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OPTIMIZATION AND MOLECULAR CHARACTERIZATION OF PHB PRODUCTION USING PEELS AS SUBSTRATES by *Acinetobacter calcoaceticus*

R. JASMINE[1*], M. KAVIYARASU[1], R. LEKHASRI[1], S. VARSHA[1] AND S. KAVYA[1]

[1]Department of Biotechnology, Bishop Heber College, Tiruchirappalli, TN, India.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

ABSTRACT

Introduction: Polyhydroxy butyrate, a family of new generation of biobased, biodegradable, eco-friendly plastic has become an alternative for petrochemical derived plastic.

Objective: These are produced by few bacteria. This study was conducted to evaluate the use of waste peels of fruits and vegetables as substrates by PHB producing bacteria.

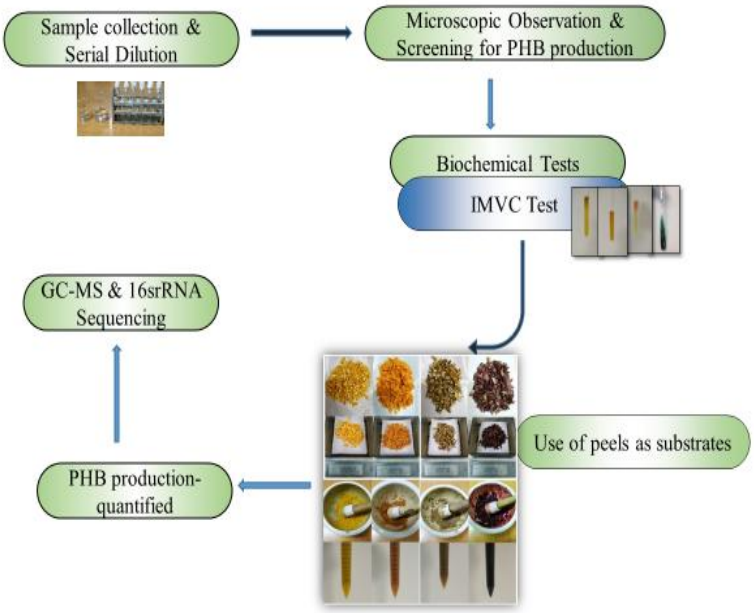
Methods: Bacteria isolated from soil of different regions were screened for PHB production by sudan black screening method. Few vegetable and fruit peels were used as substrates for PHB production, confirmed by GC-MS analysis. 16s rRNA sequencing was performed to confirm the isolate.

Results: Among the several isolates, P1, P2 and P3 were found to be efficient PHB producers. Among the isolates chosen for PHB production using cheaper agro-wastes, P3 was found to be highly productive. GC-MS analysis revealed that 1,2 benzene dicarboxylic acid to be the major compound, whose peak area was 92.29%. 16s rRNA sequencing revealed P3 isolate to be *Acinetobacter calcoaceticus.*

Conclusion: Thus our work has revealed that *Acinetobacter calcoaceticus* is a potential candidate for PHB production using various peels as substrate.

**Corresponding author: Email: jasminebhc@gmail.com;*

Graphical Abstract



Keywords: *Polyhydroxy butyrate; sudan black; agrowastes; biodegradable; Acinetobacter calcoaceticus; 16SrRNA.*

1. INTRODUCTION

Polyhydroxybutyrate (PHB) belongs to the family Polyhydroxyalkanoates, which is also known as Bioplastics. Several factors such as PHB productivity, content & yield and the cost of the carbon substrate make the production of PHB expensive. The high cost of polymer production is the major bottleneck in the commercialization of biodegradable plastics. However, the use of inexpensive and renewable carbon substrates such as agro-industrial wastes and by-products as feedstock can contribute to as much as 40-50% reduction in the overall production cost [1,2] and therefore can serve as a potential alternative to traditional carbon sources such as glucose [3,4]. One possible strategy for reducing costs is to utilize the alternative substrates such as natural products, industrial wastes and agro industrial residues for PHB production by different fermentation process [5,6]. The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics [7]. Several agro industrial residues like soy molasses, sugarcane molasses, sesame oil cake, groundnut oil cake, corn steep liquor etc., have been reported for PHB production [8]. However, to the best of our knowledge the production of PHB using vegetable and fruit peels with *Acinetobacter calcoaceticus* has not been reported yet for the production of PHB. Therefore, in this study we have selected various peels like pomegranate peel, orange peel, sweet lemon peel and beetroot peel as substrates. The aim of the present study was to explore and evaluate the potential of the strain *Acinetobacter calcoaceticus* to produce PHB from peels.

