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Biotechnological production of polyhydroxybutyrate (PHB) from *Enterobacter aerogenes*

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ABSTRACT: Synthetic polymers obtained from petrol causes air pollution only because they are not dismantled in soil for a long time. For this reason, a microbial plastic poly-β-hydroxybutyrate (PHB) has gained importance since it can be easily dismantled in nature. PHB is a widely distributed intracellular reserve substance typical of prokaryotes. PHB exists in the cytoplasmic fluid in the form of crystalline granules about 0.5 µm in diameter and can be isolated as native granules or by solvent extraction. The study aimed at screening of PHB producing strain and optimization of media parameters for increased PHB production by the strain. A Gram-negative *Enterobacter aerogenes* was identified as PHB producing strain by staining with Sudan Black B staining method. PHB production by this strain was dependent on nutrient limitation. Maltose was found to be best carbon source which gives 149 mg/L of PHB and ammonium nitrate was found to be best nitrogen source (187mg/L) for maximum PHB production. Glucose was used as a substrate and 2% glucose (146mg/L) was found to be best for PHB production. The best yield of PHB was obtained when incubated at 37˚C (193mg/L) with pH 7.0 (158mg/L) for 48 hours (185mg/L). Thus, the present data indicate that *Enterobacter aerogenes* gives best yield of PHB when incubated under optimized conditions.

***Keywords*:** Biotechnological, production, polyhydroxybutyrate, Enterobacter, aerogenes.

INTRODUCTION

Global environmental distresses and solid waste management complications have generated significant interest in the development of biodegradable plastics with the desired physical and chemical properties of conventional synthetic plastics. PHBs have found a wide range of applications as biodegradable and biocompatible polymers; however, the wide-spread replacement of conventional plastics has been limited by high production costs (Anderson, 1990; Byrom, 1987; Fiechter, 1990 and Lee, 1996).

Bioplastics, a special type of biomaterials derived from plant sources such as soybean oil, hemp oil or corn starch or microbial source, rather than traditional petrochemical derived plants. Microbial bioplastics were polyesters that are produced by a range of microorganism cultivated under various growth and nutrient conditions. These polymers usually lipids, accumulated as storage materials (as mobile, amorphous, liquid granules). Bioplastics were made from a compound called polyhydroxyalkanoate (PHA). Bacteria accumulatingPHA in the presence of excess carbon source, is similar to how humans accumulated fat deposits on their body. Poly-3–hydroxybutyric acid is the most common microbial polyhydroxyalkanoate. Polyhydroxybutyrate accumulated as energy reserve material in many microorganisms like *Alcaligenes*, *Bacillus*, *Nocardia*, *Pseudomonas*, *Rhizobium* etc. *Alcaligenes eutrophus* and *Azotobacterbeijerinickii* can accumulate upto 70% of their dry weight of PHB.

PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature (in the range of 170-180^{o}C) and a glass transition temperature in the range of 0-5^{o}C. It undergoes thermal degradation at temperature around the melting temperature (Ha and Cho, 2002; Marand, 2000).

Widespread production of PHB has far been limited due to high production costs. For PHB production to become more economically feasible, better bacterial strands as well as cheaper feedstock and purification methods are needed. Genetically modified bacteria will allow the use of cheap and abundant sources, such as household waste, agricultural and industrial waste, waste water, etc. for producing large amounts of PHB. Plastic wastes are of a major worldwide concern, these petrochemically based plastic are great havoc to environment. Due to their persistent nature they remain in environment for years. To overcome this problem the use of biodegradeable plastic is a promising alternative which ends up into water and carbon dioxide in the environment.

MATERIALS AND METHODS

Rapid screening of PHB producing Bacteria

For Screening of PHB production the method of Burdon, K.L., 2002 was followed with modifications, as described briefly, PHB producing bacteria was detected using the lipophilic stain Sudan black B. Sudan black stain was prepared as a 0.3% solution (w/v) in 60% ethanol. Smears of PHB producing bacteria were prepared on glass slides and heat fixed. Samples were stained for 10 min with Sudan black solution, rinsed with water and counter-stained with 0.5% safranin for 5 s. Stained samples were observed under oil immersion at 1000x magnification. The dark blue coloured granules into cells were taken as positive for PHB production.

