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REGULAR ARTICLE

# PRODUCTION OF POLYHYDROXYALKANOATE (PHA) USING HYDROLYZED GRASS AND *SYZYGIUM CUMINI* SEED AS LOW COST SUBSTRATES

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## ABSTRACT

Hydrolyzed Bermuda grass (*Cyanidon dactylon*) and Jambul seed (*Syzygium cumini*), were used as carbon sources for the production of Polyhydroxyalkanoate (PHA) from soil microbial isolates. The efficiency of selected isolate for PHA production utilizing the hydrolyzed substrate as carbon source was compared with *Ralstonia eutropha* (reference strain) using the same production medium. The best isolate SP-Y1 and *Ralstonia eutropha* were able to accumulate 26.76% and 28.97% of their dry cell weight when hydrolyzed grass was used as substrate and PHA accumulation increased to 41.7% and 42.2% when hydrolyzed seed was used as a sole carbon source

**Keywords:** Polyhydroxyalkonoates (PHA), grass, seed, *Ralstonia eutropha*, FTIR

## INTRODUCTION

Finding an alternative way for responding to the problems associated with plastic waste and its effect on the environment, there has been an effort made in the production and development of biodegradable plastics. Polyhydroxyalkanoates (PHA) are polyesters that accumulate as inclusions in a wide variety of bacteria and were first discovered in *Bacillus megaterium* **(Lemoigne, 1926).** It is a unique intracellular polymer accumulated under stress

conditions but with excess carbon source. Accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions, *i.e.*, when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher **(Steinbuchel, 1996)**. During starvation, PHA serves as carbon and energy source and is rapidly oxidized thereby retarding the degradation of cellular components, combating the adverse conditions as in rhizosphere **(Okon and Itzigsohn, 1992).** PHA is accumulated by numerous microorganisms which involve bacteria such as *Azotobacter*, *Bacillus, Archaebacteria*, *Methylobacteria*, and *Pseudomonas* to varying levels. *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) can accumulate PHAs upto 80% dry weight **(Lee, 1996).** Being a facultative autotroph accumulating large amounts of PHA from simple carbon sources, for example, glucose, fructose and acetic acid **(Reinecke and Steinbüchel, 2009)** it has proven to be the best characterized PHA producer **(Madison and Huisman, 1999).**

The PHA biosynthetic pathway of *Ralstonia eutropha* involves the condensation of two molecules of acetyl- CoA by β- ketothiolase to form acetoacetyl- CoA which is subsequently reduced by acetoacetyl- CoA reductase to form 3-hydroxybutyryl-CoA (3HB). The formed Monomeric 3HB are then polymerized to form Poly (3HB) by PHB synthase. The three enzymes that catalyze these reactions are encoded by genes organized as an operon in this organism, designated as PhbA, PhbB, and PhbC for ketothiolase, reductase, and synthase, respectively **(Anderson and Dawes, 1990).**

PHA has great application potential **(Sudesh and Iwata, 2008; Kanna and Srivastava, 2005).** However, there must be a several-fold reduction in the cost of carbon feedstock used for PHA production. Therefore, the identification of alternative cost-effective substrates for the production of PHA has become an important objective for the commercialization of bioplastics.

In this study, an effort has been taken to explore the PHA producing capability of micro-organisms which have been isolated from the soil and novel substrates such as Bermuda grass, Jambul seed were used as carbon sources for synthesis of PHA. Both are excellent cheap substrates for the synthesis of PHA because of their ready availability. Morphological characteristics of the isolates were identified through Gram's staining method. The PHA production capabilities of the screened isolate under the influence of two different substrates were compared with PHA prodcution of the reference strain *Ralstonia eutropha* MTCC1472. Then the extracted PHAs were analyzed for their structural properties through FTIR analysis.

## MATERIAL AND METHODS

### Isolation of microorganisms

Garden soil sample was collected at the institution (Kumaraguru College of Technology, Coimbatore, India) and used for the isolation of bacteria by serial dilution technique. Then the isolated bacterial colonies were screened and potential isolates were preserved on nutrient agar slants and glycerol stocks, until further use.

