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ACCUMULATION OF POLYHYDROXYALKANOIC ACIDS BY *AZOTOBACTER CHROOCOCCUM* MAL-201 FROM ORGANIC WASTE

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ABSTRACT

Azotobacter chroococcum MAL-201 (MTCC 3853), a free-living nitrogen-fixing bacterium accumulated intracellular poly(3-hydroxybutyric acid) [P(3HB)] accounting 69% of cell dry weight (CDW) when grown in nitrogrn-free Stockdale medium containing 2% (w/v) glucose. It also produced copolymer of poly(3-hydroxybutyrate co-3-hydroxyvalerate) [P(3HB-co-3HV)] using glucose as primary carbon source and valerate cas cosubstrate. To make the polymer production cost effective four types of waste material of different origin were tested for growth and polymer production. Stockdale medium supplemented with 1% (w/v) waste materials failed to yield good growth and polymer accumulation. Two–step cultivation was adopted for better growth and enhanced polymer accumulation. The candy factory waste was most suitable for synthesis of P(3HB) accounting 17.8 and 40.58% using single and two-step cultivation conditions respectively. Wastes of domestic and poultry origin produced P(3HB-co-3HV) with 3HV content 28.8 and 21.5 mol% respectively in two-step cultivation. Increase concentration of these wastes resulted in further upliftment of 3HV content of polymer with reduced growth and polymer accumulation. However, at optimum incubation the strain MAL-201 cells accumulated P(3HB) 48.5% of CDW (at 40h) from candy factory waste and P(3HB-co-3HV) 24.75 % of CDW with 3HV 34.65 mol % from domestic waste. Intrinsic viscosity, molecular weight and thermal degradation of the polymers accumulated in the cells grown in glucose, glucose with valerate and glucose with waste were compared.

Keywords: Poly(3-hydroxybutyric acid), Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), *Azotobacter chroococcum,* organic wastes

INTRODUCTION

Wastes with high organic contents exhausted from different food industries, poultries, agricultural and domestic activities are regarded as the main sources of environmental problems such as global warming etc. These wastes when flow out into sewage results in acute water pollution also. As a part of waste management systems, wastes of diverse types are commonly subjected to composting and sanitary land filling and recycling. Reuse of wastes depending on their nature and availability is another the valuable approach in the waste management policy, particularly for the metropolitan area.

The use of organic wastes as renewable resources for successful conversion to polyhydroxyalkanoic acids (PHAs) by microbial fermentation has gained special interest in the recent past mainly due to their biodegradable nature and close resemblance to synthetic petrochemical based plastics **(Barham and Organ, 1994).** This major group of biopolymer completely degraded by aerobic bacteria to form carbon dioxide and water or by anaerobic organisms generating methane **(Brandl *et al.*, 1988).** Microorganisms, particularly bacteria belonging to eubacteria and archaebacteria accumulate PHAs as intracellular energy and carbon reserve in presence of excess carbon source **(Anderson and Dawes, 1990).** However, the high production cost of PHA biopolymers from microbial sources compared to those of synthetic ones is one of the major drawbacks towards commercialization of these eco-friendly bioplastics.

The experiments with residual carbon substrates from agricultures and industries showed that a large number of organisms are able to utilize these wastes as their fermentation feed stock and synthesize different types of PHAs. *Azotobacter vinelandii* UWD, a mutant strain accumulated 69% (w/w) poly(3-hydroxybutyric acid) [P(3HB)] from beet molasses **(Page *et al.*, 1992)** where as the same strain synthesized 34% (w/w) poly(3-hydroxybutyrate co-3-hydroxyvalerate) [P(3HB-co-3HV)] using two-fold diluted swine waste liquor **(Cho *et al.*, 1997).** Strain *A. chroococcum* 23 **(Kizlo *et al.*, 1999)** and strain MAL-201 **(Pal Saha *et al.*, 2005)** potent amylase producers accumulated P(3HB) up to 78.5 and 68% (w/w) using potato or corn starch syrup at their stationary phase of growth. Whey, a major byproduct of dairy industries and containing approximately 4.5 (w/v) lactose when applied to the culture of *Ralstonia eutropha* **(Park *et al.*, 2002)** and recombinant *Escherichia coli* **(*Ahn et al.*, 2001)** it supported economic fermentations for P(3HB) production by them. Biorecycling of wastes of edible oil and tallow could be potentially useful in biopolymer formation by *Pseudomonus putida* KT2442 **(Ribera *et al.*, 2001),** *Pseudomonas sp.* strain DR2 **(Song *et al.*, 2008)**, *Ralstonia eutropha* H16 **(Taniguchi *et al.*, 2003)** and *R. eutropha* MTCC1472 **(Preethi *et al.*, 2012).**

In this communication we report the production of PHAs by *Azotobacter chroococcum* MAL-201 from wastes originated from candy factory (CFW), fruit processing factory wastes (FPFW), domestic activity (DW) and poultry (PW) following single-step and two-step cultivation procedures.

