of 6.2% and 5.1% of cell dry weight (CDW) respectively were considered for detailed studies.

Table 1 : Effect of carbon sources on growth and P(3HB) accumulation by the selected bacterial isolates

C source (1%, w/v)	Isolates					
	KT2105			KD2108		
	Growth, g/l	P(3HB), %CDW	Final pH	Growth, g/l	P(3HB), %CDW	Final pH
Acetate	0.61	9.81	9.74	0.57	7.7	9.68
Malate	0.62	6.20	8.89	0.59	5.12	9.71
Fumerate	0.62	4.1	8.16	0.46	1.61	8.21
Succinate	0.65	11.6	8.17	0.52	4.12	9.13
Propionate	0.64	00	8.61	0.39	3.33	8.19
Malonate	0.42	00	9.07	0.46	00	8.35
Gluconate	0.05	00	7.53	0.03	00	7.53
Butyrate	0.13	6.8	7.81	0.15	4.57	8.71
Glycerol	0.52	7.0	8.20	0.55	3.57	9.17
Citrate	0.55	5.41	8.06	0.53	4.21	9.1

The organism was grown microaerobically under a light intensity of 10,000 lux and initial pH was adjusted to 7.0. P(3HB) was estimated by method of Sleepecky & Law (1960).
CDW, Cell dry weight.

A series of carbon sources were tested for growth and accumulation of P(3HB) by the bacterial isolates KT2105 and KD2108. The organisms were found to utilize a wide range of carbon sources for growth but not for P(3HB) production. Succinate followed by acetate favours the synthesis and accumulation of P(3HB) in isolate KT2105, but in isolate KD2108, acetate was followed by malate for polymer accumulation (Table 1). Such a variation

in polymer accumulation reflectes the carbon assimilatory potential of the isolates. Libergesell *et al.* (1991) has investigated the effect of a series of different carbon sources on PHA accumulation by a number of PNSB. Succinate and acetate were found ideal for accumulation of PHA in most of the PNSB by Libergesell *et al.* (1991). Acetate was suitable for accumulation of the polyester in *Rhodobacter sphaeroides* (Brandl *et al.*, 1991).

Table 2 : Effect of nitrogen sources on growth and P(3HB) accumulation by the selected bacterial isolates

N source (0.1%, w/v)	Isolates					
	KT2105			KD2108		
	Growth, g/l	P(3HB), %CDW	Final pH	Growth, g/1	P(3HB), %CDW	Final pH
$NaNO_3$	00	00	7.0	00	00	7.0
KNO_3	00	00	7.0	00	00	7.0
$NaNO_2$	00	00	7.0	00	00	7.0
NH_4Cl	0.64	2.1	9.31	0.51	2.6	8.31
$(NH_4)_2SO_4$	0.54	3.5	8.58	0.53	3.5	8.25
Peptone	0.58	3.75	8.77	0.59	5.6	8.83
Tryptone	0.11	2.8	8.9	0.47	4.1	9.09
Casein hydrolysate	0.60	2.75	8.96	0.70	3.6	9.18
Beef Extract	0.59	2.1	8.83	0.63	3.1	8.70
Tryptose	0.61	2.4	8.87	0.45	2.2	8.99
Control (-N)	0.61	9.81	9.74	0.57	7.7	9.65

Growth conditions and estimation of P(3HB) same as under table 1
CDW, Cell dry weight

Supplementation of growth medium with various organic and inorganic nitrogenous sources was inhibitory to intracellular polyester accumulation by both isolates. Growth of isolate KT2105 was increased with NH_4Cl, while casein hydrolysate and beef extract promote growth of isolate KD2108. Accumulation of polymer showed a reverse relationship with growth (Table 2). That the polymer accumulation is influenced by nitrogen limiting conditions has been established not only in heterotrophic organisms (Page and Knosp 1989 ; Pal *et al.*, 1998), but also in phototrophic bacteria (Brandl *et al.*, 1991).

Time course of growth and P(3HB) accumulation by isolates KT2105 and KD2108 were determined under nitrogen limiting condition using acetate as the carbon source (Fig. 1). The polymer content of isolates KT2105 and KD2108 attained 9.8% and 7.7% of CDW respectively after 8 days of growth. Polymer accumulation by both isolates was initiated at the early stages of incubation and was parallel with the growth. The growth and polymer accumulation phases in both the organisms could not be segregated with time. However, there are organisms where polymer accumulation is triggered after the completion of the active growth phase (Bormann 1998). In *R. rubrum* such a pattern of grwoth and accumulation of PHA was observed. Synthesis of polyester was increased with growth and maximum accumulation was noticed in early stationary phase (Brandl *et al.*, 1989).