### Reference strain

*Ralstonia eutropha* (MTCC 1472) was obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh (India). *R. eutropha* was used as a reference strain for comparison of PHA production.

### Choice of substrate

Bermuda grass (*Cyanidon dactylon*) and Jambul seed (*syzygium cumini*) were collected locally, shredded into pieces and dried in oven at 60ºC for about 1 week and pulverized into fine particles.

### Hydrolysis of Cellulose

The method followed for the hydrolysis of substrate into glucose (zinc chloride method) was based on Cao et al., (1995). Then the glucose content was estimated by Di-Nitrosalicylic acid (DNS) method **(Miller, 1959).**

### Screening of microorganisms

Screening of PHA producing microorganisms was carried out by Nile Blue Staining method **(Ostle and Holt, 1982).** Nile blue was dissolved in acetone, and was added to the agar mediam for viable colony staining. PHA producing microorganisms were visualized as bright orange colonies under UV transilluminator **(Spiekermann, 1999).**

**Bacterial growth in production media**

The growth of SP-Y1 in production medium was monitored by taking absorbance at 660nm and the corresponding amount of PHA accumulated was determined by incubating it for five consecutive days **(Henderson and Jones, 1997).**

**Production of PHA**

The stock cultures (*R. eutropha* and the isolates) were initially revived in nutrient broth and later inoculated into mineral salts medium containing hydrolyzed grass (10g), glucose (5g), sodium chloride (5g), di-potassium hydrogen phosphate (5g), magnesium sulphate (ig) and ammonium sulphate (1g) in 1L-distilled water. The pH was adjusted to 7.4±0.05 and the cultures were incubated for 48h at 30ºC in orbital shaker **(Du *et al.,* 2001; Amirul *et al.,* 2008; Yamanaka, 2010).**

**Extraction of PHA**

After the incubation period of 48h the cultures were centrifuged at 10,000rpm for 5min. The supernatant was discarded leaving the pellet, which was air dried and weighed. The extraction was done following the method of **Santhanam and Sasidharan (2010),** where PHA was extracted using the solvent chloroform. The cell pellet was suspended in sodium hypochlorite solution and incubated at 37°C for 1-2 h for complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with distilled water and centrifuged again. Finally PHA granules in the sediment were washed twice with acetone and diethyl ether (1:1 ratio). The resultant polymer granule was dissolved in boiling chloroform and air dried to obtain PHA powder.

**Quantification of PHA**

The polymer granule was dissolved in concentrated sulphuric acid (1mg.ml$^{-1}$) and heated at 100$^{o}$C for 10 min to convert PHB into crotonic acid, which was brown coloured. The solution was cooled and the absorbance was read at 260 nm against a concentrated sulphuric acid as blank in a spectrophotometer. A standard curve was prepared with Pure PHB

(Sigma, Aldrich), concentrations ranging from 20-100µg/ml **(Law and Slepecky, 1969).** The quantity of PHB produced was determined by comparison with the standard.

**Identification of PHA granules**

The bacterial cells were stained with Nile blue stain and visualized under UV trans-illuminator. The accumulation of PHA in the form of granules could be identified from the fluorescing cells **(Amirul *et al.*, 2008).**

**Analytical procedure**

The bacterial cultures were centrifuged at 10,000 rpm for 5 min to obtain the cell pellet. The Cell pellet was dried to estimate the wet cell weight (WCW in g/ml) (Du et al 2001). Residual biomass, % of PHA accumulation and concentration of PHB **(Zakaria *et al.*, 2010)** was calculated by the following formulae.