MATERIAL AND METHODS

Bacterial strain and cultivation

Azotobacter chroococcum MAL-201 (MTCC 3853) was used through out the study and organism was maintained on agar slope of nitrogen-free Stockdale medium at 4^0C. The Stockdale medium **(Stockdale *et al.*, 1968)** with 2% (w/v) glucose and pH 7.7 was principally used for growth and P(3HB) production. A single step cultivation process was followed for P(3HB) production and for P(3HB-co-3HV) production a two-step cultivation was done where cells from early log phase (12h) of growth in Stockdale medium were harvested aseptically, washed with sterile carbon-free medium and transferred to the same medium without glucose supplemented with sodium valerate (0.2%, w/v) as cosubstrate. The 24h old liquid culture in the same medium was used as inoculum at 4% (v/v) level in each case.

Batch culture with waste

Wastes from candy factory (CFW), fruit processing factory (FPFW), domestic (DW) and poultry (PW) origin were collected from Kolkata and Howrah districts of West Bengal, India. The wastes samples were filtered and diluted to 1% (w/v) level. These diluted wastes samples used as culture medium after adjusting their pH at 7.7 and the ability of the organism for utilization of those wastes for growth and polymer production were determined under shake flask condition (140 rpm) at 32^0C. The waste samples at 0.5% (w/v) were also used with glucose (0.5%, w/v) as cosubstrate in Stockdale medium both in single-step cultivation and in two-step cultivation processes.

Analyses of wastes

Total organic carbon, total nitrogen, soluble sulfate soluble phosophate, butyric acid and valeric acid contents of the wastes CFW, FPFW, DW and PW were analyzed of before and after the bacterial growth. Total organic carbon was estimated following acid digestion method of **Walkley and Black (1934)** using concentrated sulphuric acid. For estimation of total nitrogen, sulphate and phosphate the nesslerization, turbidometric method, and ascorbic acid method were followed respectively as described by Jackson A. in 'Standard method for examination of water and waste water' by American Public Health Association, APHA (18^{th} ed., 1992). A digestion mixture was prepared containing sample (0.5g), K_2SO_4 (6g), concentrated H_2SO_4 (4ml), 10% $CuSO_4$ solution (0.5ml) and a pinch of NaCl and it was digested up to 420^0C. After cooling the digestion mixture was filtered and volume of the filtrate made up to 100ml. For nitrogen estimation a precipitation was formed by adding 0.5 ml of $ZnSO_4$ solution (10%, w/v) and 1ml of NaOH soln. (20 %, w/v).After developing the colour with EDTA and Nessler reagent in alkaline pH optical density of filtrate solution was measured at 425nm using UV- VIS spectrophotometer, Varian Cary, 50 Conc.. For phosphate estimation, to 1ml of digestion mixture filtrate acid molybdate and then ascorbic acids were added and incubated for 30min at 37^0C and the optical density of the solution was measured at 882nm. The amount of nitrogen and phosphate were calculated from calibration curves. For sulphate estimation to the filtrate of waste samples (10g/l00ml) one sulphate tablet (Orbeco analytical systems, Inc, Farmingdale NY) was suspended and turbidity representing the $SO_4^=$ (mg/L) was measured at 528 nm using Orbeco Hellige Analyst, model 975MP.

Quantitative analyses of butyric and valeric acids in wastes were made by thin layer chromatography. The concentrated chloroform extract of wastes (10 g) were spotted on silica gel layer and run with chloroform, diethyl ether and water (70:20:10) and the spots was identified with vaporized iodine. DL- butyric acid and valeric acid (SIGMA, USA) were used as standard. The qualitatively determined bands corresponding to butyric and valeric acids were eluted with chloroform, evaporated to dryness and quantified through gravimetric method.

Estimation of growth

For determining the growth of the organism cell mass was harvested by centrifugation, washed thoroughly with distilled water for several times. The cell mass was then dried to constant weight at 80 ^{0}C for 24 h and the dry weight were determined.