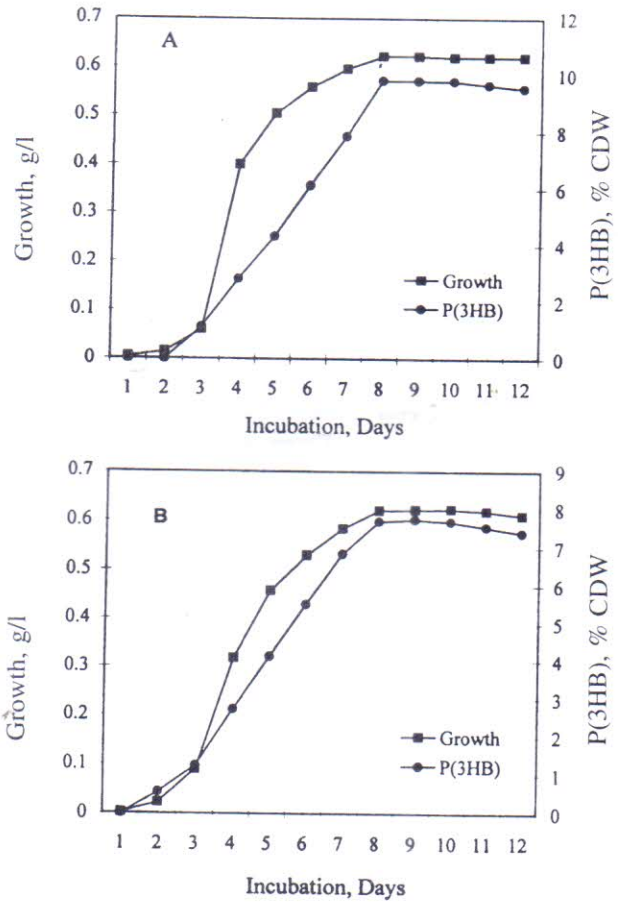

Fig. 1 : Time course of growth and P(2HB) accumulation by isolates KT2105 (A) and KD2108 (B) under phototrophic microaerobic conditions.

Polymers accumulated by both isolates were extracted with boiling chloroform and purified by repeated precipitation with diethyl ether. The purified polymers, on digestion with concentrated H_2SO_4 gave a sharp peak at 235 nm characteristic of crotonic acid (Fig. 2). Crotonic acid is formed by the dehydration of 3-hydroxybutyric acid in concentrated H_2SO_4.

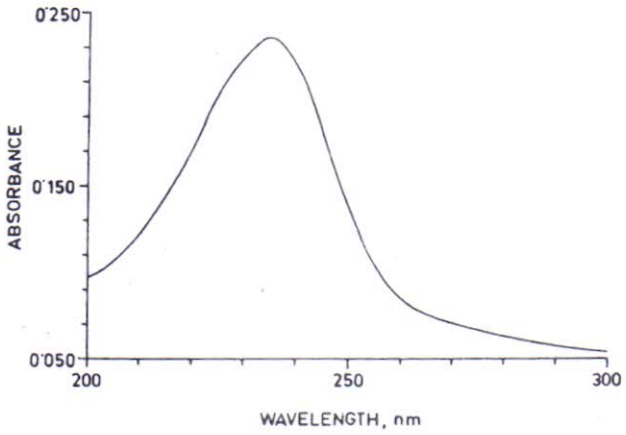

Fig. 2 : UV absorption apectra of the purified polymer accumulated by isolate KT2105 following treatment with concentrated sulphuric acid.

Infrared spectra of whole cells and pigment free cells as KBr pellets and purified polymer in sheet form revealed characteristic enolic (-OH) stretching of aliphatic compound (3440 cm^{-1}), (-CH) group of ketone (2920-2980 cm^{-1}), C = O carbonyl bonding (1730 cm^{-1}) and (-CH) group of aldehyde (1240-1370 cm^{-1}). Peaks below 1000 cm^{-1} represented the presence of (-CH_3) group. The characteristic IR spectra of both isolates were identical (Fig. 3).