Quantification of PHB production and selection of isolates

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of Jhon and Ralph, (1961). The bacterial cells containing the polymer were pelleted at 10,000 rpm for 10 min. and the pellet washed with acetone and ethanol to remove the unwanted materials. The pellet was resuspended in equal volume of 4 percent sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged and the supernatant discarded. The cell pellet containing PHB was again washed with acetone and ethanol. Finally, the polymer granules were dissolved in hot chloroform. The chloroform was filtered and to the filtrate, concentrated 10 ml hot H2SO4 was added. The addition of sulfuric acid converts the polymer into crotonic acid which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined.

Optimization of cultural parameters for maximum PHB production

Different factors affecting PHB production by the selected promising bacterial isolates was optimized i.e. time, temperature, pH, carbon source, nitrogen source and substrate concentration.

Effect of different Incubation Temperature on PHB production

The selected bacterial isolate was grown in 250 ml conical flasks containing 100 ml Nutrient broth at different incubation temperature viz. 4ºC, 18ºC, 20ºC, 22ºC, 24ºC, 26ºC, 28ºC, 30ºC, 35ºC, 37ºC and 40ºC for 48 hours. The treatments were replicated three times. After incubation at different temperatures, PHB produced by the isolate was quantified spectrophotometerically following the method of John and Ralph (1961) as stated above. Based on the PHB yields, the best incubation temperature was selected.

Effect of different Incubation time on PHB production

To observe the effects of culture conditions for maximum bacterial polymer production, cultures were incubated for different incubation periods i.e. 24, 48, 72, 96 and 120h at 37 °C.

Effect of different carbon sources on PHB production

The bacterial culture was grown in 250 ml conical flasks containing 100 ml Nutrient broth supplemented with different carbon sources viz., glucose, fructose, sucrose, maltose, arabinose, xylose and lactose at 1% level. The flasks were incubated at 37ºC for 48 hours.

Effect of different nitrogen sources on PHB production

The bacterial culture was grown in 250 ml conical flasks containing 100 ml Nutrient broth with the best carbon source, and different nitrogen sources were used viz. ammonium nitrate, ammonium citrate ammonium sulphate, glycine, tryptone, urea and ammonium di-hydrogen phosphate at 0.1g/100 ml concentration. After 48 hours, PHB yields were quantified as done previously. Based on the yield data, the best N sources was carried at.

Effect of pH on PHB production

The bacterial culture was grown in 250 ml conical flasks containing 100 ml Nutrient. Different pHs of media were maintained ranges from 2.0 to 10.0 and incubated. After 48 hours, PHB produced were quantified as done previously. Based on the data, the best pH was arrived at.

Effect of different Substrate concentration on PHB production

The culture was inoculated in the nutrient broth and incubated at glucose as a substrate with different concentrations viz 1%, 1.5%, 2%, 2.5%, 3%, 3.5% and 4% for substrate optimization. After 48hours the amount of PHB produced was determined.

ANALYTICAL METHODS

HPLC Analysis

Samples ranging from 0.01 to 500 mg of PHB-containing material were digested in 1 ml of concentrated sulfuric acid at 90°C for 30 min. The tubes were cooled on ice, after which, a 4 ml volume of 0.014 N H_2SO_4 was added with rapid mixing. Before analysis by HPLC, samples were diluted an additional 5 to 100 fold with 0.014 N H_2SO_4 containing 0.8 mg of adipic acid per ml as an internal standard and filtered through a 0.45-,um HAWP membrane filter (Millipore Corp., Bedford, Mass.) to remove particulate material. The injection volumes ranged from 10 to 50 µl. Samples were eluted with 0.014 N H_2SO_4 at a flow rate of 0.7 ml/min from an Aminex HPX-87H ion exclusion organic acid analysis column (300 by 7.8 mm) (Bio-Rad Laboratories, Richmond, Calif.) preceded by an ion-exclusion guard column of Aminex HPX-85X. Absorbance of crotonic acid was measured at 235 nm. The amount of crotonic acid produced from PHB was calculated from the regression equation derived from known crotonic acid standards.

FTIR Analysis

One mg of sample was ground well with 10 mg of spectral pure anhydrous potassium bromide crystals. The powder was made into a pellet for IR analysis. The relative intensity of transmitted light energy was measured against the wavelength of absorption on the region 400-4000cm^{-1}. IR spectra of the samples were measured at ambient conditions.

