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Agro-industrial byproducts as alternate cost-effective medium components for production of polyhydroxybutyrate

Kiran Nehra*, Priyanka Lathwal, Shivani Gupta, Parveen Kaur Sidhu & JS Rana

Department of Biotechnology, Deenbandhu Chhotu Ram University of Science & Technology, Murthal-131 039, Sonapat, Haryana, India

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Polyhydroxybutyrate (PHB), biodegradable plastics, having properties similar to conventional plastics, exhibit a high potential for replacing petrochemical-based non-degradable plastics. But a major obstacle in their large-scale commercial production is high production cost, one of the key factors responsible for which is the expensive carbon sources that are currently being used in their manufacturing process. The present work was aimed to study PHB production using cost-effective substrates as carbon sources in the production medium. Inexpensive agro-industrial byproducts (molasses, cheese whey, wheat bran and banana peel, used in different concentrations) were explored for their potential to substitute the conventional costly substrates. Compared to glucose, all the four alternate carbon sources showed an enhancement in PHB production. The mean percent increase in PHB production was in the range of 3.81% to 7.23%. However, some of the bacterial isolates showed an enhancement as high as 23.32% and 19.65%. Highest mean PHB yield was observed in molasses (135.18 mg/mL), followed by cheese-whey (133.79 mg/mL), banana peel, and least in wheat bran based production medium. On dry weight basis, PHB accumulation in cells was observed to be 64.32% and 64.29% of the total dry cell weight with molasses and cheese whey, respectively, as carbon sources. FTIR spectra of extracted PHB were found to be comparable to the spectra of standard PHB, thus, confirming the chemical nature of the extracted polymer.

Keywords: Biodegradable plastics, Bioeffluents, Bioplastics, Carbon source, Cheese whey, Molasses, Nitrogen source, PHB production

Plastic materials which form an integral part of human daily life, have today become a major cause of global environmental concern due to their non-biodegradable nature. Some of their positive qualities such as durability and resistance to degradation which were once considered as the most attractive properties of plastics; over the last few decades, are being increasingly regarded as a source of major hindrance in solid waste management^{1,2}. Additionally, as the conventional plastics are predominantly manufactured from non-renewable resources such as crude oil, they are also responsible for depletion of our finite fuel resources. More recently, these two major issues associated with conventional plastics, *viz.*, the rising environmental concerns and the realization that the petroleum resources are finite, have necessitated the need for switching to safer alternative strategies which are independent of fossil resources. Use of biodegradable plastics made from renewable resources is one such alternative strategy which can offer a

solution to the environmental hazards posed by the conventional plastics³. Therefore, in the recent past, there has been a mounting public awareness as well as scientific interest for the search for biodegradable polymeric materials which can act as an ecologically safer alternative to plastics^{4,5}. Of a vast number of biodegradable polymers which have been explored for their suitability as a good replacement for conventional plastics; polyhydroxyalkanoates (PHA), which exhibit properties similar to the conventional plastics, form a major group⁶. PHA-based plastics, in contrast to petroleum-based plastics, become a part of the carbon cycle, because decomposition of items made up of PHAs result into their oxidative breakdown and complete degradation into simple final products as water and carbon dioxide. These simple final products form the basic raw material for photosynthetic fixation of CO₂ by green plants for regeneration of carbohydrates⁷. Thus, the major advantageous characteristics of PHA are: their biodegradability, non-dependence on fossil fuels, bio-based origin and biocompatibility.

Poly- β -hydroxybutyrate (PHB) are the most common and the best characterized compounds in the

*Correspondence:

Phone: +91 130 2484128; Fax: +91 130 2484004
E-mail: nehra.kiran@gmail.com

family of polyhydroxyalkanoates⁸. PHBs are produced by both Gram-positive and Gram-negative bacteria as an intracellular storage granule serving as energy reserve material to be used during starvation period in microorganisms⁹. The PHB synthesis occurs during unbalanced growth conditions including excessive amount of carbon, and a limitation of essential nutrients such as nitrogen, phosphorous or oxygen^{10,11}. PHBs have physical and chemical properties similar to petroleum-based synthetic plastics like polypropylene, thus facilitating their applications in several areas, including packaging, agro-industrial sector and in the field of pharmaceuticals and medicine¹². But, despite several applications and advantages of these bio-polymers, the commercial production of biodegradable polymers on a large scale is still limited.

Commercial applications and wider use of PHBs have been hampered mainly due to their higher price as compared to the conventional plastics¹³. Approximately, 40-48% of the total production cost of PHB is on the raw materials. Further, the type of carbon source used in the raw materials may account for 70 to 80% of the total expenses. Thus, one of the major factors restraining commercialization of PHB production is the cost of the carbon source or the sugar substrate used in the production medium¹⁴. However, the production cost can be drastically reduced either by using inexpensive and renewable carbon sources like agro byproducts or by developing a production medium using innovative materials such as a broad range of carbon-rich household/industrial wastes as a feedstock for biomediated PHB production¹⁵. Successful commercialization of biodegradable plastic industry largely depends upon the utilization of cost-effective alternate substrates in the production medium. In this context, here we have made an attempt to reduce PHB production cost by exploiting the PHB-accumulation efficiency of a few agro-industrial wastes (molasses, cheese whey, wheat bran and banana peel used in variable concentrations as carbon substrates in the production medium) by several PHB positive bacterial isolates.

Materials and Methods

Bacterial isolates

In the present study, 21 PHB positive bacterial strains (isolated by the authors earlier from the rhizospheric area of three crops, viz., wheat, mustard and sugarcane)¹⁶ were used for evaluating the effect of

cost-effective agro-industrial byproducts as medium components on PHB production.

Standard strain

A standard PHB positive bacterial strain viz., *Cupriavidus necator* (MTCC 1472) procured from Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India, was used as the reference strain in all experiments.

Media and culture conditions

To analyze the effect of inexpensive substrates on PHB yield, all the isolates were grown using two different types of media viz., standardized and modified medium.

