International Journal of Multidisciplinary Research and Development
Online ISSN: 2349-4182, Print ISSN: 2349-5979; Impact Factor: RJIF 5.72
Received: 26-06-2019; Accepted: 28-07-2019
www.allsubjectjournal.com
Volume 6; Issue 9; September 2019; Page No. 207-211

Poly-β-hydroxy butyrate production from renewable Agri byproducts as carbon source

Swati Shalini[1*], Ajay Ade[2], Rekha Ahlawat[3], Virendra K Sikka[4]
[1, 2] National Institute of Biologicals (Ministry of Health and Family Welfare, Government of India), Noida, Uttar Pradesh, India
[3, 4] Department of Molecular Biology and Biotechnology, Chaudhary Charan Singh Haryana Agricultural University Hisar, Haryana, India

Abstract
Novel bacterial isolates producing Poly-β-hydroxy Butyrate (biodegradable plastics) were isolated from waste water and soil and were able to produce PHB at higher concentration S11 (59% w/w), S14 (58 % w/w) and S27 (57% w/w). For decreasing the production cost, agricultural byproduct such as sugarcane molasses and potato peels were used as cheap carbon sources for the fermentation process. After comparing different combinations of agricultural wastes it was found that sugarcane molasses was most suitable among them as it resulted in higher PHB production of 67% w/w on dry weight basis. For large scale PHB production the fermentation was carried out in a 10 L controlled reactor using S11 strain and it resulted in intracellular PHB accumulation of 70% PHB w/w on dry weight basis.

Keywords: PHB, agricultural byproducts, molasses, *pseudomonas species*

Introduction
Poly-β-hydroxy Butyrate (PHB) is the most popular member of bioplastics group (plastics of biological origin) since they are 100 % biodegradable thermoplastics produced by various bacterial species. Lemogine (1927) identified PHB granules in *Bacillus species* for the first time and characterized its chemical structure. After that, PHB granules have been observed in several bacterial genera, including *Azotobacter*, *Beijerinkia*, *Alcaligenes*, *Pseudomonas*, *Rhizobium* and *Rhodospirillum*. PHBs are the inclusion bodies produced by the bacteria as the storage material. And are produced when the carbon source is present in excess concentration as compared to the other nutrients in the fermentation medium (Williamson and Willkinson, 1999) [12]. Because of its high molecular mass it has similar properties like synthetic plastics. Synthetic plastics are used in almost every field of our life ranging from automobile to medicine, but they are difficult to dispose (Divya *et al*., 2010) [4]. The synthetic plastics are xenobiotic, therefore are recalcitrant towards microbial and chemical degradation as a result of which, they accumulate in the landfills, oceans and cause damage to our environment (Kumar *et al*., 2009) [6]. Eco-friendly plastics are the solution for this burning problem of increasing plastic pollutants.

In this order the attempts were made to produce bioplastics by blending synthetic polymers with starch but they were not completely biodegradable. Polyhydroxybutyrate are most appropriate bioplastics since they have the mechanical properties similar to synthetic plastic and they are completely biodegradable (De smet *et al*., 1983, Cavalheiro *et al*., 2009) [3, 2]. The novel features like nontoxicity, biocompatibility and high crystallinity make them applicable in making packaging films, paper coatings, mulching films, and various daily use items (Amara *et al*, 2005) [1]. PHBs do not elicit human immune response, therefore can be used as carrier for delayed release of therapeutic drugs (Senior and Daws, 1973).

Wide commercial applications of PHA are presently hampered because of its high cost of production. The commercial price of PHA is about ten times greater than synthetic plastics. The production cost could be reduced by utilizing low cost substrates such as agri- byproducts (molasses, wheat bran, corn steep liquor) and agro-industrial wastes (potato peels) for providing nutrition to PHB producing bacteria (Ramdas *et al*., 2010) [9]. In the present study novel PHB producing *Pseudomonas species* and *Bacillus species* were isolated from decomposed waste and drain water from different regions. These bacteria were screened on the basis of their capacity to accumulate PHB granules with help of Nile blue A stain. The selected high PHB producing strains were grown in cultivation medium consisting different agricultural wastes as carbon sources. The highest PHB producing strain was selected for pilot scale PHB production in 10 liters container using most suitable agri-byproduct and cultivation conditions.

