International Journal of Innovative Technology and Exploring Engineering (IJITEE)
ISSN: 2278-3075 (Online), Volume-9, Issue-7, May 2020

# Potential of PHA Accumulation in Escherichia Coli, Bacillus Subtilis and Pseudomonas Aeruginosa Cultured on Agro-Industrial Byproducts

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***Abstract*: Polyhydroxyalkanoates (PHAs) are type of natural polymers which are synthesized by different microorganisms to increase their survival rate during environmental change or stress conditions. The biodegradable polymers are an alternative solution to non-renewable petroleum derived plastics. The aim of this study is to produce PHAs by Escherichia coli, Bacillus subtilis and Pseudomonas aeruginosa using agro and industrial waste such as wheat bran and cane molasses. In this work, the effect of different media on each bacterium was studied. The optimum environmental condition that supported the PHA production by the three strains was inoculum concentration of 8%, pH 7.0 and temperature of 30°C. The medium was fermented for five days in orbital shaker. Each day samples were collected and analyzed. Dry cell weight and PHA accumulated was observed for each of the bacteria. On the basis of data obtained in the present work, compared to B. subtilis and E. coli, P. aeruginosa was capable to accumulate 70.27% of PHA by using Cane molasses and Wheat bran as substrate. This could be employed for industrial application after subsequent optimization of the conditions of PHA synthesis. The present study explored the potential of Pseudomonas aeruginosa to produce cost-effective PHA as an alternative to petroleum based plastics.***

***Keywords* : Polyhydroxyalkanoates, *E. coli*, *B. subtilis*, *P. aeruginosa*, cane molasses, wheat bran.**

## I. INTRODUCTION

The recent awareness by policy makers and personal preferences toward finding an alternative to petrochemical based plastics lead to explore on microbial based biopolymer polyhydroxyalkanoates (PHAs). The attention on PHA is enormous as it is biocompatible, biodegradable and has thermoplastic and mechanical properties such as versatility, elasticity, flexibily [1]. The PHA Market Research Report predicted a prospect of PHA market to reach about USD 98 million by 2024[2]. PHA is an intracellular carbon reserve for the microbes and various microorganisms have reported to produce in response to the stress conditions such as providing surplus carbon along with starved conditions of nitrogen, phosphate or sulphur [3].

**Revised Manuscript Received on May 30, 2020.**
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The high cost of production of PHA in a commercial scale limits its wide usage in various fields of application. One of the main components in the high cost of production is price of carbon source required in the fermentation [4]. Hence finding an alternative cost effective carbon substrate could improve the economic feasibility and achieve a sustainable production.

The PHA biosynthesis with numerous agro industrial waste products such as waste water from olive oil mills, apple pulp waste, dairy waste, food processing waste, plant oils, have been reported over the years[1], [5]–[8]. The utilization of agro industrial waste solves the difficulties related with waste disposal [9].

Numerous microbes from different ecological niches are reported to accumulate PHA in the cells[10]. Comprehensive studies have been conducted on Bacillus sp. as potent producer of PHA because of its high growth rate and ease of PHA extraction. Several *Bacillus* sp. such as *Bacillus cereus, Bacillus circulans, Bacillus subtilis, Bacillus firmus, Bacillus licheniformis, Bacillus sphaericus, Bacillus laterosporus* were reported to produce PHA in synthetic media as well as on waste resources[11].

Quite a few studies were conducted with recombinant *E coli* for the production of PHA[12] due to its easiness in scale up and downstream processing while native *E coli* also has given a substantial amount of PHA [13]. Several studies were conducted with *Pseudomonas* sp on different agro industrial wastes such as raw cassava starch, distillery effluent, cane molasses and corn steep liquour [14], [15].

In the present study, efforts were taken to grow three different species namely *E coli*, *B subtilis* and *P aeruginosa* on agro- industrial waste such as cane molasses and wheat bran to produce PHA in submerged fermentation in batch mode. Current study also compares the effect of media, organism and incubation period on the accumulation of PHA

## II. METHODOLOGY

### Microbial Culture

The microbes used in the study such as *Escherichia coli* (NCIM ID 5649), *Bacillus subtilis* (NCIM ID 2920)were obtained from NCL, Pune and *Pseudomonas aeruginosa* was obtained from Microbiological Lab, Coimbatore. The culture slants were stored under standard conditions of $4^0$C and the pure cultures were raised and used suitably throughout the study.

The culture was revived by growing a loopful in 50 ml of nutrient broth at 35ºC for 24 h in an orbital shaker at 120rpm. Subsequently, the 5% inoculated in 100ml of nutrient broth in 250 mL conical flasks and incubated in an orbital shaker (120 rpm) at 35ºC for 24 h. This was used as inoculum for all the experiments.

After 24 h of cultivation, cells were harvested by centrifugation at 6,000rpm at 4ºC for 15 min, washed aseptically with sterile distilled water and re-suspended into 250 ml Erlenmeyer flasks containing 100 ml of production medium.

## III. MEDIA PREPARATION

Two different basal production media were used to produce polyhydroxyalkanoates from bacteria. The media were sterilized at 121°C, 14 psi for 20 minutes.