2. MATERIALS AND METHODS

2.1 Sample Collection

The bacteria used in this study were isolated from agricultural soil, red soil and sewage sample and were screened for PHB production.

2.2 Screening for PHB Producing Bacteria

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black Dye [9]. For this screening of PHB producers, nutrient agar media supplied with 1% of glucose was autoclaved at 121^0C for 20 min at 15lbs pressure. This media was poured into sterile Petri plates and allowed for solidification. The plates were divided into 5 equal parts and bacterial isolates were spotted. These plates were incubated for 24 hours. Ethanolic solution of 0.02%

Sudan Black B was spread over the Petri plates containing colonies and was kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from colonies.

2.3 Gram Staining & Biochemical Tests

Twenty four hours old culture was gram stained and the slide was observed under microscope for gram reaction. IMViC, oxidase and peroxidase tests were also performed.

2.4 Sudan Black Staining

Cell suspension was smeared on a clean glass slide and was heat fixed and stained with drops of 3% (w/v in 70% ethanol) solution of Sudan Black B (Sigma) for 10 min, followed by immersion of the slide in xylene until it was completely decolorized. The sample was counter stained with safranin (Sigma 5% w/v in deionized water) for 10seconds, washed with water and dried. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined microscopically. In this staining, lipid inclusion granules are stained blue black or blue grey, while the bacterial cytoplasm is stained light pink.

2.5 Identification of PHB Producing Isolates

PHB producing strains were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Systematic Bacteriology.

2.6 Disruption of Cells by Chemical Methods and PHB Estimation

Nutrient broth was prepared in test tubes and inoculated with the isolates. The medium was incubated at room temperature for 24-96 hours. PHB produced was estimated every 24 hours interval. About 5 ml of culture was taken and centrifuged at 10,000rpm for 10 minutes. Supernatant was discarded and the pellet was suspended in 2.5 ml of sodium hypochlorite and 2.5 ml of chloroform and it was incubated at 30ºC for 1 hour. The above content was centrifuged at 1500 rpm for 10 minutes at room temperature. The upper hypochlorite phase, the middle chloroform containing undisturbed cells and the bottom chloroform phase with PHB were obtained. The upper and middle phases were separated and the contents were again centrifuged at 1500 rpm for 10 minutes at room temperature and the phase other than chloroform with PHB was removed carefully. Concentrated sulphuric acid was added to the chloroform phase containing PHB. It was then boiled at 100ºC in a water bath for 10 minutes. The absorbance of the samples was read at 230 nm using UV Spectrophotometer. The readings were plotted in standard graph of crotonic acid and the concentration of PHB was determined.

2.7 Crotonic acid Standard

Since PHB is converted to crotonic acid on heating with concentrated sulphuric acid, pure crotonic acid salt was used as a standard for estimation. Crotonic acid was taken in concentrations from 1-10 µg/ml and to this, 5 ml of concentrated sulphuric acid was added. The tubes were heated at 100ºC in a water bath for 10 minutes. The tubes were then cooled at 25ºC and the absorbance was read at 230 nm and a standard graph was plotted.