Assay of polymer

Quantification and compositional analysis of polymers were performed from extensively washed and oven-dried cells. The polymer was extracted from cell mass with chloroform at 60^0C, filtered through a thick glass wool bed and precipitated in chilled diethyl ether. Purification was done by repeated solution and precipitation procedure. The total amount of the polymer was determined gravimetrically and was calculated as the percentage of cellular dry weight following the method of **Ulmer *et al.* (1994).** The compositional analysis was performed with ^{1}H-NMR spectroscopic data of the deuterochloroform solution of polymer. All of the spectra were recorded at 300.13MHz on a Brucker AV 300 Supercon NMR spectrometer.

Determination of intrinsic viscosity, molecular weight and thermal degradation

The viscometric measurement was performed in chloroform solution of the purified polymer at 30^0C using Ubbelohde's dilution viscosimeter **(Bibers and Kalnins, 1999)** and mol. wt. of polymer was determined through intrinsic viscosity [η](dl/g) measurement following the Mark-Howink equation: $[\eta] = 7.7 \times 10^{-5} \times M^{0.82}$ (Bruckner et al., 1988). Thermo gravimetric analysis was performed using a thermo gravimetric analyzer (TGA) Metler Toledo TGA/sDTA 851^e with temperature ranged from -20 to 220^0C and at a scan rate of 10^0C/min.

RESULTS AND DISCUSSION

Utilization of waste for growth and polymer accumulation

The organism was grown in 50 ml of diluted (1%, w/v), filtered and sterile waste for 27h in a 250ml flask under shake flask condition at 32^0C. Growth of the isolate in all waste samples as sole source of nutrients were significantly low compared to that in Stockdale medium with 2% (w/v) glucose (considering it as control) and about 64-84% inhibition of growth were recorded. The wastes CFW and FPFW allowed the accumulation of P(3HB) accounting 17.85 and 10.48 % of cell dry weight (CDW) where as DW and PW supported the synthesis of P(3HB-co-3HV) accounting 16.5% (with 15.05 mol% 3HV) and 3.5 % (with 11.60 mol% 3HV) of CDW respectively (Table 1).

Table 1 Utilization of wastes for growth and accumulation of PHA production by MAL-201.

*Waste, %, w/v	Growth, g/l	PHA% CDW [a]	Monomer mol% [b]	
			3HB	3HV
CFW	1.25	17.85	100	0
FPFW	1.02	10.48	100	0
DW	1.05	16.5	84.05	15.05
PW	0.55	3.5	78.4	11.6
Glucose, 2%,	3.5	69.5	100	0
(Glucose, 2%, + valerate, 0.2%)	3.1	62.6	80.2	19.8

Legend: CFW - Wastes from candy factory waste, FPFW- fruit processing factory waste, DW- domestic waste and PW- poultry waste. *Wastes were suspended in distilled water (1%, w/v) and filtered. Organism was grown in sterile filtrate for 27h under shake flask condition at 32^0C. [a] Total intracellular polymer was isolated with chloroform at 60^0C and estimated gravimetrically following the method of Ulmer *et al.* (1994). [b] The mol% of monomers present in polymer were calculated following. ^{1}H-NMR (at 300.13 MHz) data analyses of the purified sample. All values are the mean of triplicate.

Utilization of waste as cosubstrate

Supplementation of wastes each at 0.5% (w/v) level to glucose (0.5%, w/v) containing Stockdale medium resulted in the increase of growth of *A. chroococcum* MAL-201 though still 20-48% inhibition of growth and 41.61 -73.4 % inhibition of polymer accumulation, compared to control were recorded. The homopolymer accounting 40.58% of CDW was recorded as maximum with candy factory waste (CFW). However, increase in 3HV as 28.8 and 21.50 mol%, occurred by the glucose-DW and glucose-PW media, respectively (Table 2).

Table 2 Utilization of waste as cosubstrate for growth and PHA

Waste * (0.5 g/l)	Growth, g/l	Growth inhibition, %	PHA,[a] % CDW	Inhibition of polymer accumulation,%	Monomer, mol% [b]	
					3HB	3HV
CFW	2.8	20.0	40.58	41.61	100	0
FPFW	2.74	21.7	22.40	67.7	100	0
DW	2.05	41.4	27.55	60.4	71.2	28.8
PW	1.82	48.0	18.85	73.4	78.5	21.5

Legend: CFW - Wastes from candy factory waste, FPFW- fruit processing factory waste, DW- domestic waste and PW- poultry waste. *In two-step cultivation waste (0.5%, w/v) was supplemented to Stockdale medium after 12h of growth and organism was allowed to grow for a total of 27h. Inhibition of growth and polymer accumulation (%) was calculated by considering the growth and P(3HB) accumulation in Stockdale medium as controls. [a, b] Same as Table1. All values are the mean of triplicate.

