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DIVERSITY OF POLYHYDROXYBUTYRATE PRODUCING BACTERIAL ISOLATES OF AGROINDUSTRIAL WASTES

J. Kasthuri and S. Poornima

Department of Zoology, The Standard Fireworks Rajaratnam College for Women, Sivakasi – 626 123, Tamil Nadu, India.

ABSTRACT

Monitoring the diversity of polyhydroxybutyrate producing bacterial isolates of 15 novel agroindustrial wastes revealed that the aquaculture industrial site seemed to harbour with a maximum of 6 positive colonies. About 67 % of the PHB producing isolates were of *Bacillus* Sp., 25 % *Acinetobacter* and only 8 % were of Enterobacter. However, *Acinetobacter* CN1 an isolate of cashew nut industry has been identified as a potential (8.4 g / L media) PHB producing strain. However, when the carbon source was substituted with one of the cheapest sources, *viz* sago waste (2 %) , *Acinetobacter* CN1 appeared to accumulate 8.5 g PHB granules / L .

Key words: Polyhydroxybutyrate, agroindustrial wastes, *Acinetobacter* CN1

INTRODUCTION

Polyhydroxybutyrate (PHB) a biodegradable thermoplastic, the most common member of the PHA family has similar physical properties like polypropylene which can also be easily biodegradable by both aerobically and anaerobically (Hankerymer and Jieerde, 1998; Luzier, 1992). It is being extracted from wide range of bacteria including heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, gliding bacteria, *Actinomycetes* Spp., Cyanobacteria, anaerobic fatty acid oxidizing Gram negative bacteria etc., (Anderson and Dawes, 1990). Further, Nickerson *et al*. (1981), Hanzlikova *et al.*(1985), Lach *et al*. (1990) and Lee, (1996) have also investigated the PHB production capacities of bacterial populations. However, the major constraint in the commercial production is its cost. 1 kg PHB costs about US$ 15–30 when compared to that of polypropylene (US$ 0.70). This can be significantly reduced by using suitable cheaper and versatile raw materials generated by the agriculture sector in the fermentation medium. Santimano *et al.* (2009) opines that the *Bacillus* Spp. strain COL / A6 isolated from humus, an econiche, are able to degrade even the more complex plant materials rich in carbohydrates in a highly effective manner.

Hence, the present investigation attempts to explore the diversity of PHB producing bacterial isolates of various agroindustrial sites / wastes and to exploit their potential for accumulation of PHB as intracellular granules and also when grown in a media in which the carbon source has been substituted with an agroindustrial (sago) waste .

MATERIALS AND METHODS

Soil samples from the surroundings of 15 different agroindustries were collected at a distance of 1 km away from the industrial sites and at the depth of 15 cm in a sterile conical flask. The soil samples were also analyzed for physicochemical properties. Soil was then extracted with water. 0.1 ml inoculum in 10^{-6} dilution was plated on to a nutrient agar plate. From the morphologically distinct colonies obtained, the pure cultures were made by streak plate technique and then subjected to screening for PHB production by Sudan black staining method (Kitamura and Doi, 1994). The positive colonies were further subjected to morphological and biochemical characterization (Cappuccino and Sherman, 2006).

The most strains examined under this programme were clustered based on Bergy's Manual of Systematic Bacteriology (Holt *et al*., 1994). The isolates were then grown in a specially designed medium (N2 Limited Minimal synthetic Medium g / L (pH: 7.0-7.2) Glucose - 20.0, Ammonium sulphate - 02.0 , KH_2PO_4 - 13.3, Citric acid - 01.7, $MgSO_4$ 7, H_2O - 1.2, Trace element solution - 10 ml, Agar agar - 15. Trace element stock solution g / L $FeSO_4.7H_2O$ - 10.00 , $CuSO_4.7H_2O$ - 1.00 , $ZnSO_4.7H_2O$ - 2.25 , $MnSO_4$ - 0.05, $Cacl_2$ - 2.00, $Na_2B_4O_7$ - 0.23, $(NH_4)_6MO_7$ - 0.10, 25 % HCl - 10 ml) to determine their PHB producing capabilities by spectrophotometrically (UV), (Bonartseva and Myschkina, 1985; Kuniko *et al.*, 1988). Further, the isolates which reported to have high yield were then further grown in a media in which glucose was substituted with 2 % sago waste and the PHB content was assayed.

RESULTS AND DISCUSSION

Examination of the physicochemical characteristics of the soil samples of agroindustrial sites **(Table 1)** revealed the pH values ranging from acidic (6.50) to alkaline (9.10) conditions. As far as the micro nutrients and trace metals are concerned, most of the samples seemed to contain no Zn and Mn. Mn was maximum in decompose of sheep yard. Cu was maximum (c 1.0 ppm) in coir pith, cashew nut industries and poultry waste area. Fe was in higher concentrations (c 15 ppm) in sheep yard, vermi compost and pharmaceutical industry samples. K was in much higher concentration than other elements. Phosphorus was generally low. Most of the samples were non-saline except that of aquaculture industry, sheep yard and cow yard, which were slightly saline in nature. Screening the soil samples for bacterial isolates **(Table 2)** revealed the highest number (8) of distinct colonies with aquaculture industrial sites. This might probably be due to the physicochemical parameters of the soil sample of this industry is found to be optimum to support the bacterial populations. Nevertheless, the lowest (2) number of colonies were reported with the rubber plantation area, wood industry and paddy waste. Interestingly, a maximum of 6 bacterial isolates of aquaculture industry showed the positive results for PHB production. Where as the coir pith industry and herbal processing unit area were reported with 2 positive colonies and only a single positive colony was exhibited by cashew nut and poultry industrial sites**.** In the present study, with Sudan Black, the positive colonies showed the

Table1 . Physiochemical Characteristics of the soil samples of various Agro Industrial sites.

