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Biotechnological conversion of agro-industrial wastewaters into biodegradable plastic, poly β-hydroxybutyrate

Anshuman A. Khardenavis, M. Suresh Kumar, Sandeep N. Mudliar, Tapan Chakrabarti *

Environmental Biotechnology Division, National Environmental Engineering Research Institute, Nehru Marg, Nagpur 440 020, India

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Abstract

Waste activated sludge generated from a combined dairy and food processing industry wastewater treatment plant was evaluated for its potential to produce biodegradable plastic, poly β-hydroxybutyric acid (PHB). Deproteinized jowar grain-based distillery spentwash yielded 42.3% PHB production (w/w), followed by filtered rice grain-based distillery spentwash (40% PHB) when used as substrates. Addition of di-ammonium hydrogen phosphate (DAHP) resulted in an increase in PHB production to 67% when raw rice grain-based spentwash was used. Same wastewater, after removal of suspended solids by filtration and with DAHP supplementation resulted in lower PHB production (57.9%). However, supplementing other wastes with DAHP led to a substantial decrease in PHB content in comparison to what was observed in the absence of DAHP.

Keywords: Biodegradable plastics; Poly β-hydroxybutyric acid; Activated sludge; Industrial wastewaters; Biopolymers

1. Introduction

Current worldwide dependence on fossil fuels for plastics manufacture (≈270 million metric tonnes of fossil fuels), the scarcity of space for disposal and growing environmental concerns for non-biodegradable synthetic plastics have fuelled research towards development of eco-friendly biopolymer materials (Grengross and Slater, 2000; Thompson, 2001). Considerable emphasis has been laid on the development of five different types of biopolymers which include fibre-reinforced composites, starch based materials, plant produced polymers, microbially produced polymers and biologically based resins, coatings and adhesives (Kolybaba et al., 2004). Of these, maximum attention has been laid on the development of microbially produced polymers, polyhydroxyalkanoates (PHA), which are linear aliphatic polyesters composed of 3-hydroxy fatty acid monomers and polylactic acid (PLA).

Poly β-hydroxybutyric acid (PHB) is the most extensively studied PHA, produced in nature in the presence of excess carbon by bacteria as storage granules providing food, energy and reducing power (Pfeffer, 1992; Salehizadeh and Van Loosdrecht, 2004). PHB has properties similar to petroleum derived synthetic plastics like polypropylene (PP) and is completely biodegradable in the environment. However, the production cost of PHB is nine times higher in comparison to synthetic plastics as it involves production of biomass with expensive carbon sources (Serafim et al., 2004). This has limited the use of PHB to specialized areas like surgery and medicine. Efforts on cost reduction have been directed towards increase in PHB content by developing better bacterial strains and efficient fermentation and recovery systems (Lee, 1996; Wang and Lee, 1997; Choi et al., 1998). Another approach involves the use of excess activated sludge from a wastewater treatment plant as a source of PHB and renewable carbon resources derived from agriculture or industrial wastes as substrate for PHB accumulation (Braunegg et al., 1978; Chua and Yu, 1999; Suresh Kumar et al., 2004). In addition to these approaches, researchers have prepared blends

* Corresponding author. Tel.: +91 712 2249757; fax: +91 712 2249961.
E-mail address: t_chakrabarti@neeri.res.in (T. Chakrabarti).

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of starch and PHB (PHB:starch 70:30) which, in addition to reducing the final cost of product would result in a product having properties similar to that of pure PHB (Godbole et al., 2003). These approaches have a dual advantage of saving cost on biomass generation and volume reduction of waste activated sludge by extracting PHB. The saving on waste activated sludge disposal cost following volume reduction could reduce PHB production cost thereby attributing economic advantage to the process.

The present work was carried out to evaluate the PHB inducing efficiency of carbonaceous agro-industrial wastewaters in waste activated sludge in presence and absence of di-ammonium hydrogen phosphate.

2. Methods

2.1. Materials

Analytical grade chemicals and reagents procured from Hi-Media Laboratories Pvt. Ltd., (India) and Ranbaxy Fine Chemicals (India) were used for preparation of media and chemical analyses. Chromatography grade solvents, obtained from Merck Ltd., (India), were used for extraction and analysis of intracellular PHB granules. Standard PHB was purchased from Aldrich Chemical Co., (USA).