**Residual Biomass** (g/ml) = WCW (g/ml) - Dry weight of extracted PHA (g/ml).

$$\textbf{PHA Accumulation } (\%) = \frac{\text{Dry weight of PHA extracted } \left(\frac{\text{g}}{\text{ml}}\right) \text{x100}}{\text{WCW } (\frac{\text{g}}{\text{ml}})}$$

**PHB Mass** (µg/ml) = Obtained from Standard PHB assay

**Analysis of extracted PHA**

KBr was added to the PHA samples, evaporated and analyzed using Fourier Transform Infra Red Spectroscopy, (Model: FTIR-8400S Shimadzu) to identify the functional groups, structure and purity. The peaks were observed from 4000-400 $cm^{-1}$ **(Oliveira *et al.*, 2007; Pandian *et al.*, 2010).**

## RESULTS AND DISCUSSION

### Isolation and screening of microorganisms containing intracellular PHA

Various microorganisms isolated from the soil were screened for their PHA production, which was confirmed by the orange fluorescence emitted (fig 1) after staining with Nile blue dye, under UV transilluminator **(Landazuri and Maldonado, 2008).**

**a) Colony plate of isolate SP-Y1 b) Nile blue stained colony plate**

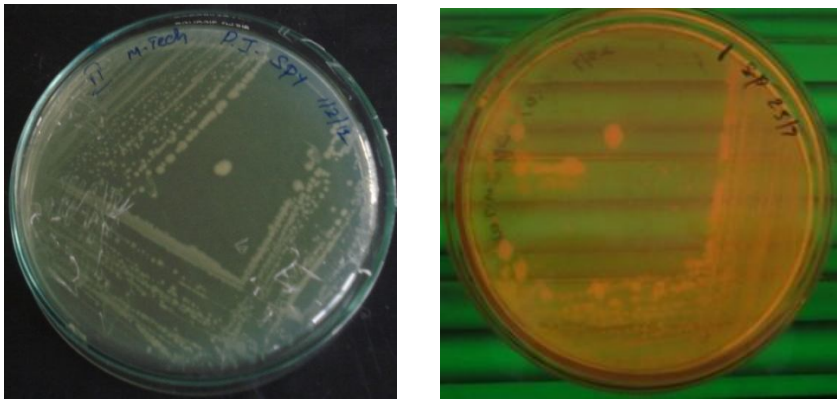


**Figure 1** Isolation and Screening of the PHA producing organisms

### Estimation of Glucose in the hydrolyzed substrate

The glucose content in the hydrolyzed substrates (Grass and seed) was estimated using DNS method. From the standard curve obtained from DNS assay, it was inferred that the amount of glucose was found to be 4100 µg/ml and 4150 µg/ml for grass and seed respectively.

### Selection of best isolate for PHA prodcution

The isolates were compared with the reference strain *R. eutropha* for their PHA production by utilizing glucose as a sole carbon source in their growth medium. From the results obtained, it was inferred that the isolate SP-Y1 produced 0.82 g/L of PHA and it was selected for further studies. PHA production by SP-Y1 was comparitively higher as shown in fig 2.