Material and Methods
Isolation of bacterial strains and screening for PHB production
Hundreds of bacterial strains were isolated from decomposed soil and sewage water samples obtained from different location in Haryana, India. Isolation of bacterial strains was carried out by serial dilution in 0.8% saline solution and plated on nutrient agar medium. PHB producing strains were screened on minimal medium containing Nile blue A stain. PHB granules were then stained by Nile blue A which is an oxazine dye and produces orange fluorescence when excited with UV light at wavelength 460nm (Ostle and Holt 1982). Screening media was constituted with glucose 3 g/l, KNO_3 0.5 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$.7H2O 0.2 g/l, NaCl 0.1 g/l and $CaCl_2$ 0.02 g/l.

Identification of bacterial strains
Bacterial strains were partially characterized by performing

different microbiological along with chemical experiments as per "Bergey's manual of systematic bacteriology".

Determination of biomass

In order to determine the dry cell weight, the suspension cultures were filtered using Whatmann filter paper and centrifuged at 8000 rotation per minutes till 10 minutes. Harvested cell pellets were Kept in oven at 65 °C and dried till constant weight was obtained. The constant weight noted at dry cell mass.

PHB extraction and estimation in diverse isolates

In order to perform the extraction procedure of PHB, chloroform along with sodium hypochlorite method (Slepecky and Law, 1961) [10] was used. Chloroform and sodium hypochlorite caused cell lysis, the suspension got separated into two phases organic and aqueous. PHB got dissolved in the organic phase when it was heated and chloroform got evaporated leaving PHB as a residue. The 500 µl of PHB residue was dissolved in chlroform and the solution was further mixed with 6 ml of conc. H_2SO_4. The mixture was boiled at 100°C till 10 minutes which lead to crotonic acid production (Sharma, 2006) [11]. The PHB concentration was directly correlated with crotonic acid concentration observed by UV spectrophotometer at 235 nm. The absorbance was then compared with internal standards of crotonic acid.

Large scale production of PHB using agri-byproducts as carbon source:

The carbon and nitrogen sources were replaced with various organic and inorganic compounds in order to identify the most suitable substrate responsible for enhanced PHB accumulation. With the purpose of making PHB production economical, agro industrial byproducts were tried as carbon source. Large scale fermentation was carried out in various vessels containing 10 liters medium. Media constituted of molasses 3 g/l + $KN0_3$ 0.65 g//l + K_2HPO_4 0.5 g//l + $MgSO_4.7H_2O$ 0.2 g//l + NaCl 0.1 g//l + $CaCl_2.2H_2O$ 0.02 g//l. The temperature was maintained at 37 °C throughout the cultivation period. 1% inoculum was used to initiate the fermentation. Agitation was was done at 200 rpm to provide adequate mixing and aeration pH of the medium was maintained at 7.0 before autoclaving. The cultivation was carried out till 6 days after inoculation for maximum accumulation of PHB.

Results and Discussion

Isolation of bacterial species and their selection for PHB production

Various bacterial species were isolated by spreading serially diluted samples on Nutrient agar media. Later on, the isolated single cell colonies were transferred to minimal medium and screened for their PHB accumulation capacity. On the basis of fluorescence studies (Figure 1) six bacterial strains were selected for detailed analysis, and media optimization studies. After further analysis it was found that medium containing 0.65 g/l KNO_3 with 2 g/l glucose was most suitable for PHB production. Later on glucose was replaced with sugarcane molasses and potato peels in different ratios as carbon source for pilot scale production of PHB at 10 liters. Strain no. S9, S11, S14, S22, S23, S27 were producing maximum fluorescence were selected for further studies.