2.2. Biomass growth medium

Biomass for PHB accumulation was collected in the form of activated sludge from an effluent treatment plant belonging to a dairy and food processing industry and was subjected to selective enrichment of the PHB producing bacterial biomass by aeration in synthetic medium containing (in g l^{-1} of distilled water); CH_3COOH, 20; $(NH_4)_2HPO_4$, 0.754; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.4; and trace element solution, 1 ml (which contained in mg ml^{-1} of distilled water Na_2SO_4, 25 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; $MnSO_4 \cdot 4H_2O$, 4.06 mg; $ZnSO_4 \cdot 7H_2O$, 4.40 mg; $CuSO_4 \cdot 5H_2O$, 0.79 mg; $CaCl_2 \cdot 2H_2O$, 73.4 mg).

2.3. Characterization and preparation of wastewaters as carbon substrate for PHB production

Wastewaters from a food processing industry (producing mainly potato chips, wafers and sweets) and starch rich grain-based alcohol industries (rice grain-based and jowar grain-based distillery spentwash) were characterized and used as substrates for PHB production. Usually, prior to fermentation, starch is hydrolyzed by commercial enzymes, which represent a significant cost in glucose production process. In the present investigation, different starch rich wastewaters were directly used for production of PHB in the following forms:

(a) *Type 1-raw*: wastewaters were used in the form in which they were obtained from the industry.

(b) *Type 2-filtered*: wastewaters in which the suspended solids were removed by filtering through 0.45 μ Whatman glass fibre microfilter.

(c) *Type 3-deproteinized*: wastewaters, where pH of wastewaters was first adjusted to 7.0 with 1N NaOH/1N H_2SO_4 and subsequently boiled to precipitate the proteins which were removed by centrifugation at $1500 \times g$ for 20 min in a cooling centrifuge (Remi C-24, Remi Instruments Ltd. India). The supernatant was then filtered using 0.45 μ Whatman glass fibre microfilter.

Filtration and deproteinization were carried out in order to reduce the total organic nitrogen content in the wastewaters thereby achieving an increase in C:N ratio of the wastewaters. Each wastewater in all the above forms, was used for PHB production in batch experiments in absence and presence of external nitrogen source (DAHP) along with the other media components but by omitting the carbon source (acetate) from the synthetic wastewater mentioned above. pH was adjusted at 7.0 for all the combinations.

2.4. Culture conditions

The enriched activated sludge (0.23 g dry weight equivalent) was added to 100 ml of each of the three types of wastewaters (viz. 1, 2 and 3) in 250 ml conical flasks and incubated on a rotary shaker at 150 rpm at 30 ± 2 °C. Sample (30 ml) was withdrawn at 48 h (midway) and 96 h (end of experiment) intervals and analyzed for biomass and PHB concentration in biomass. The calculation for PHB and biomass concentration for 96 h sample was done based on 70 ml volume which remained at the end of 48 h sampling and values were extrapolated to per litre for calculation of PHB content. Initial PHB content was determined directly from enriched sludge, which was added initially to wastewater as a source of PHB accumulating biomass.

2.5. Analytical procedures

Microbial growth was monitored by measuring the absorbance of culture broth at 600 nm in a UV–Visible spectrophotometer (Spectronic, Genesys-2, USA) against a distilled water blank. Sludge biomass concentration was measured gravimetrically and defined as gram dry weight per liter of culture broth. Carbon content in the different wastewaters was estimated by measuring the chemical oxygen demand (COD). COD equivalent of acetate i.e. 1.066 was used for converting carbon from volatile fatty acid (VFA) in different wastewaters into COD values. Total and reducing sugar content in the wastewaters were estimated by anthrone (Morris, 1948; Clegg, 1956) and dinitrosalicylic acid (Miller, 1959) reagents respectively.

The presence and characterization of PHB in raw sludge biomass from source was confirmed by FT-IR (Hong et al., 1999) and ^1H NMR (Jan et al., 1996). A Bruker Vector- 22 FT-IR (France) controlled with an IBM compatible PC

running OPUS software (version 2.2) was used with the following scanning conditions; absorbance spectra at wavenumber values between 4000 and 400 cm^{-1}, KRS-5 as window material and a spectral resolution of 4 cm^{-1}. ^1H NMR spectra was recorded on a Bruker AM 400 MHz Spectrometer (France) at 30 °C in $CDCl_3$ as solvent. PHB sample for FT-IR and ^1H NMR analysis was extracted from raw sludge from the industry by the method described by Hahn et al. (1993) with slight modification. Sludge biomass was pelletized by centrifugation (1500 × *g*, 10 min, 10 °C) followed by washing with acetone and ethanol to remove moisture and sludge impurities. This was followed by lysis of sludge biomass by 5% NaCl (since sodium hypochlorite resulted in degradation and reduction in molecular weight of extracted polymer, it was replaced with NaCl). The PHB released from sludge biomass was extracted into hot chloroform. The chloroform soluble PHB was separated from cell debris by filtration and subjected to FT-IR and 1H NMR analyses.