Time profile of growth and polymer accumulation from wastes in two-step cultivation

Cells grown in Stockdale medium for 12h were harvested aseptically and washed with sterile carbon-free Stockdale medium. The cell mass was resuspended in equal volume of sterile waste (0.5%, w/v)as the sole source of nutrients and was allowed to grow for polymer synthesis. The organism attained its maximum growth at 40, 45, 50 and 45h of incubation in CFW, FPFW, DW and PW, respectively (Figure1). At these stages of growth, the polymer content accounted for 43.5, 34.8, 31.0 and 20.75% of CDW, respectively Moreover, the polymers synthesized from CFW and FPFW were homopolymers of 3-hydroxybutyric acid while those from DW and PW were copolymers of 3-hydroxybutyric acid and 3-hydroxyvaleric acid. Maximum copolymer was derived from domestic waste, which constitutes 34.65 mol% of 3-hydroxyvaleric acid.

The chemical compositions of waste medium before and after growth (60h) of MAL-201 have been determined (Table 3). The CFW and FPFW were rich in organic carbon and 78-85% of which utilized by the bacteria during its growth. The wastes DW and PW contained high amount of nitrogen, soluble phosphate and soluble sulphate. Alkanoic acids were absent in CFW and FPFW. The DW and PW contained butyric acid 217 and 421 mg/l, and valeric acid 134 and 120 mg/l, respectively. The contents of organic carbon, alkanoic acids including sulphate and phosphate in media fall sharply after growth of MAL-201. However, nitrogen in culture filtrate increased.

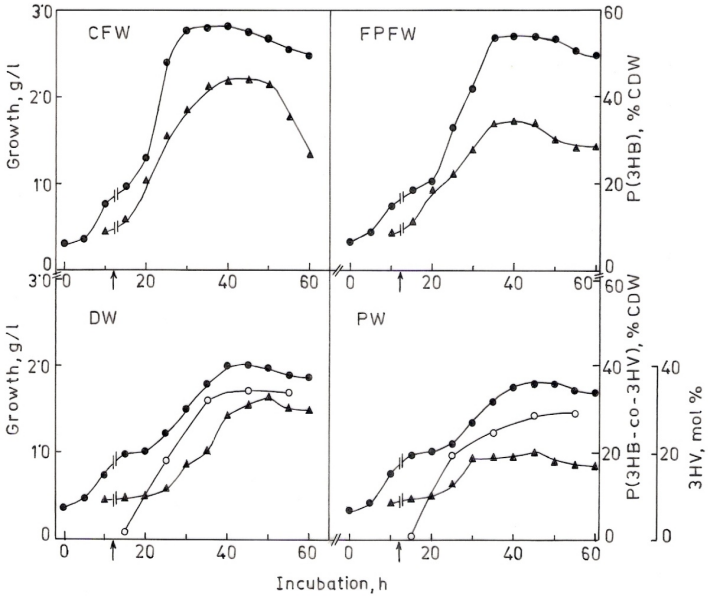

Figure 1 Time profile of growth (●), polymer accumulation (▲) and its 3HV mol% (○) of *A. chroococcum* MAL-201 in two-step cultivation. Glucose and diluted waste (0.5 %, w/v) were used as the substrates respectively, at first and second step of cultivation under shake flask condition (120 rpm) at 32^{0}C. CFW - candy factory waste, FPFW- fruit processing factory waste, DW- domestic waste, PW- poultry waste.

Table 3 Composition of wastes filtrate (0.5%) before and after the growth of *A. chroococcum* MAL-201

Waste	A						B					
	Carbon, g/l	mg/l					Carbon, g/l	mg/l				
		N	S	P	B	V		N	S	P	B	V
CFW	3.7	0.043	0.129	0.015	0	0	0.08	0.425	0.015	0.006	0	0
FPFW	2.85	0.105	0.052.	0.016	0	0	0.53	0.115	0.025	0.005	0	0
DW	4.2	112.0	44.0	40.0	217	134	1.15	142.5	3.3	9.0	39.6	14.5
PW	1.35	228.0	57.5	34.0	412	120	0.75	284.5	4.25	11.0	53.5	24.6

Legend: CFW - Wastes from candy factory waste, FPFW- fruit processing factory waste, DW- domestic waste and PW- poultry waste. Analyses of diluted waste were performed before (A) and after (B) the growth of MAL-201. N- Total nitrogen, S -soluble sulphate, P- soluble phosphate, B butyraic acid, V-valeric acid.