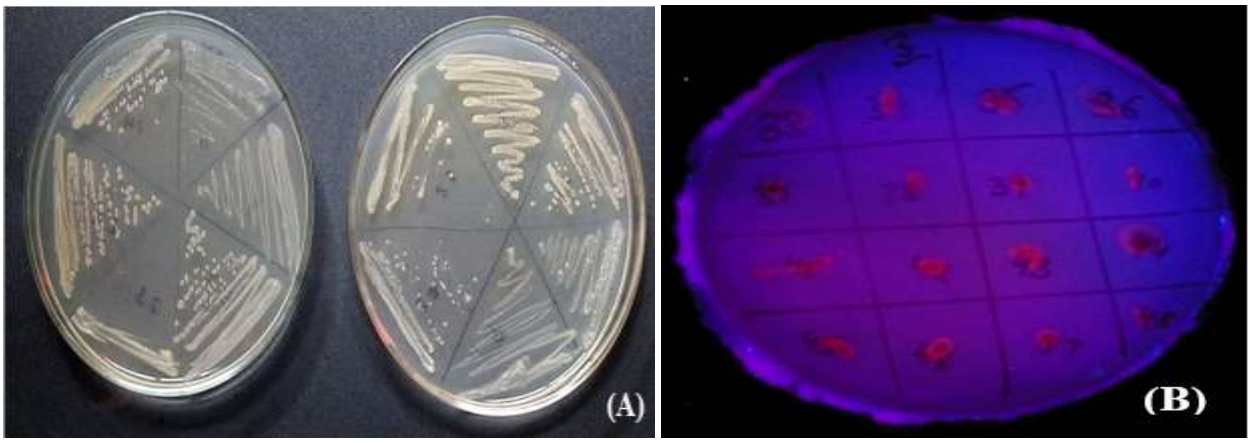

Fig 1(A): Isolation of bacterial strains (B): Screening on NILE blue A stain for PHB accumulation

Partial characterization of selected bacterial strains

The Bacterial strains were partially characterized on the basis of test mentioned in "Bergey's manual of systematic bacteriology" On the basis growth on diverse selective media (Table 1) and biochemical tests, it was found that the gram positive isolates S9, S22 and S23 were spore forming so they were suspected to belong to genus *Bacillus species* and the gram negative strains S11, S14 and S27 were producing pink pigment so they were suspected to as *Pseudomonas species* (Figure 2).

Table 1: Partial characterization of selected bacterial isolates by biochemical tests

S. No.	Strain	Gram staining	Screeing on YEMA + Congo red	Oxidase test	Catalase test	Screening on Kings'B	Screening on Peptone Broth	Spore formation test
1	S9	+ve	Pink	-ve	+	White	+	+ve
2	S11	-ve	Pink	+ve	+	Pink	++	-ve
3	S14	-ve	Pink	+ve	+	Pink	++	-ve
4	S22	+ve	Pink	-ve	+	White	+	+ve
5	S23	+ve	Pink	-ve	+	White	+	+ve
6	S27	-ve	Pink	+ve	+	Pink	++	-ve

++ = Heavy growth + = Presence of growth / positive assay

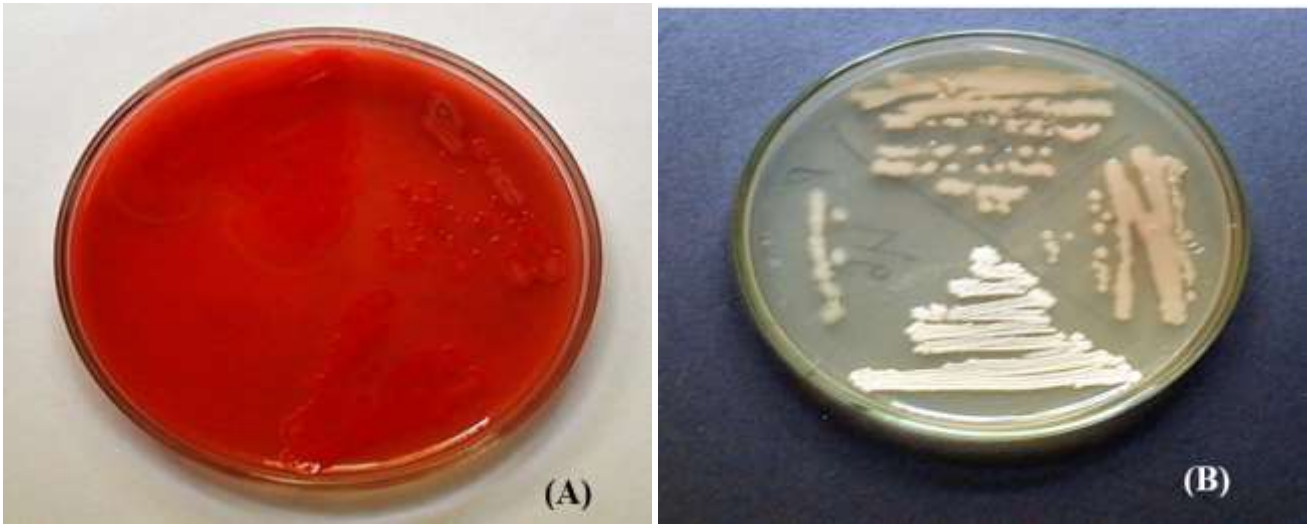

Fig 2: The growth of selected bacterial strains on YEMA medium (A) and Kings B Medium (B)