The PHB level in the sludge was determined spectrophotometrically by the method of Law and Slepecky (1960) and was reported in percent on a dry weight basis. Productivity of PHB was defined as the concentration of PHB expressed as g l^{-1} h^{-1}. $Y_{p/s}$ and $Y_{x/s}$ were the yield of product (p) and biomass (x) defined as the weight of PHB and biomass produced per unit weight of substrate/COD (s) consumed. C:N ratios were calculated from the total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) of the wastewaters and expressed as gram per gram on a dry weight basis. All physico-chemical analyses were performed as per standard methods (APHA, AWWA, WEF, 2001).

3. Results and discussion

The activated sludge was inhabited by PHB producing bacteria and also by other non-PHB producing organisms such as ciliates, rotifers, nematodes and oligochaetes (Rastak et al., 1993). The relative abundance and occurrence of the different organisms varied with the ratio of food (F) (Chemical Oxygen Demand-COD/Biochemical Oxygen Demand-BOD) to microorganisms (M) (Volatile Suspended Solids-VSS/Mean Liquor Suspended Solids-MLSS) and high F:M ratios favoured the increase in bacterial biomass in comparison to other organisms (Mishoe, 1999; Chua et al., 2000). This selectively enriched sludge could serve as a low cost source of PHB producing biomass.

3.1. FT-IR analysis

The PHA extracted from raw activated sludge from the treatment plant had the same C–H and carbonyl stretching bands as standard PHB as revealed by FT-IR analysis. Absorption bands occurring at 2983–2875 cm^{-1} indicated the presence of aliphatic CH_3 and CH_2 groups and at 1724 cm^{-1} represented the carbonyl of esters. Two C–O characteristic bands of carboxylic esters were seen at 1057 and 1228 cm^{-1}. The absorption bands at 1382 and 1455 cm^{-1} represented aliphatic C–H symmetrical and asymmetrical bending vibrations characteristic of methyl groups. The presence of absorption bands at 1724 cm^{-1} and 1280 cm^{-1} in extracted PHB sample were characteristic of C=O and C–O stretching groups and were identical to PHB (results not shown).

3.2. ^1H NMR analysis

The extracted polymer (40 mg) were dissolved in 1 ml $CDCl_3$ followed by ^1H NMR analysis. Three groups of signals characteristic of polymer PHB were seen in the spectrum (results not shown). A doublet at 1.53 ppm represented the methyl group (CH_3) coupled to one proton while a doublet of quadruplet at 2.75 ppm resulted from methylene group (CH_2) adjacent to an asymmetric carbon atom bearing a single proton. The third signal was a multiplet at 5.52 ppm, which was attributed to a methyne group (CH). From the contribution of various groups to the NMR spectra, it was concluded that the waste activated sludge from food processing industry could directly serve as an inexpensive source of biodegradable polymer and that the bacterial biomass in sludge produced PHA exclusively in the form of PHB.

3.3. Characterization of wastewater

Physico-chemical characterization of different wastewaters and activated sludge is presented in Table 1. Of the three wastewaters, rice grain-based distillery spentwash had the highest carbon and nitrogen content followed by jowar grain-based distillery spentwash and food processing wastewater. Khardenavis et al. (2005) showed C:N ratio 50:1 (g g^{-1}) to be optimum for maximum PHB accumulation in activated sludge in 96 h. Hence, medium components mentioned in Section 2 without the carbon (acetate) were added to the different wastewaters for fortification. The addition of DAHP (0.160 g l^{-1} N) as external nitrogen source was necessary only in case of rice-grain based distillery spentwash where the initial C:N ratios were in the range of 101:1–113:1. However, such addition of DAHP resulted in a decrease in the C:N ratios of jowar grain-based distillery spentwash and food processing wastewater (Table 2). Also, it was seen that filtration and deproteinization did not have a considerable impact on C:N ratio which indicated that these operations were not needed for the wastewaters under investigation to modify their C:N ratios.