Waste suspended in distilled water (0.5%, w/V) and filtered. Sterile filtrate was used as culture medium in second step of two-step cultivation. Organism was grown under shake flask condition at 37^{0}C.

The composition of waste, before and after growth of MAL-201 determined following the 'Standard methods for examination of water and waste water' by APHA (18th ed., 1992). Total organic carbon quantified following the acid-digestion method of Walkley and Black (1934).

Qualitative and quantitative estimation of alkanoic acids performed through TLC method.

All values are the mean of triplicate.

Effect of concentration variation of wastes

Two-step cultivation with wastes further showed that growth and polymer synthesis by *A. chroococcum* MAL-201 was strongly dependent on the concentrations of waste substrates. Increase in CFW concentration up to 1.5% (w/v) generated P(3HB) accounting 48.9% of CDW where as other wastes, specially DW and PW affected both growth and polymer accumulation (Figure 2). However, 3HV content in P(3HB-co-3HV) reached the level of 39.5 and 31.2 mol % when MAL-201 was grown in DW and PW at 1.0 and 1.5 % (w/v), respectively.

Intrinsic viscosity, molecular weight and thermal degradation of polymers

The intrinsic viscosity, molecular weight and thermal degradation of the purified polymers obtained from two-step cultivation were determined (Table 4). The P(3HB) originated from CFW showed highest intrinsic viscosity (15.9 dl/g) and mol. wt. (3026 KDa) and copolymers of P(3HB-co-3HV) derived from DW, PW were with less intrinsic viscosity and mol. wt . The temperature required for thermal degradation of P(3HB) polymers originated wastes appeared higher than those of P(3HB-co-3HV) copolymers derived from DW and PW (Table 4).

Table 4 Intricsic viscosity molecular weight and thermal degradation of polymers

Substrate	Polymer	Intrinsic [a] viscosity dl/g	Mol. wt.,[b] KDa	Thermal [c] degradation, ^{0}C
Glucose	P(3HB)	15.5	2937	280
Glucose+valerate	P(3HB-co-19.8mol%3HV)	13.2	2398	268
CFW	P(3HB)	15.9	3026	284.5
FPFW	P(3HB)	14.0	2558	276.8
DW	P(3HB-co-34.65mol% 3HV)	10.6	1845	257.7
PW	P(3HB-co-31.2mol%3HV)	11.0	1931	276.8

Legend: CFW - Wastes from candy factory waste, FPFW- fruit processing factory waste, DW- domestic waste and PW- poultry waste. Polymer isolated from extensively washed and dried biomass using chloroform at 60^{0}C and purified with diethyl ether. [a] The viscometric measurement was made in chloroform solution of purified polymer at 30 ^{0}C with Ubhelohde's dilution viscosimeter (Biber *et al*., 1999). [b] Mol. wt. of polymer was estimated through intrinsic viscosity measurement using Mark-Howink equation. [c] Data of thermal degradation of polymers were obtained from TGA analyses. All values are mean of triplicate.