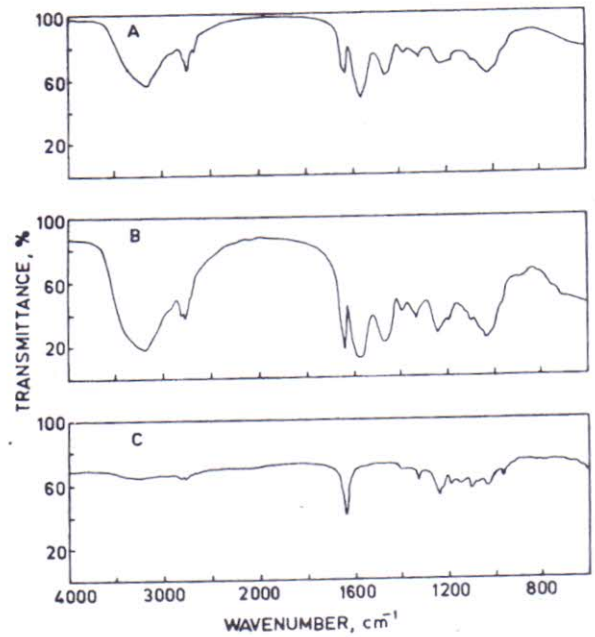

Fig. 3 : Infrared absorption spectra of whole cells (A), pigment free cells (B) and purified polymer (C) from isolate KT2105.

^{13}C NMR spectra of the purified polymer dissolved in $CDCl_3$ showed characteristic chemical shifts at 20.17, 41.20, 68.20 and 169.54 ppm (Fig. 4). which assigned the presence of (-CH_3), (-CH_2), (-CH) and (C=O) groups respectively. These characteristic signals are in good agreement with those of hydroxybutyric acid and confirm the homopolymeric nature of the compound obtained from isolate KT2105.

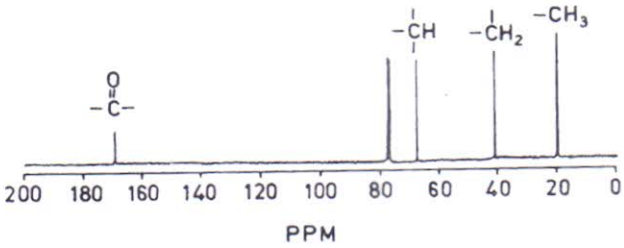

Fig. 4 : ^{13}C NMR spectra of the purified polymer accumulated by the isolate KT2105

Table 3 : Morphological, physiological and biochemical characteristics of the selected bacterial isolates

Characters	KT2105	KD2108
Cell shape	Ovoid	Small rod
Cell size	(1.5 x 1.2) μ	(1.1 x 0.6) μ
Gram nature	Gram negative	Gram negative
Motility	+	+
Color of cell suspension	Red	Purple red
Aerobic growth	+	+
NaCl rquirements	–	–
Vitamin requirement	Thiamin, Biotin, Niacin	p-ABA
Mode of reproduction	Binary fission	Budding
Cellular absorption maxima	380nm, 500nm, 590nm, 805nm, 870nm	340nm, 380nm, 490nm, 590nm, 810nm, 865nm
Bacteriochlorophyll type	Bchl a	Bchl a
Absorption maxima of carotenoides	350nm, 485nm, 515nm	350nm, 480nm-485nm
Electron donor utilized	Acetate, propionate, butyrate, valerate, caproate, caprylate, lactate, malonate, malate, succinate, fumerate, tartarate, citrate, aspartate, arginine, glutamate, gluconate, glucose, fructose, sorbitol, glycerol, methanol, ethanol, propanol	Formate, acetate, propionate, butyrate, valerate, caproate, caprylate, lactate, malonate, malate, succinate, fumerate, tartarate, citrate, aspartate, glutamate, gluconate, glucose, fructose, sorbitol, glycerol, methanol, ethanol, propanol, sulfide, thiosulfate
Cannot utilize	Formate, benzoate, thiosulfate, sulfide	Arginine, tartarate
Tentative identification	*Rhodobacter* sp.	*Rhodopseudomonas* sp.

The selected bacterial isolates, KT2105 and KD2108 were characterized in terms of their morphological, physiological and biochemical features following standard microbiological methods. The isolates are Gram negative, motile which develop deep red coloration in culture and can grow aerobically in dark, characteristic feature exhibited by many phototrophs. Absorption spectra of whole cells, bacteriochlorophylls and carotenoids failed to show any remarkable variation amongst the selected isolates. The isolates, however, showed distinct variation in morphology, electron donor utilization and growth factor requirements. Isolate KT2105 which is ovoid in shape requires thiamin, biotin and niacin but cells of KD2108 are rod shaped and require only para-aminobenzoic acid (pABA) as growth factor. Among the 28 electron donor tested, isolate KT2105 cannot utilize formate, benzoate, thiosulfate and sulfide while arginine and tartarate are not utilized by KD2108. These characteristic features of isolates were compared with those described in Bergey's Mannual of Systematic Bacteriology (1969) and ioslates KT2105 and KD2108 were tentatively identified as *Rhodobacter* sp. KT2105 and *Rhodopseudomonas* sp. KD2108 respectively.

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