Standardized medium

PHB yield was first estimated by growing different isolates under standard medium conditions involving conventional carbon (C) and nitrogen (N) sources. These culture conditions were previously optimized in the host laboratory and constituted: minimal salt medium (MSM)¹⁷ supplemented with 2% glucose as the carbon source and 0.1% ammonium sulphate as the nitrogen source, having a pH of 7.0, incubation temperature of 30°C, and incubation time period as 48 h¹⁸.

Modified medium

The second set of conditions involved estimation of PHB yield by raising the isolates on a modified culture medium which included the replacement of expensive carbon (glucose) and nitrogen (ammonium sulphate) sources in the standardized medium with cost-effective agro-industrial byproducts as the alternate carbon (molasses, cheese whey, wheat bran and banana peel) and nitrogen (urea) sources, but maintaining the incubation time period, pH and temperature conditions similar to the standardized medium¹⁸.

Alternate substrates for PHB production

At present, most of the industrial PHB production units make use of glucose as the sugar substrate. However, if less expensive materials were to be used in PHB production medium, the substrate cost could be reduced, and the use of PHBs would become more feasible. On the other hand, improper disposal of industrial wastes is contributing to the deterioration of the environment and is leading to several health problems. If this waste could be put use to some fruitful purpose, it might help in reducing the environmental deterioration. In this study, we therefore evaluated the potential of agro-industrial

wastes as medium supplements for commercial biosynthesis of bioplastics.

In the present work, four different locally available bioeffluents (molasses, cheese whey, wheat bran, and banana peel) were studied as alternates for glucose as the C-source in the culture medium used for PHB production by promising bacterial strains. Molasses were procured from a sugar mill in Panipat, India; cheese whey from National Dairy Research Institute (NDRI), Karnal, India; wheat bran from Krishna flour mill, Panipat, India; and for banana peel, peel of fresh bananas bought from the local market were used as the alternate C-sources. Urea, procured from the local market was used as the N-source in the production medium as a replacement of ammonium sulphate in the standardized medium. Four combinations of these alternate substrates were made: (i) MSM + molasses as C-source and urea as N-source, (ii) MSM + cheese whey as C-source and urea as N-source (iii) MSM + wheat bran as C-source and urea as N-source, and (iv) MSM + banana peel as C-source and urea as N-source.

Further, to determine the optimum concentration of cost-effective alternate sources, the effect of different C:N ratios on PHB production was also determined. For this, cultures were inoculated in MSM supplemented with different concentrations of the alternate C and N source (C/N ratios as 2:1, 10:1, and 20:1) for each of the four different combinations; thus, resulting in 12 different combinations of the production medium (three for each of the four major combination group). The bacterial isolates were grown in 100 mL of each of the sterilized combination of alternate substrate, incubated at 30°C for 48 h on a rotary shaker (150 rpm), and thereafter, PHB production was quantified by broadly following the method of Law & Slepecky¹⁹. The PHB yield was quantified spectrophotometrically, and based on the yields, the best cost-effective alternate C/N combination and ratio was determined.

PHB extraction was carried out using the dispersion method of sodium hypochlorite and chloroform method of Law & Slepecky¹⁹ with minor modifications²⁰; and quantification was done by two methods: the modified Law & Slepecky method, and by determining dry cell mass²⁰. A comparison was made between the PHB yield of isolates grown on optimized medium with standard carbon (2% glucose) and nitrogen (0.1% ammonium sulphate) sources, and the PHB yield obtained by growing the isolates on a

medium containing cost-effective alternate carbon (molasses, cheese whey, wheat bran and banana peel) and nitrogen (urea) sources.

Estimation of PHB using modified Law & Slepecky Method

This method is based on the principle that PHB can be converted quantitatively to crotonic acid by heating in concentrated sulfuric acid, which can then be estimated by measuring the UV absorption at 235 nm. PHB production by all the 21 PHB positive isolates and the standard strain (MTCC 1472) was quantified by calculating PHB yield from the standard curve prepared by using commercial poly- β -hydroxybutyrate (Sigma-Aldrich). For the extraction of poly (3-hydroxybutyrate), the bacterial pellets were lysed in sodium hypochlorite at 37°C for 30 min, centrifuged at 10000 rpm, and the residue was washed twice with each of the following: water, acetone, and ethanol. The residue was then extracted with boiling chloroform and filtered through Whatman No. 1 filter paper. The chloroform extract was converted to crotonic acid (having maximum UV absorbance at 235 nm) by addition of concentrated H₂SO₄; and the amount of PHB was finally estimated spectrophotometrically in terms of crotonic acid formed by reading the absorbance at 235 nm against a concentrated H₂SO₄ blank. The quantity of PHB produced was determined by referring to the standard curve and was expressed as PHB yield (mg/mL).

Estimation of PHB on dry weight basis

For PHB estimation on dry weight basis, the total cell dry weight (total biomass) of each isolate was first determined by harvesting, washing, and finally drying to a constant weight at 60°C. For this, each bacterial culture was pelleted by centrifugation at 6000 rpm, the cell pellet was dried, and the dry cell weight (DCW) was recorded in units of g/L for further calculations at later stages. The PHB was then extracted by modified Law and Slepecky method as detailed in the above section. The chloroform extract, instead of being treated with concentrated sulfuric acid, was collected in pre-weighed test tubes, and the dissolved PHB was recovered by evaporating the chloroform. The dry weight of PHB was measured by taking the difference of the test tube containing the PHB and the pre-weighed empty test tube. The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry cell weight and expressed as % DCW.

$$\text{PHB accumulation (\%)} = \frac{\text{Dry wt. of extracted PHB (g/L)}}{\text{Total DCW (g/L)}} \times 100$$

FTIR analysis for characterization of PHB

The presence and chemical nature of the extracted polymer was confirmed by conducting FTIR analysis^{21,22}. FTIR is an analytical technique that collects spectra based on the temporal coherence measurements from an infrared source. It is primarily used for identifying unknown substances by producing an infrared absorption spectrum that can identify chemical bonds present in the molecule²². Standard PHB (Sigma Aldrich, USA); PHB extracted from the standard strain (MTCC 1472); and PHB extracted from all the 21 isolates by growing them on each of the four different combinations of alternate C and N sources, were analyzed through FTIR. For this, 1.0 mg of the extracted PHB was mixed with FTIR grade KBr to form a pellet, and the pellet was then subjected to FTIR analysis in FTIR spectrophotometer (Perkin Elmer, frontier DRS-diffuse reflectance sampling accessory). The spectra were recorded in 400-4000 cm⁻¹ range.