2.8 Estimation of PHB from the Tested Isolates

The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight PHB accumulation (%) = Dry weight of extracted PHB (g/L) × 100 / DCW (g/L)

2.9 GC-MS

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer instrument employing a column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df,composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV. Helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) where the injector temperature was 250°C and ion-source temperature was 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C/min, then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

2.10 Molecular Characterization by 16s rRNA Amplification

The selected culture was inoculated in to 50 ml of culture medium and incubated until it reached the OD_{600} of 1 to 2. The cells were harvested by centrifuging at 1250 g at ambient temperature. The cell pellet was resuspended in 467 µl TE buffer and

further incubated with 33 µl of lysing buffer (30 µ1 of 10% SDS and 3 µl of 20 mg/ml proteinase K) for 1 hr at 37 ° C. The lysate was extracted with an equal volume of 25:24:1 phenol: chloroform: isoamyl alcohol. The aqueous phase was transferred to a 1.5 micro centrifuge tube and added with 1/10 volume of 3M sodium acetate and incubated at -20ºC for 30 min. After the incubation 0.6 volumes of isopropanol was added and mixed gently and centrifuged at 14000 g for 20 min at 4° C. The DNA pellet was washed with 1 ml of 70% ethanol twice by spinning at 14000 g for 20 min at 4° C. The DNA pellet was resuspended in 100 µl TE buffer and stored at 4°C overnight. The quality and quantity of the DNA was estimated by OD ratio of 260/280, 260/230 nm through 8 port Nanodrop (Thermo Fisher Scientifics, USA) and 1% agarose gel respectively.

3. RESULTS AND DISCUSSION

3.1 Selection of PHB Producing Bacterial Isolates from Soil Samples

Among the several bacterial isolates from soil, three (P1,P2,P3) showed positive result for PHB production by sudan black dye as shown in (Figs. 1&2). Hence, they were chosen for the study.

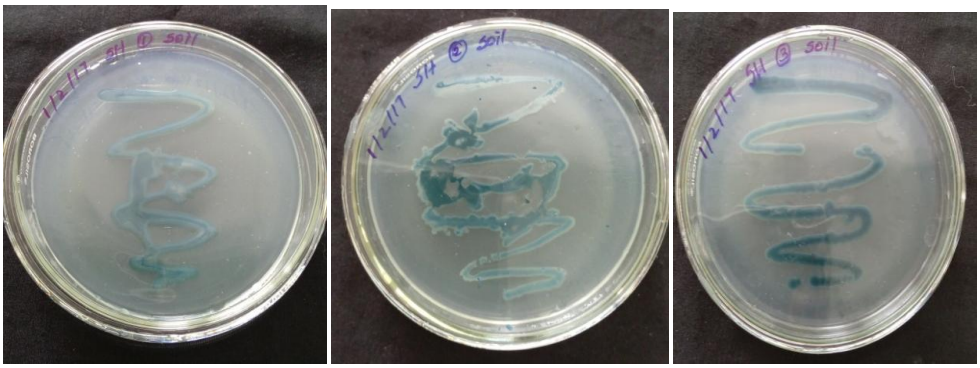
3.2 PHB Production

Polyhydroxy butyrate production of the 3 isolates was determined in both minimal broth and also in minimal broth supplemented with 2% glucose. The PHB production after 24hr incubation was estimated by determining the OD at 235nm as shown in Table 4. Among the 3 bacteria chosen for the study, P-3 was the predominant producer of PHB. This increase in PHB production is depicted in table (Table 1).

Table 1. Comparison of PHB production using selected bacterial isolates (48h incubation)

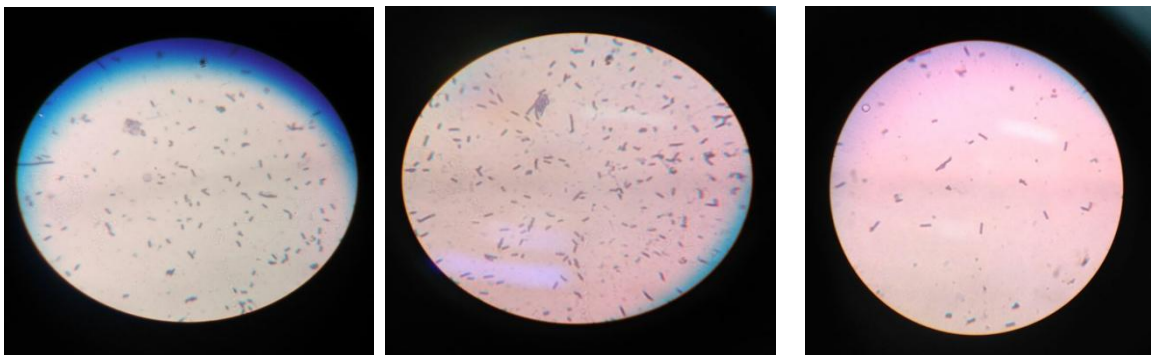
Strains	Cell pellet wt. (gm/l)	PHB expressed as Crotonic acid (OD 235)
P-1	0.86±0.011	0.11±0.005
P-2	1.03±0.012	0.19±0.001
P-3	3.5±0.012	0.39±0.003