RESULTS AND DISCUSSION

EFFECT OF DIFFERENT INCUBATION TIME ON PHB YIELD

Bacterial culture has been incubated at different time ranges from 48 hours to upto 144 hours. Data obtained have shown that 48 hours was found to be optimum for PHB production which gives 185mg/L of PHB yield. At 144 hours least production of PHB 135mg/L have been observed which depicts with increase of incubation time, yield of PHB have decreased.

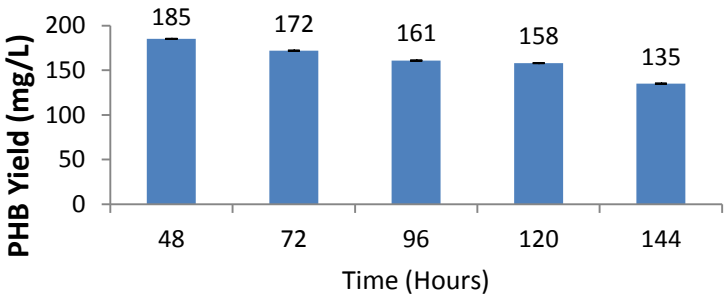

Figure 1. PHB yield (mg/L) at different Incubation Time (hours)

EFFECT OF DIFFERENT TEMPERATURE RANGES ON PHB YIELD

Bacterial culture have been inoculated at different temperatures ranges from 4˚C to upto 40˚C. Data obtained have shown that 37 ˚C have found to be optimum for PHB production which gives 193mg/L of PHB yield. At 4 ˚C least production of PHB 6mg/L has been observed.

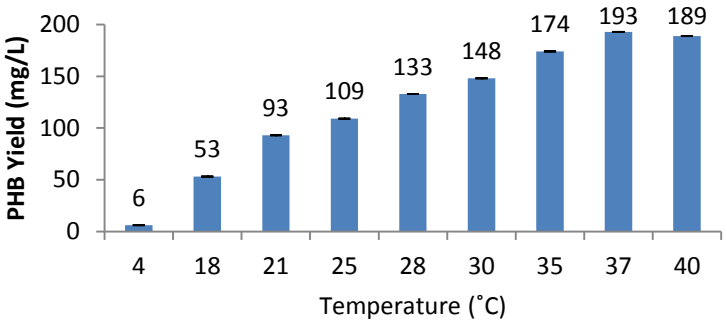

Figure 2. PHB yield (mg/L) at different incubation Temperatures (˚C) by E. aerogenes

EFFECT OF DIFFERENT pH LEVELS ON PHB YIELD

Out of the different pHs of media tested ranges from 2-.0 to 10.0, 7.0 pH was found to be optimum for maximum PHB production by E. aerogenes.

Least PHB production was observed at pH 2.0 that was 66mg/L. At pH 7.0, the highest PHB of 158mg/L was observed which was significantly higher.

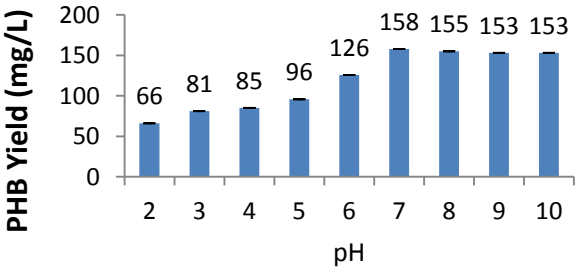

Figure 3. PHB yield (mg/L) at different pH by E.aerogenes

EFFECT OF DIFFERENT CARBON SOURCES ON PHB YIELD

Among the different carbon sources tested to evaluate their effects on PHB yield, maltose was found to be the best carbon source. It yielded a mean PHB of 149mg/L.

This was followed by sucrose with a mean PHB of 138mg/L. Other sugars tested such as glucose, fructose, lactose, arabinose and xylose have also given little yield of PHB.