Results

The present study was designed as an attempt to economize the process of PHB production so as to enable it to compete with petrochemical derived

polymers. For this, the potential of a few cost-effective substrates as alternate C and N sources in the culture medium was examined; results of the study are detailed in the following sections.

PHB production under different culture conditions

The PHB yield of all the 21 PHB positive isolates and the standard strain *Cupriavidus necator* (MTCC 1472) grown under standard culture conditions, and in culture medium containing cost-effective substrates as alternate C and N sources (as detailed in the methodology section), was determined using the modified Law and Slepecky method. The PHB yield was calculated by referring to the standard curve (expressed in mg/mL), and on dry weight basis (expressed as total % DCW), and the results are expressed in Tables 1 & 2, respectively.

PHB production under optimized culture conditions with conventional C- and N-sources

Under standardized culture conditions involving glucose as the C-source, the PHB yield of all the 21 isolates was found to vary between 97.48 mg/mL (PaM-2 isolate) to 139.80 mg/mL (KW-4 isolate); and between 50.29% (FtM-8 isolate) to 72.73% (HM-2 isolate) (Tables 1 & 2). The average PHB yield of all

Table 1 — PHB yield (mg/mL) of the isolates on standardized medium and on medium with different combinations of alternate C & N sources

Isolate	PHB Yield (mg/mL) and (% increase/decrease in PHB yield in different combinations of alternate C/N sources over PHB yield in optimized medium)								
	Optimized medium	Molasses with Urea (20:1)		Cheese Whey with Urea (20:1)		Wheat Bran with Urea (20:1)		Banana Peel with Urea (20:1)	
	PHB Yield (mg/mL)	PHB Yield (mg/mL)	% Increase/ Decrease	PHB Yield (mg/mL)	% Increase/ Decrease	PHB Yield (mg/mL)	% Increase/ Decrease	PHB Yield (mg/mL)	% Increase/ Decrease
FtW-2	98.61	101.40	2.83	109.39	10.93	98.05	-0.57	100.88	2.30
PaM-2	97.48	97.71	0.002	96.71	-0.79	92.12	-5.50	99.34	1.91
FS-4	89.90	90.06	0.18	97.69	8.67	92.89	3.33	98.72	9.81
AW-1	136.60	138.61	1.47	128.05	-6.26	133.10	-2.56	139.13	1.85
KW-4	139.80	149.59	7.00	148.46	6.19	142.74	2.10	147.22	5.31
PnW-4	134.13	144.28	7.57	144.44	7.69	131.81	-1.73	137.63	2.61
RoW-1	136.04	136.40	0.26	142.12	4.47	143.05	5.15	142.12	4.47
RoW-4	132.27	136.09	2.89	146.04	10.41	142.27	7.56	144.70	9.40
SW-3	114.90	137.48	19.65	133.77	16.42	128.05	11.44	130.26	13.37
AM-2	127.27	138.66	8.95	133.10	4.58	116.96	-8.10	133.77	5.11
FtM-8	122.58	135.73	10.73	126.91	3.53	119.75	-2.31	133.30	8.75
FM-1	119.75	142.12	18.68	129.13	7.83	118.66	-0.91	127.43	6.41
HM-2	124.80	136.81	9.62	136.24	9.17	126.24	1.15	138.87	11.27
RM-1	135.21	147.63	9.19	142.74	5.57	141.96	4.99	138.61	2.51
RoM-1	128.15	140.73	9.82	133.10	3.86	144.28	12.59	130.78	2.05
KS-1	131.45	142.89	8.70	138.66	5.48	117.12	-10.90	131.60	0.11
KS-3	139.75	149.03	6.64	149.34	6.86	137.32	-1.74	144.59	3.46
MS-6	137.32	144.28	5.07	136.76	-0.41	136.09	-0.90	138.77	1.06
RoS-4	133.25	141.96	6.54	145.37	9.10	142.89	7.23	139.54	4.72
SiS-3	135.37	144.44	6.70	147.12	8.68	143.82	6.24	140.88	4.07
KaS-3	130.52	142.89	9.48	144.59	10.78	139.28	6.71	137.32	5.21
Mean	125.95	135.18	7.23	133.79	6.32	128.02	1.58	132.16	5.03
MTCC 1472	141.24	149.90	6.13	149.81	6.06	144.75	2.48	147.29	4.28

Table 2 — PHB yield (%DCW) of the isolates on standardized medium and on medium with different combinations of alternate C & N sources