Note: Values are represented as Mean ± Standard Deviation



P-1 **P-2** **P-3**

Fig. 1. Screening of PHB producers from different samples



P-1 **P-2** **P-3**

Fig. 2. Sudan black staining of three isolates

3.3 Optimization of PHB Production Using Different Carbon Sources

The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry cell weight. Residual biomass was estimated as difference between the dry cell weight and dry extract of PHB and the production of PHB using different sugars as carbon source was noted (Table 2). PHB production using various peels as carbon source was analyzed and P-3 was the most efficient strain (Table 3).

The major factor limiting the commercial use of PHB is the cost of its production. The two significant areas increasing the production cost are the substrate used for the polymer production and the downstream processes. The use of cheaper carbon sources would bring down the polymer cost. Agricultural wastes like beet and cane molasses, malt extract, corn syrup, wheat bran, starch and dairy wastes like cheese whey with or without ammonium sulphate supplement have been used as raw materials for PHA production. PHB has a wide range of potential applications because of its desired features such as biocompatibility, biodegradability and negligible cytotoxicity to the cells. Hence, the potential application of PHB as replacement for petrochemical based polymers is gaining popularity in various fields involving packaging, medical and coating materials. These desirable properties in compounding and blending have broadened their performances as potential end-use applications. Development of PHB as potential substitute material to some conventional plastics has drawn much attention due to the biodegradable and biocompatible properties of PHB. Because of its good biodegradability and biocompatibility, PHB has potential use in advanced drug delivery systems. However, PHB production strains are still needed.

Table 2. Effect of different carbon sources on PHB production (µg/ml)

Media	P-1	P- 2	P-3
Minimal Broth	1.2	0.7	2.3
Minimal Broth+ 2% glucose	1.9	1.1	2.2
Minimal Broth+ 2% lactose	0.8	1.1	3.4
Minimal Broth+ 2% sucrose	1.1	0.9	2.3

Table 3. Effect of different peels as carbon sources on PHB production (µg/ml)

Different Peels	Concentration	Isolate 1	Isolate 2	Isolate 3
	2%	2.5	5.3	4.2
Pomegranate	5%	2.1	0.91	5.8
	10%	1.2	0.91	6.2
	2%	5.9	4.2	8.1
Orange	5%	3.9	7.8	2.4
	10%	12.2	4.2	1.1
	2%	6.8	4.6	3.9
Sweet lemon	5%	5.4	6.1	8.3
(Sathukudi)	10%	0.7	4.1	7.8
	2%	4.1	1.1	8.4
Beet root	5%	3.9	7.8	6.1
	10%	7.8	4.1	8.1