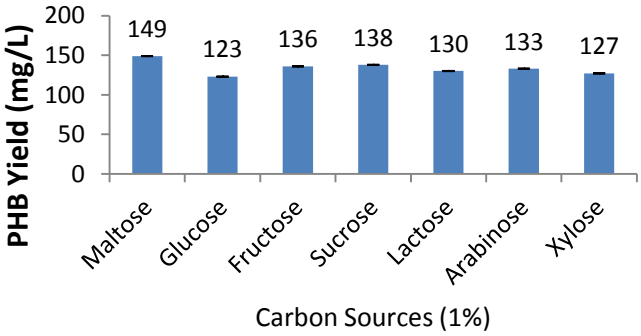

Figure 4. Effect of different Carbon sources (1%) on PHB yield (mg/L)

EFFECT OF DIFFERENT NITROGEN SOURCES ON PHB YIELD

Amongst different N sources, ammonium nitrate was found to be the best N source with PHB yield of 187mg/L.

It produced a mean PHB of 187mg/L. The next promising N sources were glycine with 154mg/L and ammonium sulphate with 148mg/L PHB yields. Ammonium citrate and ammonium di-hydrogen phosphate was found to be the least supporter of PHB production.

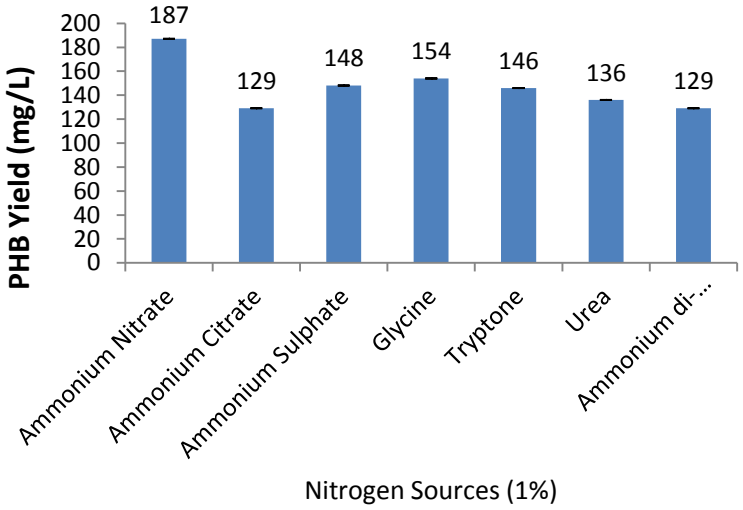

Figure 5. Effect of different Nitrogen sources (0.1%) on PHB yield (mg/L)

EFFECT OF DIFFERENT SUBSTRATE CONCENTRATIONS ON PHB YIELD

Glucose has been used as a substrate in this work. Different concentrations of substrate has been employed such as 1.0, 1.5, 2.0, 2.5.3.0, 3.5 and 4.0. Graph in fig. 6 shows that 2.0% of substrate concentration was found to be optimum for PHB production which gives PHB yield of 146mg/L. Least production of PHB was found to be at 1.0% which gives yield of 108mg/L.

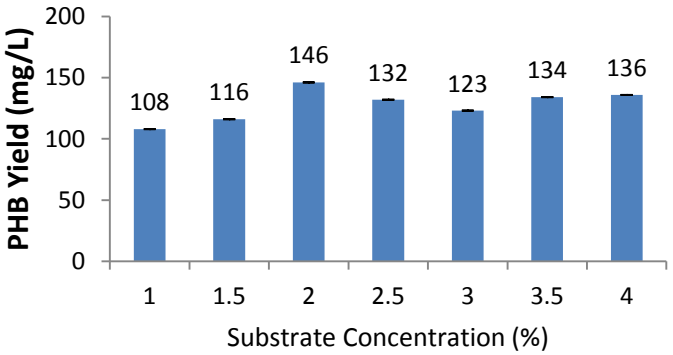

Figure 6. Effect of different Substrate concentrations (%) on PHB yield (mg/L)

HPLC ANALYSIS

Analysis of recrystallized crotonic acid in 0.014 N H_2SO_4 showed one peak with a retention time of 13.781 min. The treatment of crotonic acid with concentrated sulfuric acid (30 min, 95°C) caused no change in the elution pattern or signal intensity. The analysis of purified PHB after conversion to crotonic acid by sulfuric acid is displayed in Fig. 7b and analysis of standard is displayed in Fig. 7(a).