Strains	PHB Yield (% Total dry cell weight) and (% increase/decrease in PHB yield in different combinations of alternate C/N sources over PHB yield in optimized medium)								
	Optimized medium (20:1)	Molasses with Urea (20:1)		Cheese Whey with Urea (20:1)		Wheat Bran with Urea (20:1)		Banana Peel with Urea (20:1)	
	PHB Yield (% DCW)	PHB Yield (%DCW)	% Increase/ Decrease	PHB Yield (%DCW)	% Increase/ Decrease	PHB Yield (%DCW)	% Increase/ Decrease	PHB Yield (%DCW)	% Increase/ Decrease
FtW-2	52.80	60.31	14.22	60.01	13.66	60.12	13.86	61.23	15.97
PaM-2	59.78	63.59	6.37	60.37	0.99	62.98	5.35	62.55	4.63
FS-4	61.34	63.83	4.06	62.58	2.02	63.54	3.59	63.71	3.86
AW-1	61.31	64.28	4.84	64.57	5.32	63.68	3.87	63.01	2.77
KW-4	72.32	73.42	1.52	72.36	0.06	72.59	0.37	71.55	-1.06
PnW-4	58.81	59.67	1.46	65.66	11.65	60.45	2.79	64.52	9.71
RoW-1	58.53	59.86	2.27	58.86	0.56	58.84	0.53	59.70	2.00
RoW-4	67.53	69.79	3.35	69.82	3.39	69.01	2.19	69.32	2.65
SW-3	63.09	65.63	4.03	64.70	2.55	63.58	0.78	63.58	0.78
AM-2	58.79	60.21	2.42	60.45	2.82	59.87	1.84	59.82	1.75
FtM-8	50.29	52.97	5.33	51.48	2.37	52.36	4.12	52.64	4.67
FM-1	63.15	64.25	1.74	63.85	1.11	64.03	1.39	63.75	0.95
HM-2	72.73	72.93	0.27	73.21	0.66	72.91	0.25	72.53	-0.27
RM-1	65.05	66.87	2.80	66.14	1.68	65.47	0.65	65.34	0.45
RoM-1	62.63	64.97	3.74	63.59	1.53	64.44	2.89	62.89	0.42
KS-1	64.81	67.52	4.18	65.34	0.82	66.92	3.26	67.59	4.29
KS-3	57.47	59.79	4.04	59.42	3.39	59.23	3.06	58.67	2.09
MS-6	71.01	71.96	1.34	71.83	1.15	72.35	1.89	71.41	0.56
RoS-4	58.39	60.35	3.36	60.58	3.75	58.95	0.96	61.28	4.95
SiS-3	65.11	69.42	6.62	68.45	5.13	67.38	3.49	69.37	6.54
KaS-3	54.20	59.21	9.24	66.84	23.32	55.23	1.90	60.88	12.32
Mean	61.86	64.32	4.15	64.29	4.18	63.52	2.81	64.06	3.81
MTCC 1 ^c	76.49	77.52	1.35	77.82	1.74	76.92	0.56	75.29	-1.57

the 21 PHB positive isolates on this medium was recorded as 125.95 mg/mL and 61.86% total DCW. Although, PHB yield of the reference strain MTCC 1472 was observed to be slightly higher (141.24 mg/mL and 76.49%) than the isolates, but still, several isolates showed a PHB yield quite close to that of the standard strain. The maximum PHB yield (in mg/mL) was observed by the isolate KW-4 (139.80 mg/mL), closely followed by KS-3 (139.75 mg/mL) and MS-6 (137.32 mg/mL). However, in terms of total dry cell weight, the maximum PHB accumulation was observed by HM-2 (72.73%), followed by KW-4 (72.32%) and MS-6 (71.01%).

PHB production using diverse agro-industrial byproducts as alternate substrates

The effect of bioeffluents including molasses, cheese whey, wheat bran and banana peel as alternate carbon sources, and urea as alternate nitrogen source (in different C/N ratios *viz.*, 2:1, 10:1 & 20:1 in the culture medium) was examined on PHB production by all the 21 PHB positive isolates. Results revealed that the PHB production by most of the isolates with these bioeffluents as C-source in the culture medium was moderately higher than the PHB yield obtained using the standardized medium with glucose as the

C source (Tables 1 & 2, and Figs. 1 & 2). The maximum percent increase in PHB production was observed when the culture medium was supplemented with molasses in place of glucose as the C source, followed by cheese whey, banana peel, and least with wheat bran as the C source.

Several isolates showed a high percent increase in PHB yield with alternate sources. Two isolates SW-3 and FtW-2 exhibited a consistently high percent increase (11.44-19.65% and 13.86-15.97%, respectively) in PHB accumulation with all the four alternate carbon sources; however, maximum increase was observed with molasses as C source in the medium. The standard strain MTCC 1472 also showed an increase in PHB production with all the four alternate C sources, thus, strengthening the possibility of exploitation of these bioeffluents as potential C sources in the PHB production medium. The detailed results obtained with each of the four different alternate substrates are presented in subsequent sections.

PHB yield of isolates on the alternate substrate molasses and urea

The data presented in Fig. 1A depicts the comparison of PHB accumulation by the isolates when raised in a medium with conventional C and N

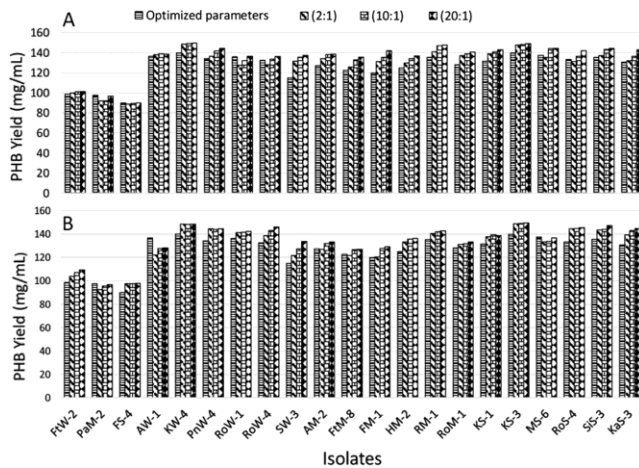


Fig. 1 — Effect of use of alternate substrates (in different concentrations) in the culture medium on PHB production: (A): Molasses as C source and urea as N source; and (B) Cheese whey as C source and urea as N source