Selection of suitable cultivation condition for PHB accumulation

On the basis of preliminary florescence studies six bacterial strains S9, S11, S14, S22, S23 and S27 were selected for media optimization studies. For enhanced PHB accumulation different carbon as well as nitrogen substrate were examined. Various nutrient medium composition were inoculated with selected strain and analyzed for biomass as well as PHB production. One liter cultivation medium was inoculated with selected strains and kept in incubator shaker fixed at 28°C

for 6 days. The longer cultivation period was kept to ensure the maintenance of stationary phase.

The cells were then harvested for biomass estimation and PHB extraction. Highest PHB concentration was reported in the case of S11 bacterial (59% w/w) followed by S14 (58% w/w) as shown in figure 3). Least amount of PHB was observed in the case of S23 (29% w/w). On the basis of results obtained, S11 strain was selected for further media optimization and PHB production.

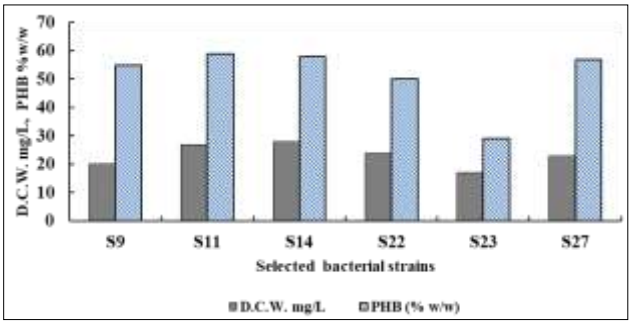

Fig 3: PHB production and biomass accumulation by selected strains on minimal medium

Selection of suitable nitrogen substrate

Various nitrogen substrates were compared in order to study their effect on biomass accumulation as well as PHB concentration. It was observed that the carbon substrates were required in excess concentration so that the supplementary carbon residue could get converted into PHB granules. Nitrogen source in the form of KNO_3 (0.65 g/l) was found better for PHB production since it enhanced the PHB accumulation by 13% as compared to organic nitrogen source (Table 2).

Selection of suitable agri-byproducts

For cutting down the cost of PHB production cheaper agri-byproducts were analyzed as carbon substrate. On the basis of experiments performed in the present study it was observed that potato peels with molasses led to higher PHB accumulation as the crotonic acid formation enhanced by 14%. Potato peels with molasses also led to increased dry cell weight accumulation by 4% (Table 2). Therefore, it was concluded by present study that agri-byproducts were better carbon substrate for biomass growth as well as PHB production.

Table 2: PHB production by selected S11 strain using glucose and agri-byproducts as carbon source along with organic and inorganic nitrogen sources

S. No.	Carbon Substrate (g/l)	Nitrogen Substrate (g/l)	Dry cell weight (g/l)	PHB conc^n (mg/l)
1	Glucose 3	KNO_3 (0.65)	2.06	15.5
2	Glucose 3	Y.E (1)	0.992	12.6
3	Potato peels (2) + Glucose (1)	Y.E (1)	2.06	15.5
4	Molasses (3)	Y.E (1)	0.992	12.6

Comparison of various agri-byproduct concentration for higher PHB production

Agricultural waste produced as industrial byproducts for example sugarcane molasses, potato peels in various combinations along with inorganic nitrogen substrate were compared for PHB production. It was observed after the present study that Molasses (3 g/l) was the most suitable carbon substrate for PHB formation. Higher biomass as well as PHB concentration was obtained when molasses only was utilized as carbon substrate. When only potato peels were used as carbon substrate it led to decreased biomass. Therefore, it was concluded from the present study that molasses had a beneficiary impact on biomass accumulation as well as PHB concentration (Table 3).