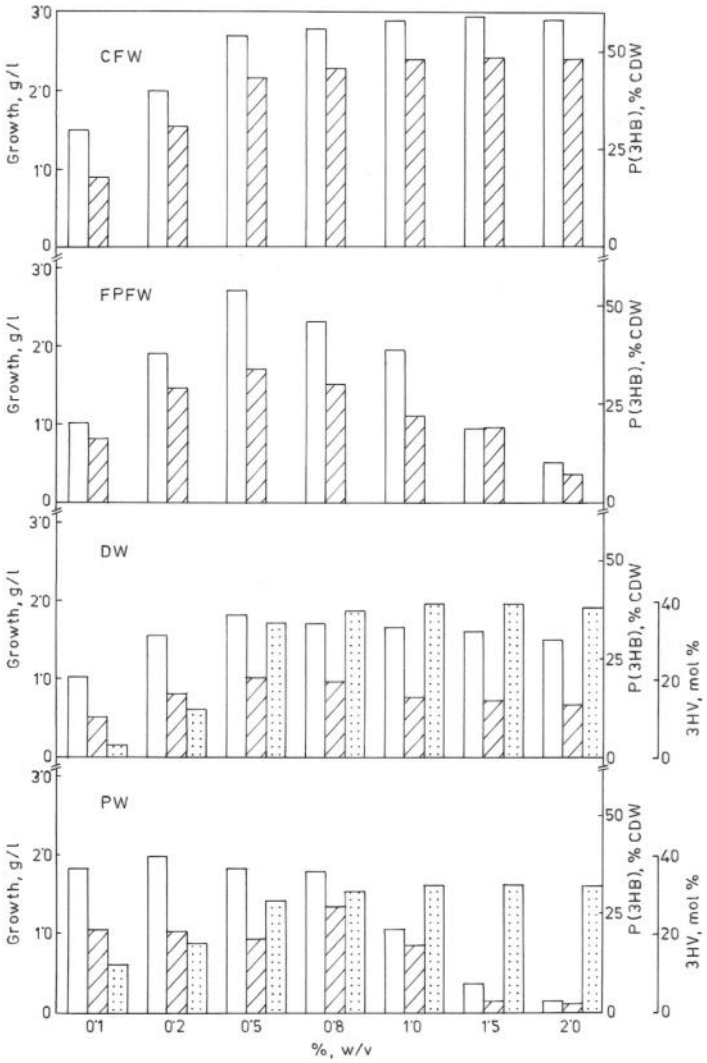

Figure 2 Effect of concentration variation of wastes on growth (□), polymer accumulation (▨) and on their 3HV mol% (▩) during two-step cultivation of *A. chroococcum* MAL-201. CFW - Wastes from candy factory waste, FPFW- fruit processing factory waste, DW- domestic waste and PW- poultry waste.

DISCUSSION

Azotobacter chroococcum MAL-201 has been reported to accumulate P(3HB) and P(3HB-co-3HV) accounting 70% and 67% of CDW, respectively. The present finding clearly indicated that the cultivation of MAL-201 in waste filtrate did not appear as suitable medium for growth as well as accumulation of polymers (Table 1). Supplementation of individual waste in Stockdale–glucose medium in single-step cultivation still resulted in the reduction of growth and synthesis of polymer than those on control medium (Table 2). However, increased accumulation of 3HV in P(3HB-co-3HV) from DW and PW could be attributed by the increase in the enhanced uptake of valeric acid from waste.

In two-step cultivation, glucose initiated the early log phase of growth and optimum incubation time for growth and polymer accumulation shifted from 27 h **(Pal *et al.*, 1998)** due to the change of substrate used in second step. The diauxic growth pattern was recorded when diluted waste was introduced as medium. However, the identities of carbon substrate present in wastes were not determined in this experiment. Reduction in growth and polymer content, particularly by DW and PW occurred due to the presence of alkanoic acids and high amount of inorganic salts. Moreover, low carbon contents of those wastes decreased the growth of organism (Table 1, 2 and 3). Such inhibitions by the alkanoic acids at higher concentration have been studied in *Azotobacter vinelandii* **(Page *et al.*, 1992)** where 25 and 21% inhibition of growth were found respectively by butyric and valeric acid at 10mM concentration.

Researches on polyhydroxyalkanoic acids have established that mol. wt. of polymers mostly depend on their 3HV contents **(Scandola *et al.*, 1992; Savencova *et al*., 2000; Pal Saha *et al.*, 2006)** and carbon substrate used in growth medium **(Preusting, *et al.*, 1990, Chen and Page, 1994; Kizlo *et al*., 1999).** In our experiments similar results of variable mol. wt. and thermal degradation nature were obtained when MAL-201 was grown in glucose, glucose-valerate and wastes medium for PHA synthesis (Table 4). All of the values were comparable to those of the homopolymer and copolymer synthesized by MAL 201 in control media.

CONCLUSION

Today there is a major legislative requirement regarding the recyclable products. The world consumers of plastics looking for a material that has the same versatility and same advantages of plastics. It is a challenge to the researchers dealing with polyhydroxyalkanoates to produce this alternative product from nonconventional cheaper substrate or from wastes using a suitable microbial strain to make the process less expensive.

The production of PHA by strain *A. chroococcum* MAL-201 using wastes looks promising, as at present their market price is practically zero. In spite of the low polymer contents of cells, compared to that of control, these investigations suggest a way to convert the organic compounds of wastes into useful green products and to reduce the cost of polymer production by the strain *Azotobacter chroococcum* MAL-201.

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