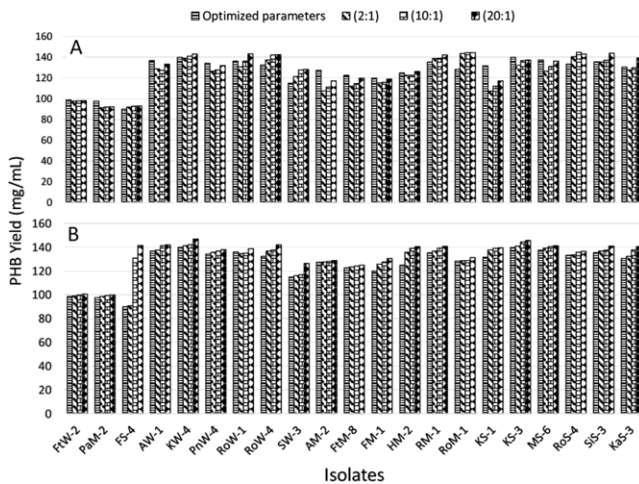


Fig. 2 — Effect of use of alternate substrates (in different concentrations) in the culture medium on PHB production: (A) Wheat bran as C source and urea as N source; and (B) Banana peel as C source and urea as N source

sources, and in culture medium having inexpensive substrates molasses and urea as the C and N sources. All the isolates reported an enhancement in PHB production when molasses was used as a replacement of glucose in the culture medium. The mean PHB yield of the isolates on standard medium with conventional C-source was found to be 125.95 mg/mL, whereas the mean PHB yield of the isolates on MSM medium supplemented with molasses and urea with C/N ratios as 2:1, 10:1 and 20:1 was found to be 128.35 mg/mL, 130.26 mg/mL, and 135.13 mg/mL, respectively, corresponding to a mean percent increase of 7.23% at 20:1 C/N ratio. The dry PHB

weight of all the 21 PHB isolates also showed a mean percent increase of 4.15% at 20:1 C/N concentration of molasses and urea combination in the culture medium. The maximum PHB accumulation by all the isolates as estimated by both crotonic acid as well as dry weight estimation method was thus reportedly obtained at a C/N concentration of 20:1. Amongst the different isolates, KW-4 (149.59 mg/mL and 73.42% DCW), KS-3 (149.03 mg/mL), HM-2 (72.93% DCW), MS-6 (71.96% DCW and 144.28 mg/mL) and SiS-3 (144.44 mg/mL and 69.42% DCW) were observed to exhibit a significantly superior PHB yield when compared to other isolates. However, a higher percent increase in PHB accumulation was observed by the isolates SW-3 (19.65%), FM-1 (18.68%) and FtW-2 (14.22%). When compared with other alternate substrates, molasses showed highest PHB accumulation, thus exhibiting a high potential for replacing glucose in the production medium.

PHB yield of isolates on alternate substrate cheese whey and urea

Data pertaining to PHB yield of the bacterial isolates in presence of cheese whey and urea is presented in Fig. 1B. Among different C/N concentrations, 20:1 gave the maximum mean PHB yield. It produced a mean PHB of 133.79 mg/mL and 64.29% DCW. The next promising concentration was 10:1 with 129.04 mg/mL PHB yield. The C/N ratio of 2:1 was found to be the least supporter (126.49 mg/mL) of PHB production. However, all three concentrations exhibited a mean PHB yield higher than the yield obtained with the standard medium with conventional carbon source. The isolates KS-3, KW-4, HM-2, MS-6 and SiS-3 on 20:1 C/N ratio resulted into high PHB accumulation (149.34 mg/mL, 148.46 mg/mL, 73.21% DCW, 71.83% DCW and 147.12 mg/mL, respectively). All the other isolates (except PaM-2, AW-1 and MS-6) also showed an increase in PHB production with cheese whey as compared to glucose as the C-source in the medium, the maximum increase always being observed at 20:1 C/N ratio. The mean % increase in PHB production with cheese whey and urea in 20:1 concentration ratio was recorded as 6.32% and 4.18%. The standard strain *Cupriavidus necator* (MTCC 1472) also showed an increase of 6.06% and 1.74% with cheese whey and urea as compared to the production medium having glucose and ammonium sulphate as C and N sources.

PHB yield of isolates on alternate substrate wheat bran and urea

The results presented in Fig. 2A indicate that among the three different C/N concentrations (2:1,

10:1 and 20:1) evaluated for PHB production, maximum PHB production was observed with 20:1 C/N ratio. Among the different isolates screened for PHB production, RoM-1 gave the maximum productivity. RoM-1 was the highest PHB producer at all three C/N concentrations, producing a mean PHB yield of 144.28 mg/mL at 20:1 C/N concentration. It was followed by the isolate RoW-1 which gave a PHB yield of 143.05 mg/mL; however, some other high producers included SiS-3 (143.82 mg/mL and 67.38% DCW), KW-4 (142.74 mg/mL and 72.59% DCW), HM-2 (72.91% DCW) and MS-6 (72.35% DCW). PaM-2 isolate was the least PHB producer at all levels. The mean PHB yield of the isolates on standard medium was found to be 125.95 mg/mL, whereas the mean PHB yield of the isolates on MSM medium supplemented with inexpensive substrates wheat bran and urea at C/N ratios 2:1, 10:1 and 20:1 were found to be 126.58, 127.04 and 128.02 mg/mL, respectively. At 20:1 C/N ratio, an average percent increase of 2.81 and 1.58% was recorded (Tables 1 & 2). Compared to the other two combinations: molasses with urea, and cheese whey with urea; the combination of wheat bran and urea exhibited a lower increase in PHB yield over the standard medium containing the expensive substrates *viz.*, glucose and ammonium sulphate, some of the isolates showing almost no increase in the PHB yield. Thus, compared to the other combinations, wheat bran and urea combination was found to be least effective for replacement of conventional C- and N- sources in the PHB production medium.