3.4. Production of PHB from rice grain-based distillery spentwash

Table 2 summarizes the results for PHB production with rice grain-based distillery spentwash. Maximum of 67% PHB (of sludge dry weight) was accumulated in 96 h with

Table 1
Physico-chemical characterization of activated sludge and different industrial wastewaters

Parameter	Activated sludge	Food processing wastewater	Jowar grain-based distillery spentwash	Rice grain-based distillery spentwash
Colour	Brown	White	Creamish	Creamish
pH	7.70	5.24	3.60	2.90
Total acidity ($g\,l^{-1}$)	–	0.73	2.30	7.10
Total alkalinity ($g\,l^{-1}$)	0.85	–	–	–
Mixed liquor suspended solids-MLSS ($g\,l^{-1}$)	7.38	1.75	2.00	7.64
Total dissolved solids-TDS ($g\,l^{-1}$)	1.40	2.10	8.35	26.11
Chemical oxygen demand-COD, soluble ($g\,l^{-1}$)	0.60	3.60	10.80	35.00
Raw ($g\,l^{-1}$)	16.00	6.40	15.20	36.00
Volatile fatty acids-VFA ($g\,l^{-1}$)	ND[a]	1.70	0.65	1.46
Total sugar ($g\,l^{-1}$)	ND[a]	2.75	0.225	5.40
Reducing sugar ($g\,l^{-1}$)	0.015	0.025	0.100	2.00
Total Kjeldahl nitrogen ($mg\,l^{-1}$)	767.00	56.00	126.00	140.00
Nitrate-N (NO_3^-) ($mg\,l^{-1}$)	ND[a]	8.00	27.80	54.00
Phosphate-P (PO_4^{3-}) ($mg\,l^{-1}$)	23.90	19.80	64.20	41.60
Sulphate (SO_4^{2-}) ($mg\,l^{-1}$)	60.00	0.55	1.40	ND[a]
Chloride (Cl^-) ($mg\,l^{-1}$)	258.00	260.00	80.00	180.14

[a] Not detected.

Table 2
Production of PHB with different agro-industrial wastewaters

Type of wastewater	Rice grain based spentwash[a]	Jowar grain based spentwash[a]	Food processing wastewater[a]
Raw	36.5 (101:1)	34.7 (47:1)	28.3 (55:1)
Raw + DAHP	67.0 (47:1)	27.9 (21:1)	23.9 (14:1)
Filtered	40.1 (109:1)	36.2 (51:1)	39.1 (45:1)
Filtered + DAHP	57.9 (48:1)	26.0 (18:1)	26.9 (10:1)
Deproteinized	11.7 (113:1)	42.3 (57:1)	31.6 (48:1)
Deproteinized + DAHP	25.9 (53:1)	36.3 (17:1)	26.9 (9:1)

[a] Values in the bracket indicate respective C:N ratios in different wastewaters.

raw wastewater in presence of DAHP as additional nitrogen source. PHB accumulation decreased slightly to 58% when filtered wastewater supplemented with extra nitrogen was used. In the absence of the inorganic nitrogen source, these wastes resulted in lower PHB production of 36–40% (w/w). Further, deproteinized wastewater, giving the lowest PHB accumulation both in the absence and the presence of external nitrogen source was not suitable for PHB production.

3.5. Production of PHB from jowar grain-based distillery spentwash

Deproteinized jowar grain-based distillery spentwash resulted in 42.3% PHB content in the absence of DAHP in 48 h, which was around 36.2% and 34.7% in case of filtered and raw forms of this wastewater, respectively (Table 2). Supplementing these wastes with DAHP led to a further decrease in PHB accumulation to 36.3%, 26% and 28% for deproteinized, filtered and raw wastewater, respectively. Further incubation beyond 48 h resulted in complete consumption of carbon in the waste followed by reduction in PHB content for all the forms of this wastewater.

3.6. Production of PHB from food processing industry wastewater

Table 2 shows PHB production with food processing industry wastewater with and without additional inorganic nitrogen source. PHB accumulation was 39.1% in 48 h of incubation for filtered (type 2) wastewater in the absence of DAHP; however, other forms of this waste (type 1 and 3) resulted in lowest PHB production values between 24% and 31.6% (w/w). Carbon content of food processing industry wastewater was the lowest among the three types of wastewaters, and was completely exhausted in 48 h, leading to a decrease in intracellular PHB content on further incubation beyond 48 h.