S.No.	Soil Sample	pH	E.C (dS/m)	O.C (%)	P (ppm)	K (ppm)	Fe (ppm)	Zn (ppm)	Cu (ppm)	Mn (ppm)
1.	Coir pith Industry (CP)	6.87	3.05	1.30	0.05	192.00	08.024	0.004	1.042	0.00
2.	Cashew nut Industry (CN)	6.67	1.45	0.00	0.48	160.00	06.196	0.001	1.032	0.00
3.	Poultry waste(PO)	7.22	0.98	2.28	1.37	472.00	08.120	0.002	1.070	0.00
4.	Rubber Plantation area (RPA)	6.50	0.17	0.58	1.46	041.82	04.850	6.206	0.822	0.00
5.	Pharmaceutical Industry (PH)	7.53	0.91	2.23	0.37	135.00	15.720	0.004	0.812	2.13
6.	Agro compost (AC)	8.25	1.98	2.59	2.50	300.00	06.100	0.006	0.860	0.00
7.	Wood Industry (WI)	7.55	0.64	2.96	1.50	039.00	05.426	0.000	0.876	0.00
8.	Paddy Waste (PW)	6.84	0.16	0.02	1.15	018.47	11.488	52.24	0.804	0.00
9.	Herbal Processing Unit (HPUA)	6.80	2.11	2.70	0.23	179.00	06.292	0.000	0.960	0.00
10	Vermi Compost (VC)	6.90	3.77	0.00	1.85	250.00	15.912	0.000	0.869	0.00
11	Aqua culture Industry (AI)	7.80	4.25	0.00	2.27	021.67	06.582	0.000	0.776	1.016
12	Vermi Processing Unit (VP)	7.85	2.00	2.76	0.73	047.20	06.292	0.000	0.784	3.632
13	Sheep Yard (SY)	9.10	6.91	2.59	1.34	290.00	14.278	0.000	0.730	0.00
14	Cow Yard(CY)	9.03	6.72	2.23	3.83	260.00	21.240	0.000	0.784	0.00
15	Sheep Yard decompose(SYC)	7.14	2.42	0.00	3.05	156.00	08.024	0.000	0.736	9.108

Table 2. PHB producing bacterial isolates / colonies obtained from various agroindustrial sites.

S.No.	Samples	Collected Sites	Total No of colonies obtained	No of colonies Showed Positive results for PHB
1.	CP	Marugur, Kanya Kumari	**8** (CP1-CP8)	**2** (CP1&CP3)
2.	CN	Kulasekaram, Kanya Kumari	**5** (CN1-CN5)	**1** (CN1)
3.	PO	Salem	**4** (PO1-PO4)	**1 (** PO4)
4.	RPA	Thiruvattaru, Kanya Kumari	**2** (RPA1-RPA2)	Nil
5.	PH	Orchid Pharma ,Chennai	4 (PH1-PH4)	Nil
6.	AC	Thevarkulam, Sivakasi	**3** (AC1-AC3)	Nil
7.	WI	Nagercoil	**2** (WI1-WI2)	Nil
8.	PW	Kudan kulam ,Trinelveli	**2** (PW1-PW2)	Nil
9.	HPUA	Zigma Herbal Remedies,Aralvaiemozhi	**7** (HPUA1-(HPUA7)	**2** (HPUA 2&5)
10.	VC	Scott College, Nagercoil	**3** (VC1- VC3)	Nil
11.	AI	Anchigramam , Kanya Kumari	**7** (AI1-AI 7)	**6 (**AI1 – AI 6)
12.	VP	Scott Colleges, Nagercoil	**3** (VP1-VP3)	Nil
13.	SY	Arunachalapuram ,Sivakasi	**4** (SY1-SY4)	Nil
14.	CY	Arunachalapuram ,Sivakasi	**3** (CY1- CY3)	Nil
15.	SYC	Thevarkulam ,Sivakasi	**3** (SYC1-SYC3)	Nil

colour range from Grey to Blue. Lawson and Tonhazay (1980) have also reported the presence of PHB in *Acinetobacter* Spp. by staining with Sudan Black. Where as Gavin *et al*. (1993) have observed the PHB granules of *Acinetobacter* as electron transparent bodies when stained with Sudan black and Nile blue.