PHB yield of isolates on alternate substrate banana peel and urea

The alternate substrate banana peel is a desirable carbon source for PHB production as it is relatively cheaper as compared to other sugars. Fig. 2B shows mean PHB production ability of all the isolates on this alternate substrate as 126.19, 128.77, and 132.16 mg/mL, at concentrations of 2:1, 10:1 and 20:1, respectively. The results also depict an increase in mean PHB yield (132.16 mg/mL and 64.06% DCW) on this medium at 20:1 C/N concentration, as compared to the mean production (125.95 mg/mL and 61.86% DCW) on standardized medium (Tables 1 & 2). The isolate KW-4 recorded the highest PHB yield of 147.22 mg/mL at a concentration ratio of 20:1 which is superior to its PHB yield (139.80 mg/mL) with the standard medium. Several other isolates also showed a promising PHB yield at 20:1 C/N ratio of banana peel and urea. These included: KS-3 (144.59 mg/mL),

RoW-4 (144.70 mg/mL and 69.32% DCW), SiS-3 (140.88 mg/mL and 69.37% DCW), HM-2 (72.53% DCW) and MS-6 (71.41% DCW). A mean percent increase in PHB production by all the isolates was observed as 5.03% and 3.81%, thus, depicting the potential of banana peel as an inexpensive source of carbon in the culture medium.

FTIR analysis for characterization of PHB

FTIR is a powerful tool which can be used to deduce the chemical structures of polymers as every chemical compound in the structure is responsible for its own distinct input to the absorbance/transmittance spectrum. FTIR analysis has been reported to be used to determine the chemical nature of the PHB extracts in previous studies too²³. On similar lines, the PHB polymer extracted from the standard strain (MTCC 1472), and from all the 21 isolates by growing them on each of the four different combinations of alternate C and N sources was characterized by subjecting it to FTIR analysis. Fig. 3 represent the FTIR spectra of the PHB extracted from two high yielding isolates (KW-4 and KS-1); and from the standard strain *Cupriavidus necator* (MTCC 1472), grown on two best combinations (molasses with urea, and cheese whey with urea); and the FTIR spectra of the pure PHB (Sigma Aldrich, USA).

The FTIR spectrum of the extracted polymer from all the isolates was recorded in the range of 4000 cm^{-1} to 400 cm^{-1} . The different spectral peaks obtained signify specific rotations around carbon atoms specific to certain functional groups. The transmittance bands obtained at 1724 cm^{-1} (in spectra of all the isolates) represent the C=O stretch of the ester group (ester carbonyl) present in the molecular chain of highly ordered crystalline structures such as PHB. Representative bands of the C-O-C groups were also observed in the spectral region between 1150 to 1300 cm^{-1} (1184, 1229/1230, and 1279 cm^{-1}). Transmittance regions from 2800 to 3100 cm^{-1} (2977, 2934 cm^{-1}) corresponded to the stretching vibration of C-H bonds of the methyl (CH_3) and methylene (CH_2) groups. Some other characteristic bands for PHB were found to be present at 1101, 1057 (C-O), 980 and 515 cm^{-1} . These peaks corresponded to the peaks obtained for PHB extracted from the standard PHB producing strain *Cupriavidus necator* MTCC 1472 and also to the peaks obtained for the standard pure PHB (Fig. 4); thus, confirming that the extracted polymer is PHB.