Table 3 summarizes PHB production from different inexpensive substrates as studied by different researchers. Traditionally, whey has been the most extensively studied waste for PHB accumulation by a variety of microorganisms and, so far, recombinant *Escherichia coli* with whey waste as substrate has yielded 80% PHB (Kim, 2000 and Park et al., 2002). Similarly, *Azotobacter vinelandii* UWD was reported to grow to 2.0 g dry cell weight per litre and produced 34% P(3HB-co-HV) in twofold diluted swine waste liquor, which increased to 58.3% when the waste was supplemented with $30\,g\,l^{-1}$ glucose (Cho et al., 1997). Alcoholic distillery wastewater rich in sugars and nitrogen compounds was also found to be a potential feedstock for PHB production and *Actinobacillus* sp. EL-9 accumulated 42% P(3HB) of the dry cell weight (Son et al., 1996). PHB production from activated sludge has also been investigated with synthetic wastewater (65.8% of dry cell

Table 3
PHB production by different microorganisms from inexpensive substrates and wastewaters

Source microorganism/ substrate	Culture mode	Time (h)	PHB content (%)	Productivity $Y_{x/s}$ ($g\,l^{-1}\,h^{-1}$)	PHB yield $Y_{p/s}$ ($g\,g^{-1}$ COD)	References
Azotobacter chroococcum/ starch	Batch	58	73.9	0.0149	0.174	Kim (2000)
Recombinant *E. coli*/whey	Fed-batch with O_2 limitation	52	80.0	0.48		Kim (2000)
Whey	Fed-batch with air as O_2 source	26	70.0	1.35		Park et al. (2002)
Ralstonia eutropha/Tapioca hydrolysate	Fed-batch	59	58.0	1.03		Kim and Chang (1995)
Food scraps	Batch, anaerobic–aerobic		72.6	0.226	0.258	Du and Yu (2002)
Azotobacter vinelandii UWD/ molasses	Fed-batch	36	66.0	0.610	0.290	Page and Cornish (1993)
Alcaligenes latus DSM 1124/malt waste	Fed-batch	69	70.1	0.328		Yu et al. (1999)
Soy waste		69	32.6	0.086		
Activated sludge/malt waste	Fed-batch	69	43.3	0.095		Yu et al. (1999)
Municipal wastewater only	Batch	72	31.0			Chua et al. (2003)
Municipal wastewater + acetate	Batch	72	21.0			Chua et al. (2003)
Fermented food waste	Sequential–batch	60 days	51.0		0.05	Rhu et al. (2003)
Synthetic wastewater	Batch	96	65.8	0.0038	0.028	Khardenavis et al. (2005)
Anaerobic wastewater	Batch	96	58.0	0.0003	0.0047	Khardenavis et al. (2005)

weight) and anaerobic wastewater (58%) (Khardenavis et al., 2005).

In the present study, highest biomass concentration of 6.6 $g\,l^{-1}$ (dry weight) was produced in 96 h in raw rice grain-based distillery spentwash accumulating 2.7 $g\,l^{-1}$ PHB which corresponded to a productivity of 0.028 $g\,l^{-1}\,h^{-1}$ and a yield of 0.212 (g PHB g^{-1} COD consumed). Deproteinized jowar grain-based distillery spentwash and filtered food processing wastewater gave lower PHB and biomass accumulation resulting in a lower productivity of 0.021 and 0.013 $g\,l^{-1}\,h^{-1}$, respectively. However, the PHB yield for jowar grain spentwash was comparable to rice grain spentwash (0.216 g PHB g^{-1} COD consumed) while it was lower in case of food processing wastewater (0.184 g PHB g^{-1} COD consumed). Though the PHB content achieved in this study (67%) was lower than that obtained with pure cultures (up to 80%), the results were amongst the highest obtained so far with activated sludge using wastewater as substrate.

4. Conclusions

The ready availability of starch-based industrial wastes and their renewable nature merit their use as substrates for PHB production from activated sludge. This would not only utilize the excess sludge generated and reduce the load on landfills, but would also contribute to reduction in the cost of PHB production by avoiding sterile conditions and pure carbon sources for maintenance and growth of pure cultures. PHB content is the most important factor affecting the production cost of PHB due to its effect on PHB yield and recovery efficiency, followed by cultural conditions and carbon substrates used. In this study we have been able to reach a high PHB content of 67% (w/w) with rice grain-based distillery spentwash and activated sludge with a yield of 0.212 (g PHB produced g^{-1} COD consumed) at C:N ratios near to previously reported values.

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