The results of both the morphological and biochemical characterization of PHB producing bacterial isolates revealed that out of the total isolates, 67 % were of *Bacillus* Sp., 25 % , *Acinetobacter* and only 8 % of the isolates were *Enterobacter* **(Table 3).** Interestingly, about six isolates (AI1-AI6) of aquaculture industry were exclusively *Bacillus* Spp. Only where as the PHB producing isolates of coir pith industry were characterized as *Bacillus* CP1 and *Acinetobacter* CP3. Nevertheless, the wastes of the herbal processing units were reported with *Bacillus* HPUA2 and *Enterobacter* HPUA5 where as the PHB producing isolates of cashew nut and poultry industries were identified as *Acinetobacter* CN1 and PO4, respectively.

PHB producing efficiency of bacterial isolates **(Table 4)** ranged from 3.1 to 8.4 g/L media. *Acinetobacter* CN1 exhibited maximum PHB production (8.4 g / L media) followed by *Acinetobacter* CP3 and *Bacillus* AI 6 (0.2) and then by *Bacillus* AI 5 (6.1). The remaining *Bacillus* HPUA2 and HPUA5 (3.8 & 3.6), *Bacillus* CP1 (3.2), *Bacillus* AI 2 and AI 3 (3.2) and AI 4 (3.1 g / L media) were on the lower side. The high PHB yielding *Acinetobacter* CN1 has recorded 8.5 g / L media when grown in a medium where glucose was substituted with sago industrial waste.

The accumulation of PHB by *Acinetobacter coleoaceticus* var. *lwoffi* isolated from activated sludge has been demonstrated by Lotter and Dubery (1986). Subsequent investigations have also showed that a number of *Acinetobacter* strains are capable of synthesizing PHB via carbon storage route (Lawson and Tonhazy, 1980; Lotter and Murphy, 1988). Recently, the PHA synthase gene has been exercised from an *Acinetobacter* strain isolated

from a modified activated sludge treatment plant (Schembri *et al*., 1994). Steinbuscheil and Schlgel (1991) reported an alternative mechanism for regulation of PHB synthesis in *Acinetobacter* Spp. which involves the activation of the PHB biosynthetic gene under conditions of phosphate starvation.

Table 3. Morphological and biochemical characteristics of bacterial isolates.

S.No.	Morphological Studies			Biochemical Studies														
	PHB producing bacteria	Cultural Characteristics		Gram staining	Indole	MR	VP	Citrate	Oxidase	Catalase	Starch	Triple sugar iron	Motility	Capsule	Spore	Maconkey agar	Urease	Organism Identified
1	CP1	white	rhizoid	G+b	-	+	-	-	-	+	+	Alkali	+	-	central	-	+	*Bacillus*
2	CP3	yellow	dotted	G-b	-	+	-	+	-	+	-	A-bud Al - slant	-	-	-	sigmoid	+	*Acinetobacter*
3	CN1	white	filamentous	G-b	-	+	-	+	-	+	-	A-bud Al - slant	-	-	-	sigmoid	+	*Acinetobacter*
4	PO4	white	raised	G-b	-	-	-	+	-	+	+	Alkali	-	-	-	Fine colony	+	*Acinetobacter*
5	HP2	white	beaded	G+b	-	-	-	-	-	-	+	A-bud Al - slant	+	-	central	-	+/-	*Bacillus*
6	HP5	white	circular	G+b	-	+	+	+	-	+	-	A-bud Al - slant	+	-	-	mucoid colony	-	*Enterobacter*
7	AI1	white	circular	G+b	-	-	-	+	-	-	-	A-bud Al - slant	+	-	-	-	+/-	*Bacillus*
8	AI2	white	iregular	G+b	-	+	-	+	-	+	+	A-bud Al - slant	+	-	central	-	-	*Bacillus*
9	AI3	yellow	circular	G+b	-	-	-	+	-	+	+	Alkali	+	-	central	-	+	*Bacillus*
10	AI4	white	circular	G+b	-	+	-	-	-	-	+	Alkali	+	-	central	-	+	*Bacillus*
11	AI5	white	beaded	G+b	-	+	-	-	-	-	+	A-bud Al - slant	+	-	central	-	+	*Bacillus*
12	AI6	white	circular	G+b	-	-	-	-	-	+	+	A-bud Al - slant	+	-	central	-	+/-	*Bacillus*

Gavin *et al*. (1993) have reported the effects of limitation of various nutrients such as ammonia, phosphate and sulphate in a defined minimal salt medium on PHB production by the strains RA3117 and RA 3757 of *Acinetobacter* Spp. isolated from activated sludge and reported with more number of PHB granules only when phosphate was limited.

However, the present approach of utilization of agro-industrial wastes for the synthesis of a valuable product, PHB. a potential biodegradable replacement for persistent conventional plastics will not only ensure the reduction in manufacturing cost but also would solve the problems associated with waste disposal.

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Table 4. Quantitative Estimation of PHB.

S.NO.	Samples	Concentration g/L
1.	CP1	3.2
2.	CP3	6.2
3.	CN1	8.4
4.	HP2	3.8
5.	HP5	3.6
6.	PO4	6.8
7.	AI1	6.0
8.	AI2	3.2
9.	AI3	3.2
10.	AI4	3.1
11.	AI5	6.1
12.	AI6	6.2

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