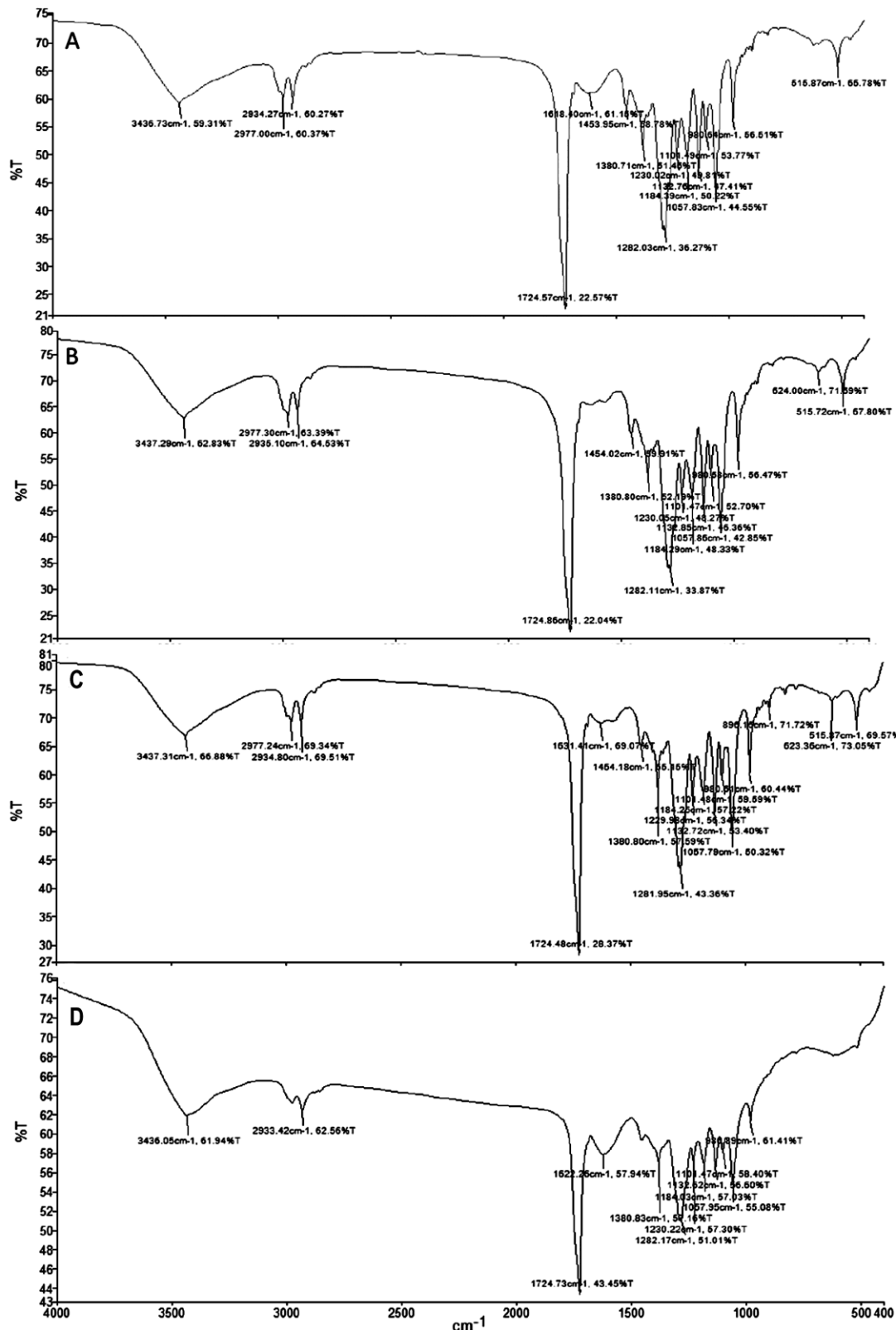


Fig. 3 — FTIR spectra of PHB extracted from the isolate (A & B) KW-4; (C & D) KS-1 grown in different production mediums: (A & C) Production medium containing molasses and urea; and (B & D) Production medium containing cheese whey and urea

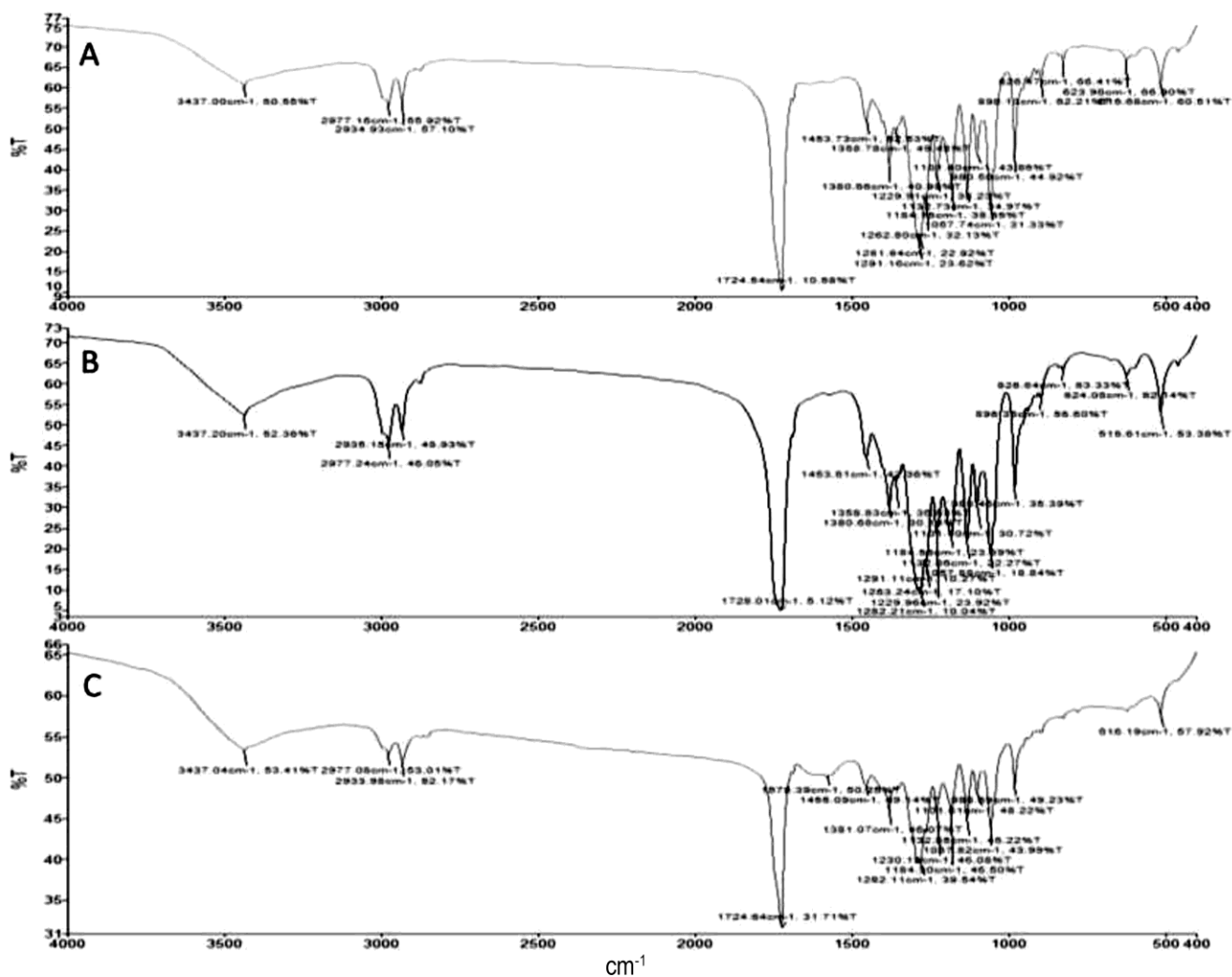


Fig. 4 — FTIR spectra of control and standard: PHB extracted from the standard strain *Cupriavidus necator* (MTCC 1472) grown in production medium containing (A) Molasses and urea; (B) Cheese whey and urea; and (C) Standard pure PHB (Sigma Aldrich)

Discussion

Plastic can be regarded as one of the greatest inventions and an indispensable commodity of human life ever since it has developed into a major industry²⁴. Plastic goods are affordable and reduce the wastage of many valuable resources, thus contributing towards a sustainable development. However, the non-degradable nature of these plastics has resulted into their large-scale accumulation in the environment, which has become a cause of major global concern. The use of biodegradable PHB as a substitute for non-biodegradable petroleum-based plastics is an attractive solution to this problem²⁵, but, it costs substantially more than its fossil fuel based counterpart, thus, limiting its widespread use²⁶. The high production cost of this biopolymer is therefore, a major stumbling block in its commercialization. This

high production cost mainly depends upon the methods adopted for its synthesis and the composition of media²⁷ used for the large-scale growth of PHB producing bacteria. Selection of a proper and cost-effective media is a vital factor as it not only provides optimal conditions for production of PHBs by different strains of bacteria, but also results into an end product that is economically feasible²⁸.