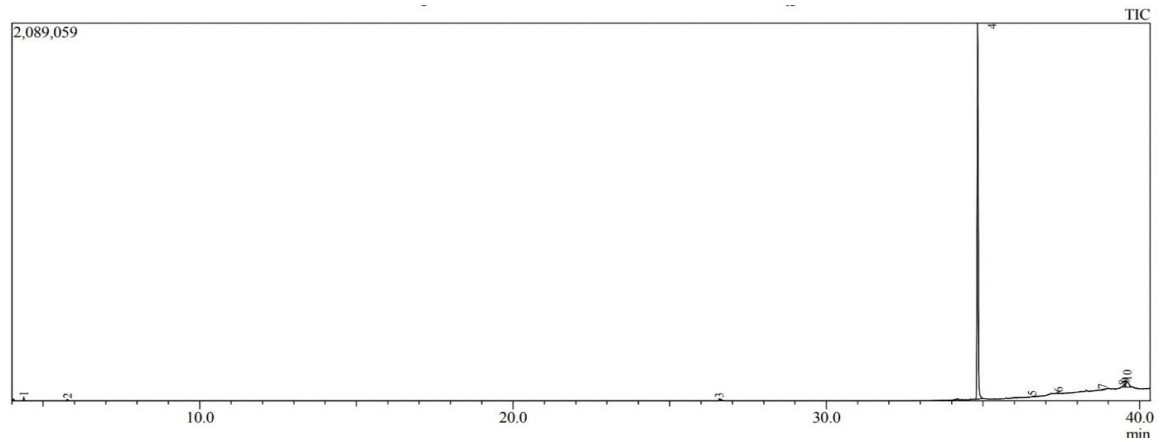


Fig. 3. Chromatogram of PHB by Acinetobacter sp.

Table 4. Components of PHB by GC-MS analysis

Peak	RT	Peak area	Area percentage	Compound name
1	4.383	36487	0.68	1-BUTANOL, 3-METHYL-, ACETATE
2	5.78	20459	0.38	BUTANE, 1,1-DIETHOXY-3-METHYL-
3	26.59	33796	0.63	DECANOIC ACID
4	34.83	4960832	92.29	1,2-BENZENEDICARBOXYLIC ACID
5	36.57	18968	0.35	2-FURANMETHANOL, 3-ETHENYLTETRAHYDRO-, CIS-
6	37.41	16391	0.3	2-FLUORO-6-TRIFLUOROMETHYLBENZOIC ACID, 4-PENTADECYL ESTER
7	38.81	17688	0.33	PROLINE
8	39.47	53840	1	3,4-DIDEHYDRO -1-HYDROXY-6.BETA,8A.BETA-DIMETHYL-5-METHYLENE-TRANS-PERHYDRONAPHTHALENE-1-CARBOXALDEHYDE
9	39.55	23985	0.45	CIS-(2-BENZYLOXYCYCLOPENT-3-ENYL)METHYL ACETATE
10	39.60	192910	3.59	7-HEXADECENAL, (Z)

The lipophilic staining with Sudan Black B (SB staining) reportedly has high sensitivity in PHB screening. The Nitrogen source depletion leads to the cessation of protein synthesis which in turn leads to the inhibition of TCA cycle enzymes (citrate synthase and isocitrate dehydrogenase) and consequently slows down the TCA cycle. As a result, the acetyl Co-A routes to PHB synthesis. The main limitation in the commercial production of PHB as biodegradable plastic is its high production costs compared to the production of petrochemical based synthetic plastics.

Among the three sugars used in the experiment, the results did not reveal any preferential utilisation of the sugars by the isolates. Lactose was found to enhance PHB production than glucose and sucrose for P3, whereas isolate P1 was seen to prefer glucose and Pe 2 had no such preferences. Among the 3 isolates, P3 was found to produce maximum PHB, where the maximum concentration was 8.1-8.4 µg/ml using beet root, sweet lemon and orange peels, while the production using pomegranate peel was 5.8 µg/ml. Enhanced production of 12.2 µg/ml of PHB using orange peel at 10% concentration was observed in P3. The values reveal that PHB production was not concentration dependent.

In the presence of sulphuric acid and methanol, the PHB was methanolysed and analyzed by GC-MS. GC MS graph shows that the major peak at (m/z 150) is of phthalate, which is a type of plastic, and compound name is (1,2-Benzenedicarboxylic acid), whose peak area is 92.2%.

The structure of components could be clarified by the help of GC-MS analysis and the identification of the compounds is depending on their retention peak. However, the use of GC-MS for characterization the extracted PHA has been reported by others [10-12].

4. CONCLUSION

The results of this study confirmed that cheaply available agro-residues can be used for the production of PHB serving triple purposes of reducing the cost of biodegradable plastics, reducing environmental pollution problems caused by conventional plastics and solving disposal problem of the agricultural wastes.: Thus our work has revealed that Acinetobacter calcoaceticus is a potential candidate for PHB production using various peels as substrate.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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