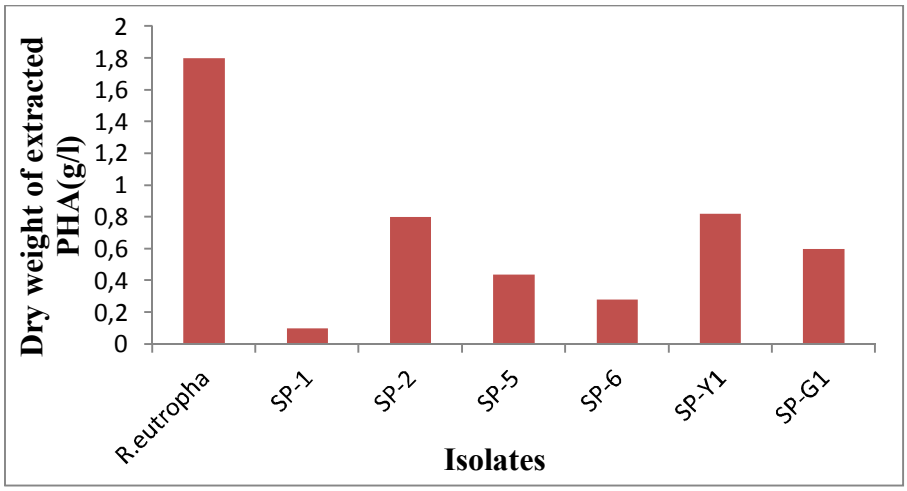



**Figure 2** Comparision of PHA production by the screened isolates

**Bacterial growth in production media**

When the bacterial culture was incubated for five days and monitered for PHA accumulation, it was found that PHA accumulation was in proportion to the bacterial density and cell weight (Table 1). Maximal accmulation was observed on 2nd and 3rd day of growth respectively, with grass and seed as carbon sources. Similar result was found in literature were maximal accumulation was achieved in the 2nd or 3rd day for *Ralstonia eutropha* depending on the nature of media used for PHA production **(Henderson and Jones, 1997; Santhanam and Sasidharan, 2010)** and PHA accumulation was also reported to be proportional to the bacterial density.

**Table 1** Relationship between bacterial growth and PHA accumulation

| Incubation period (days) | Hydrolyzed Grass | | Hydrolyzed Seed | |
|---|---|---|---|---|
| | Absorbance at 660nm | % PHA Accumulation | Absorbance at 660nm | % PHA Accumulation |
| 1 | 0.166 | 1.114 | 0.018 | 10.39 |
| 2 | 0.222 | 16.54 | 0.026 | 11.22 |
| 3 | 0.123 | 11.89 | 0.114 | 11.73 |
| 4 | 0.039 | 11.40 | 0.02 | 7.07 |
| 5 | 0.022 | 10.85 | 0.016 | 2.165 |

**Extraction and screening of PHA**

*R. eutropha* and the best isolate SP-Y1 were grown in mineral salts media augmented with hydrolyzed straw alone and a combination of glucose and hydrolyzed grass, for PHA production. The extracted PHA was an ivory white coloured powder. It was found to be sparingly soluble in water. PHA presence was confirmed by staining with Nile Blue, which emits orange fluorescence under UV transilluminator. Similar kind of studies using fluorescent microscopy for visualizaton of intracellular PHA accumulation have been cited **(Amirul *et al.,* 2008)**.

**Effect of different substrates on PHA prodcution**

The extracted PHA of SP-Y1 and *R.eutropha* were quantified and residual biomass, % of PHA accumulation and mass were determined. From the results, it could be inferred that the PHA accumulation was in proportion to the wet cell weight, as earlier stated in literature **(Du *et al.,* 2001; Zakaria *et al.,* 2010)**. Pure cultures of SP-Y1and *R.eutropha* showed greater efficiency of PHA production when utilizing hydrolyzed seed as sole carbon source compared to that of grass as a sole carbon source and to that of mixed substrates. For *R. eutropha*, % of PHA accumulation and PHA mass were found to be 41.77 and 97 µg/ml, respectively, whereas for SP-Y1 they were found to be 42.26 and 100 µg/ml, respectively (Table 2). Although significant amounts of carbohydrates were found in the hydrolyzed sources (4100 µg/ml and 4150 µg/ml) there were other residues still present, which made them slightly complex and the microorganisms were unable to adapt to this situation when mixed substrate was used, thus the PHA accumulation decreased in both microorganisms **(Yang *et al.,* 2010).**

**Table 2** Comparison of PHA production between pure culture and mixed cultures utilizing different combinations of substrates