The PHB can be produced from a large variety of substrates including: low-cost renewable resources²⁹ (for example, sucrose, starch, cellulose, triacylglycerols), fossil resources (methane, mineral oil, lignite, hard coal), byproducts (molasses, whey, glycerol), and chemicals (propionic acid, 4-hydroxybutyric acid)³⁰. In previous researches, attempts have been made to cut down the cost of production of bioplastics by using several alternate carbon sources, so that their

use can be enhanced³¹⁻³². Some of the low cost carbon and nitrogen sources which have been tried for PHB production by earlier workers include molasses and corn steep liquor³³, various pretreated molasses³⁴, date seed oil and date molasses³⁵, sugarcane vinasse and molasses³⁶, whey³⁷, banana pseudostem³⁸, pineapple peel³⁹, damaged food grains, pea shells, cassava waste⁴⁰, palm oil mill effluent⁴¹, beer brewery wastewater containing maltose⁴², starch⁴³ and dairy wastes like cheese whey⁴⁴. But, there is still a great scope for further research in this direction, as; a perfect cost-effective medium has not yet been commercialized⁴⁵. In the present study, an attempt was therefore made to minimize the cost of PHB production by using different agricultural and industrial wastes for PHB production. Four different combinations of alternate substrates used included: MSM medium supplemented with each of the four alternate carbon sources, viz., molasses, cheese whey, wheat bran and banana peel (0.2, 1 & 2%), along with urea (0.1%) as a common nitrogen source in all the combinations. The effect of all four combinations on PHB production was studied in 21 PHB positive isolates.

Molasses is the low value, unpalatable and final residual syrup generated in sugar-refining mills after repeated sugar extraction by carrying out crystallization of sugarcane or sugar beet juice. Depending on the grades and sources, sugar molasses cannot be further used in foods or feeds and, thus, become ideal for consideration as an inexpensive carbon source for fermentative processes. In the present study, molasses (2%) with urea (0.1%) when used as an alternate substrate for PHB production gave a PHB yield of 135.18 mg/mL and 64.32% DCW. In a similar previous study conducted by Gouda *et al.*⁴⁶, maximum PHB production was observed with 3% molasses, however, similar to the present study, maximum yield of PHB (46.2% per mg cell dry matter) was obtained with 2% molasses. They reported corn steep liquor to be the best nitrogen source for PHB synthesis (32.7 mg per cell dry matter). However, in the present study, using urea as the nitrogen source resulted into a higher PHB accumulation.

Wheat bran is a byproduct of the wheat milling process and it accounts for 8-12 % of the wheat kernel. In the present study, wheat bran with urea when used as substrate gave 128.02 mg/mL and 63.52% DCW of mean PHB production at C/N ratio of 20:1. Similarly, Shamala and coworkers⁴⁷ observed

that bacterial growth and polymer production were enhanced with the supplementation of hydrolysates of wheat bran (WBH) or rice bran (RBH) individually or in combination (5–20 gL⁻¹, based on weight of soluble substrates-SS). However, contrary to this result; in the present study, it was observed that although wheat bran produced PHB accumulation higher than glucose, but compared to the other three alternate carbon sources tested; wheat bran resulted into a lower PHB accumulation.

During cheese production at the food processing industries, about 80-90% of the processed milk volume is converted to whey as a byproduct⁴⁸. Yellore & Desai⁴⁴ showed that *Methylobacterium* sp. ZP24, isolated from a local pond, is able to grow in a medium containing 12 g L⁻¹ lactose as a sole source of carbon, giving 5.25 g L⁻¹ biomass yield and poly-3-hydroxybutyrate (PHB) up to 59% of its dry wt. in 40 h. The isolate was also able to utilize cheese whey and produce 1.1 g L⁻¹ PHB. Addition of ammonium sulphate increased the production of PHB from whey to 2.5-fold. In the current study also, use of cheese whey with urea gave enhanced PHB production of 133.79 mg/mL as compared to the PHB yield of 125.95 mg/mL in medium with glucose and ammonium sulphate as sole source of carbon and nitrogen respectively. Similar results were seen when MSM medium was supplemented with banana peel and urea giving enhanced PHB yield of 132.16 mg/mL as compared to the optimized medium.

An important observation of the present study was that the results of PHB accumulation using cost-effective alternate substrates showed a high consistency among the different isolates; as, a similar pattern was observed with not just one or two isolates, but with most of the 21 bacterial strains; thus, confirming the PHB accumulation inducing efficiency of these alternate carbon sources.

Conclusion

Results of the present investigation demonstrate that all the four agro-industrial byproducts (molasses, cheese whey, wheat bran and banana peel in combination with urea as the nitrogen source) tested in this study hold a high potential for substituting glucose as carbon source in the production medium, and for being used as inexpensive and effective alternative options for PHB production. However, molasses and cheese whey were found to be a better substrate than banana peel or wheat bran; and the best combination was observed to be molasses and urea in a ratio of 20:1,

wherein we were able to reach a PHB accumulation as high as 73.42% with a yield of 149.59 mg/mL, which was higher than the PHB accumulation and yield by these isolates on pure glucose. A higher amount of PHB accumulation was observed by all the bacterial isolates when they were grown in medium containing the alternate carbon and nitrogen sources tested in the present study, as compared to when the isolates were grown on medium containing the conventional carbon and nitrogen source; indicating the capability of the usage of these alternate sources by a broader range of bacterial isolates. Thus, the results of the present study strengthen and support the usage of agro-industrial wastes as raw materials for effective PHB production. If utilized on commercial scale, the consumption of these agro-industrial wastes as potential substrates for PHB production, would not only help in reducing the overall cost of PHB production, but would also help in mitigating the environmental pollution.

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Conflict of interest

Authors declare no conflict of interests.

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