A comparison of the two chromatograms showed that each contained one major peak whose retention time was identical to that of internal standard i.e adipic acid. The total dilution of the samples in Fig. 7(a) and 7(b) is 100-fold. Thus, from HPLC analysis the concentration of PHB has been determined which comes out to be 0.4679%.

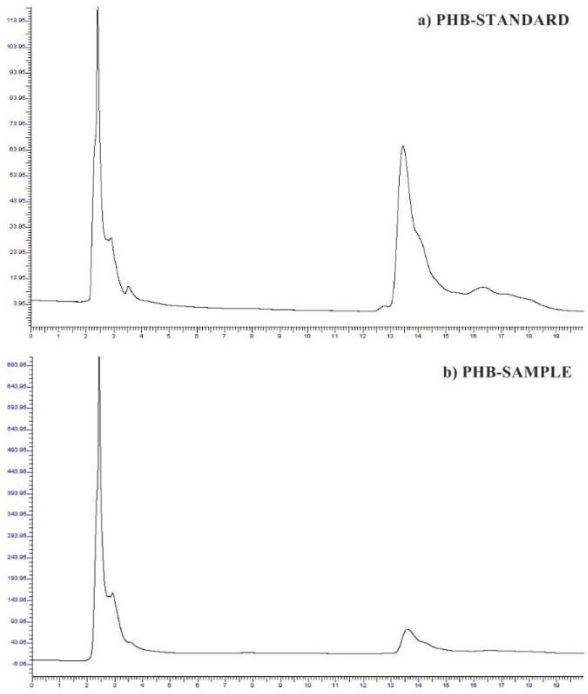

Figure 7. Chromatograms of HPLC Analysis: (a) Chromatogram of Mono-Hydroxybutyric Acid (b) Chromatogram of Poly-β-Hydroxybutyric acid

DISCUSSION

OPTIMIZATION OF PROCESS PARAMETERS

Optimization of fermentation conditions has long been used to enhance yields and productivities of many bioprocesses. Hence, in order to maximize PHB production by E. aerogenes, various factors such as time, temperature, pH, carbon source, nitrogen source, and substrate concentrations were optimized.

EFFECT OF INCUBATION TEMPERATURE ON PHB PRODUCTION

To find out the optimum temperature required for maximum production of PHB, E .aerogenes has been incubated at different temperatures ranging from 4˚C to 40˚C. It has been observed that it gives best yield of PHB at 37˚C (193mg/L).

This data supports the information provided by Bergey's Manual of Determinative Bacteriology. According to the manual, the optimum temperature for growth of E. aerogenes is 34 - 40°C.

EFFECT OF INCUBATION TIME ON PHB PRODUCTION

To find out the optimum incubation time required for the production of PHB by E. aerogenes, culture have been incubated at different time interval ranging from 48 hours to 144 hours. It has been observed that E. aerogenes gives the best yield of PHB at 48 hours of incubation.

Yamane, 1996 studied the production of PHA by A. latus using sucrose as the feed substrate. Higher cell concentration (142mg/L) was obtained in a short culture time (18 h) and PHB content at the end of the culture time was 50%. They concluded that the inoculum size reduced the culture time.

EFFECT OF DIFFERENT pH ON PHB PRODUCTION

To find out the optimum pH for best production of PHB, Culture has been inoculated into the media with different pH ranging from 2 to 10 and incubated at 37˚C for 48 hours. It has been find out that ph 7.0 is the optimum pH at which E. aerogenes gives best production of PHB (158mg/L).

This was in agreement with Aslimet al., 2002 who observed that the PHB in Rhizobium strain grown on yeast extract mannitol broth adjusted to pH 7.0, the amount of PHB was 0.01 to 0.5 g/L culture and the percentage of PHB in these cells was between 1.38 and 40 percent of cell dry weight. Tavernleret al., 1997 also investigated the effect of different nitrogen, carbon sources and different pH values on the production of exopolysaccharide and PHB by strains of Rhizobium meliloti. They reported that these two strains showed higher PHB content at pH 7.0.

EFFECT OF DIFFERENT CARBON SOURCES ON PHB PRODUCTION

Different carbon sources like glucose, fructose, sucrose, maltose, arabinose, lactose and xylose (1%) were amended to nutrient medium and the bacterial culture was grown in them. Maltose was found to be the best carbon source. It yielded the mean PHB of 149mg/L. This was followed by sucrose (138mg/L) and fructose (136mg/L). But, glucose gives the lowest yield of PHB (123mg/L).