Table 3: Effect of Agri-byproduct in cultivation medium for obtaining higher PHB concentration

S.No.	Carbon Source (g/l)	Wet Biomass (g/l)	Dry Biomass (g/l)	PHB (mg/l)	PHB (% w/w)	Incremental % PHB production
1	Potato peals (2) + Glucose (1)	4.602	0.294	15.4	54	28
2	Potato peals (2) + Molasses (1)	4.306	0.332	18	56	50
3	Molasses (3)	5.261	0.335	20	60	67
4	Potato peels (3)	2.957	0.26	12	46	0

PHB production using molasses and KNO3 in cultivation medium at 10 liters

After preliminary optimization studies for selection of most suitable nutrients and cultivation conditions the scale up was done in bigger vessel containing 10 liters media using S11 strain. The fermentation resulted in 0.5 g/l of dry weight accumulation with PHB production of 70% w/w on dry weight basis. The PHB estimation by crotonic acid method detected the presence of 25 mg/l PHB in dried cell pellet (Figure 3).

Fig 4: PHB production using different agricultural waste as nutrients 1 litre Erlenmeyer flask and 10 liters Culture Vessel.

In a similar study, the effect of different carbon sources was studied on PHB accumulation and maximum concentration of PHB was obtained when cane molasses and glucose were used as sole carbon sources (Gauda *et al*., 2001) [5]. Kumar (2009) [6] isolated 146 rhizobacteria and screened them for PHB production using a combination of medium based on agri-byproducts containing molasses and potato peels along with mustard cake as substrates resulted in PHB production up to 70.2%.

Conclusions

While screening the strains for PHB production different combinations of carbon and nitrogen sources were tried and it was found that 2 g/l glucose as carbon source and 0.65 g/l potassium nitrate were most suitable for intracellular PHB accumulation. Six best PHB accumulating strains were selected and partially characterized. The selected strains were analyzed for their PHB production capacity and they produced 59-29% PHB dry weight of cell pellet. S11 was found to accumulate highest concentration of PHB and therefore, was used for further studies in order to select cheaper carbon source for PHB production. Various agri-byproducts were utilized for selection of cheaper substrates for PHB production and it was found that molasses at concentration of 3 g/l was the most suitable carbon substrate.

References

1. Amara AA, Rehm BH. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochem. J. 2003; 374(2):413-421.
2. Cavalheiro JMBT, Almeida MCMD, Grandfils C, da Fonseca MMR, Production of polyhydroxyalkanoates (PHAS) from waste materials and by products by submerged and solid-state fermentation. Bioresource technology. 2009; 100(23):5996-6009.
3. De Smet M, Eggnik G, Witholt B, Kingma J, Wynberg H, Characterization of intracellular inclusions formed by Pseudomonas oleovorans during growth on octane. Journal of Bacteriology. 1983; 154(2):870-878.
4. Divya G, Archana T, Manzano RA, Polyhydroxy Alkanoates–A sustainable Alternative to Petro–Based Plastics. Journal of Petroleum & Enviranmental Biotechnology, 2013.
5. Gauda, MK, Swellam, AE, Omar SH. Production of PHB by a bacillus megaterium strain using sugarcane

molasses and corn steep liquor as sole carbon and vitroya sources. *Microbial Rs.* 2001; 156:201-207.
6. Kumar U, Sharma A, Sikka VK, Maximization of poly-β-hydroxy butyrate production by rhizo-bacteria using ecofreindly agri-byproducts. The South Pacific Journal of Natural Science. 2009; 27:35-37.
7. Lemoigene M. Production d'acid 3-oxybutyrique par certain bacterias du grouped du *B. subtilise* CR Acad Sci. Paris. 1927; 176:1761-1762.
8. Ostle AG, Holt, JG. Nile blue as a fluorescent stain for PHB. Appl. Environ. Microbial. 1982; 44:238-241.
9. Ramdas N, Soccol C, Pandey A. A statistical Approch for Optimization of Polyhydroxybutyrate Production by Bacillus sphaericus NCIM 5149 under submerged Fermentation Using Central Composite Design. Applied Biochemistry and Biotechnology, 2010; 162(4):996-1007.
10. Slepecky RA, Law JH. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium.* J Bacteriol. 1961; 86:37-42.
11. Sharma A, Identification and manipulation of rhizobial stains and genes for polyhydroxy butyric acid production. Ph. D thesis submitted to CCS HAU, Hisar, 2006.
12. Williamson D. Willkinson J, The Isolation and Estimation of the Poly-β-hydroxy-butyrate Inclusions of Bacillus Species. Journal of General and Applied Microbiology. 1958; 19(1):198-209.