| Microorganism | Substrate | Dry weight of Extracted PHA (g/ml) | Wet cell weight (g/ml) | Residual Biomass (g/ml) | % PHA Accumulation | PHA mass (µg/ml) |
|---|---|---|---|---|---|---|
| *Ralstonia eutropha* | G+HG | 0.003 | 0.036 | 0.033 | 8.5 | 25 |
| | G+HS | 0.003 | 0.104 | 0.1 | 2.38 | 11 |
| | HG | 0.043 | 0.151 | 0.107 | 28.97 | 73 |
| | HS | 0.044 | 0.105 | 0.061 | 41.77 | 97 |
| | Mixed substrate (HG&HS) | 2.76x10$^{-}$3 | 0.034 | 1.581 | 8.03 | 22 |
| SPY-1 | G+HG | 0.006 | 0.039 | 0.033 | 16.03 | 49 |
| | G+HS | 0.002 | 0.025 | 0.023 | 9.52 | 38 |
| | HG | 0.029 | 0.106 | 0.078 | 26.76 | 52 |
| | HS | 0.06 | 0.142 | 0.082 | 42.26 | 100 |
| | Mixed substrate (HG&HS) | 2.64 x10$^{-}$3 | 0.03 | 1.391 | 8.67 | 29 |

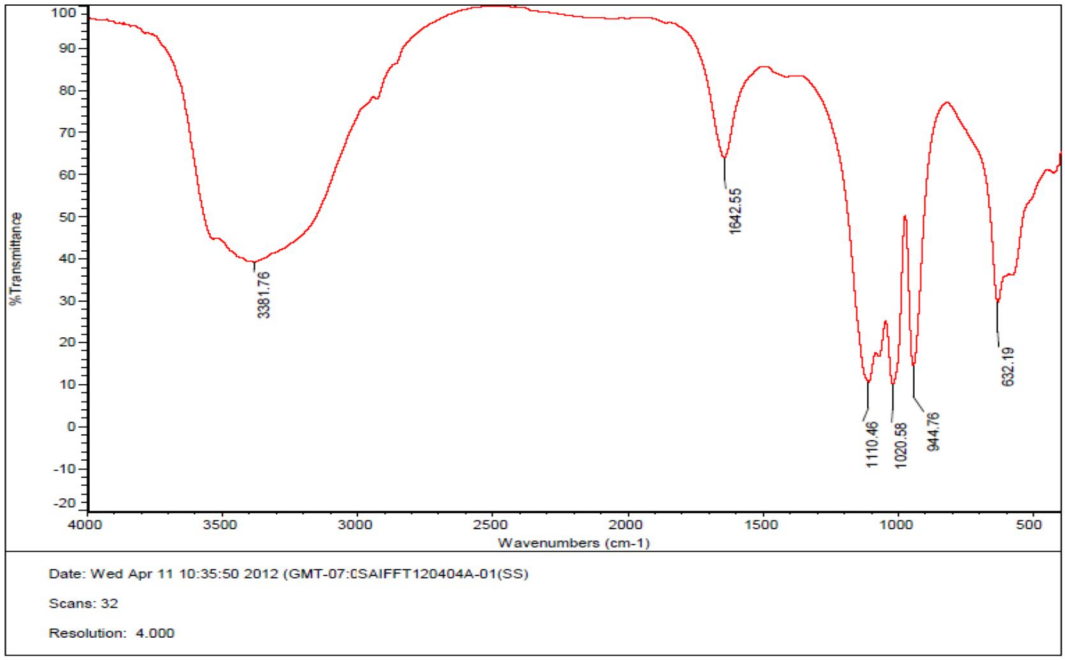
**Legend:** G – Glucose, HG –Hydrolyzed Grass and HS –Hydrolyzed Seed

**Identification of functional groups in PHA samples**

The extracted PHA was analyzed for identification of its functional groups through FTIR analysis. From the FTIR spectra (ig- 3 a, b), it can be concluded that the peaks observed in HS sample were similar to the standard PHB spectra. According to results published earlier **(Sandhya *et al.*, 2012)**, the peak at 3381 $cm^{-1}$ indicates strong bond of H stretching originated by terminal OH groups found in (HS) sample, the spectra matches similar results cited in literature **(**Chen ***et al.*, 2009)** the peak at 1642 $cm^{-1}$ indicates a weak C=O stretching for conjugated carbonyl or amide group or may be due the C=C stretching, while the peak at 1110 $cm^{-1}$ accounts for C-O stretching (strong bond) other peaks correspond to the presence of Alkyl halides (1020 $cm^{-1}$, 944 $cm^{-1}$, 632 $cm^{-1}$) **(Arcos-Hernandez *et al.*, 2010; Sandhya *et al.*, 2012)**. Similar peaks were observed from the FTIR spectral result of sample HG, C=O stretching at 1637 $cm^{-1}$, additional peaks found in the region 1430 $cm^{-1}$, 1373 $cm^{-1}$ and 1320