Working with different carbon sources in MSM broth, Khanna and Srivastav (2005) observed higher PHB yield on fructose by A. eutrophus. They reported that glucose and fructose, being monosaccharides were readily utilized by bacteria and, hence, have supported growth and subsequently PHB production. The complex molecules like starch and lactose were not utilized.

Yuksekdag, 2003 reported that thehighest PHB synthesis was found in B. subtilis strain and B. megaterium strain when glucose was used as the carbon source. The production of PHB in B. megateriumwas studied by Hori, 2002 and found the highest value of PHB contents when glucose was used.

In our experiment, E.aerogeneshas given highest PHB yield on maltose which is a complex carbon source. As the complexity of the carbon source has increased, PHB yield has also increased.

EFFECT OF DIFFERENT NITROGEN SOURCES ON PHB PRODUCTION

To study the effects of N and to select the best nitrogen source for maximum PHB production, different nitrogen sources like ammonium nitrate, ammonium citrate, ammonium sulphate, glycine, tryptone, urea and ammonium di-hydrogen phosphate were included in the nutrient medium (1 g/l), with the best carbon source (maltose1%).

Nutrient limitation is necessary to trigger PHB accumulation, and generally ammonia is used as the critical control factor for uncoupling the growth of cells and PHB production (Wang and Lee, 1997). A recombinant E. colistrain gave the maximum PHB content (about 60% PHB of DCW) at a specific combination of yeast extract and peptone (Mahishi, 2003).

Mercan, 2002 investigated the effect of different nitrogen and carbon sources and PHB production in two strains of Rhizobium sp. They noted that the strains produced less PHB in yeast exractmannitol (YEM) broth media with different carbon (glucose, sucrose, arabinose) and nitrogen (L-cysteine, L-glycine, DL-tryptophan, protease peptone, potassium nitrate) sources, while the highest level of PHB accumulation was observed in the media with L-cysteine, L-glycine.

EFFECT OF DIFFERENT CONCENTARTIONS OF SUBSTRATE ON PHB PRODUCTION

Glucose has been used as a substrate for PHB production. Different concentrations of substrate has been employed i.e. 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0%. It has been find out that 2% of substrate (glucose) is optimum for maximum production of PHB which gives 146mg/L.

Saha I., 2005 observed that AzotobacterchroococcumMAL-201, accumulated PHB. The polymer yield accounted to 69 per cent of cell dry weight when grown in N2-free stock dale medium containing 2 per cent (w/w) glucose

HPLC ANALYSIS

In particular, HPLC analysis is especially applicable to experiments following PHB accumulation in physiologically developing material or verifying that mutants lack PHB. Such experiments may involve limited amounts of biological material with rapidly changing or vastly differing quantities of PHB. Methods requiring multiple steps in the isolation and purification of PHB often result in losses of material. In addition, measurement of PHB-derived crotonic acid at nonselective wavelengths without prior fractionation could introduce further errors. This may account for the differences in the percentage of PHB recovered by methods which incorporate fractionation of PHB before its measurement (HPLC, 84%, and GC analysis, 88%) and methods which do not (spectrophotometry, 93%). Slepecky and Law noted that certain compounds can interfere with the assay of crotonic acid at 235 nm. Many organic acids and bases as well as heterocyclic compounds absorb near 210 nm, and many of these compounds are common metabolites. Variation in their concentrations is not unexpected in experimental samples. Thus, an analytical method like HPLC, which includes fractionation before measurement, is required. For routine analysis of PHB, the HPLC method provides a wide range of detection (0.01 to 14 ,ug of crotonic acid). Its sensitivity is an order of magnitude greater than that of Law and Slepecky. This enhancement is because of a highly sensitive detection system and because the absorbance maximum of crotonic acid is near the wavelength used in these detectors.

HPLC (ion-exclusion column for organic acids, isocratic mode) and UV detection, provides a simple technique for routine analysis of PHB. It provides the advantages of (i) fractionation of materials in the digest before detection of crotonic acid, (ii) greater sensitivity and accuracy across a wide concentration range, and (iii) easy and rapid sample analysis.

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