$cm^{-1}$, refers to –C-H- bending **(Oliveira *et al.*, 2007)**. Intense peak at 3406 $cm^{-1}$ indicating OH stretching, 1106 $cm^{-1}$ peak representing stretching for C-O, Alkyl halides peaks at 1065 $cm^{-1}$, 1023 $cm^{-1}$, 948 $cm^{-1}$ and 632 $cm^{-1}$).

**3a) SPY-1 utilizing HS as substrate**




**3b) SPY-1 utilizing HG as substrate**

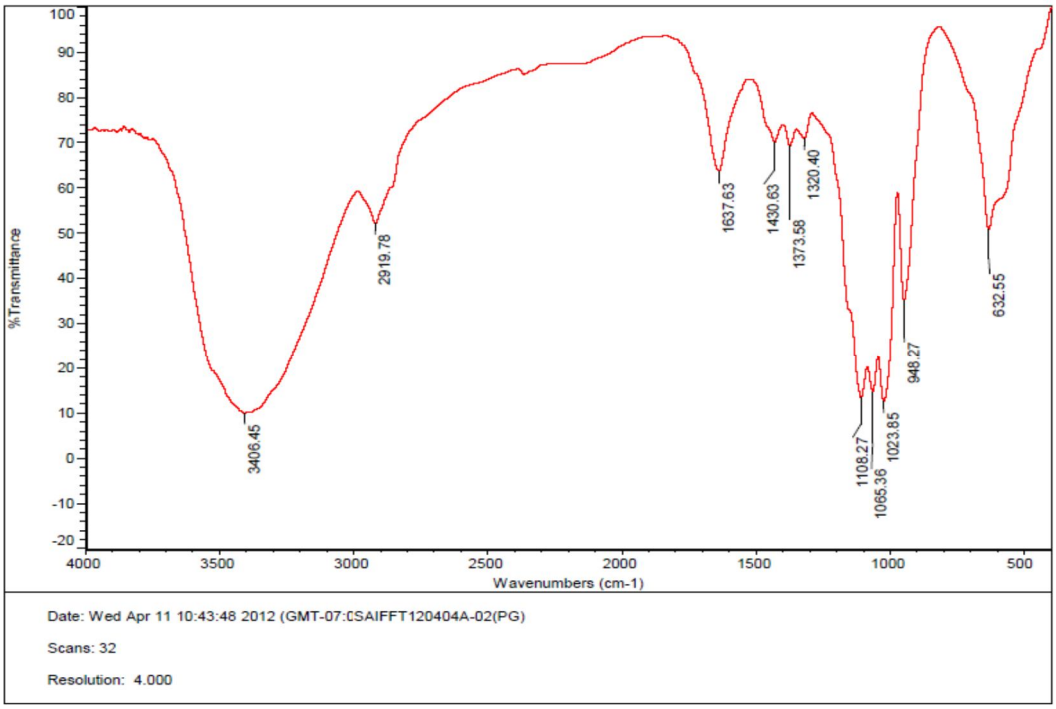



**Figure 3** FTIR analysis of extracted PHA from SPY-1

## CONCLUSION

The extent of PHA accumulation by bacteria depends on the characteristics of the producing species and effectiveness in utilization of carbon sources available to them. When the best PHA producing isolate and *R. eutropha* were grown in Mineral Salts Media with hydrolyzed grass and seed as carbon sources, both the strains produced significant amounts of PHA (97µg/ml and 100µg/ml for hydrolyzed seed, respectively. Further optimization of the parameters via design of experiment (DOE) and scaling up will lead to significant levels of production of PHAs. Thus, the seed and grass can be utilized as cheap carbon sources in harnessing PHA.

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