1. Production medium containing (g/L): NH4Cl 1.0, NaHCO3 0.5, KH2PO4 2.0, Na2 HPO4. 2H2O 2.0, MgSO4.7H2O 0.5, CaCl2. 2H2O 0.01, Fe (NH4) citrate 0.05, trace elements solution: 5.0 mL of the solution (containing ZnSO4.7H2O 0.08, MnCl2.4H2O 0.03, H3BO3 0.3, CoCl2.6H2O 0.2, CuCl2.2H2O 0.01, NiCl2.6H2O 0.02, Na2MoO4.2H2O 0.03).
2. The basal production medium for batch culture containing (g/L): Na2HPO4.2H2O 2.2, KH2PO4 1.5, MgSO4.7H2O 0.2, and ammonium acetate 1.75 [16].

Complex nutrients obtained from agro industrial waste are

## IV. SUGARCANE MOLASSES

PHA production by *B. subtilis*, *P. aeruginosa* and *E. coli* was studied using sugarcane molasses as a carbon source. Sugarcane molasses obtained from El Rashidi Ei Mizan Confectionery, Egypt and was used as an alternate carbon source.

Pretreatment of sugar cane molasses was carried out by following method:

Cane molasses was diluted with an equal quantity of double distilled water. Clarified liquid was obtained from the by centrifugation at 2000 rpm for 10 min after an incubation of 5h at 40°C. The pH of clarified liquid was adjusted to 3.0 with 0.1N $H_2SO_4$ and the solution was kept undisturbed for 1.5 h and then separated by centrifuged at 3000 rpm for 15 min. The supernatant was stored at 4°C for further use in fermentation media[17].

The fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml basal media with 8% of the pretreated cane molasses. The flasks were inoculated with 8.0 ml inoculum and incubated at 30 ºC in an orbital shaker (120 rpm) for 120 h.

### Wheat Bran

PHA production by *B. subtilis*, *P. aeruginosa* and *E. coli* was studied using Wheat bran which was obtained from Sresta Natural Bioproducts Pvt. Ltd., Telangana, India. In the current study, wheat bran was added at a concentration of 10% and the preparation of wheat bran for the fermentation was by grinding it to the powder form.

Fermentation

The culture medium was inoculated with 8% of the seed culture and incubated in orbital shaker at 120 rpm at 30°C for 120 h. The pH was maintained at 7.0 by the manual addition of NaOH (10%), or HCl (10%). Samples (10 ml) were taken from the growing cultures periodically every 24 h under aseptic conditions to determine bacterial growth and accumulated PHA. All experiments were carried out in triplicates.

## V. ANALYTICAL PROCEDURES

### Biomass Quantification

The culture sample collected periodically from the growing culture was centrifuged at 6000 rpm, 4°C for 15 min to separate the spent culture media and the cells were washed with sterile distilled water to remove the media residuals. The cells were then dried in hot air oven at $105^0$C until a constant weight was attained and the biomass dry weight was recorded.

### Extraction And Quantification Of Polyhydroxyalkanoates

To the dry biomass obtained, 10 ml of sodium hypochlorite (1% w/v) was added and the mixture was kept at 40°C for 2 h after proper mixing. It was then separated by centrifugation at 6000 rpm for 20 min and the pellet was washed with acetone (10ml) and subsequently with distilled water. Further the pellets were reconstituted in chloroform and dried at 60°C for 20 h. the weight of the pellet was measured [13].

PHA yield was calculated as follows:

$$PHA\ \% = \frac{Weight\ of\ PHA}{Weight\ of\ the\ dry\ cell} X100$$

### Statistical Analysis

The experiments were conducted in triplicates and the values in the study are expressed as mean ± standard deviation. Statistical analyses were performed with Microsoft Excel using analysis of variance at a confidence interval of 95%.

## VI. RESULTS

The study was conducted to compare three different selected microorganisms namely *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* to produce polyhydroxyalkanoates in different media made of agro industrial wastes. The microbes were preserved at $4^0$C and were revived in nutrient broth prior to the study.

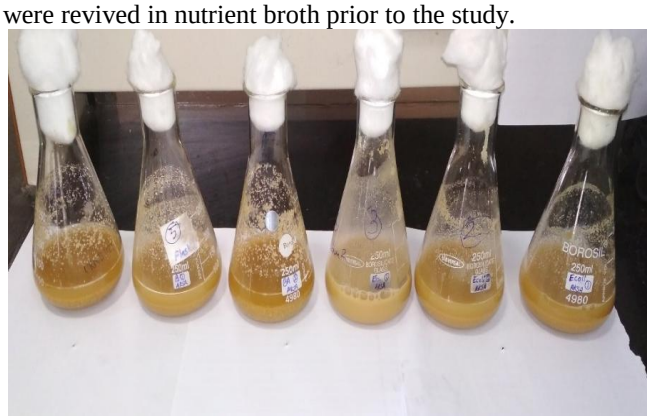

**Fig 1: PHA production in different media batch culture**

**Batch fermentation**

The different media were prepared and inoculated with the microbes (Fig 1) in the batch mode of incubation period of 120h. About 10 ml of samples were withdrawn aseptically in every 24 h to measure the growth and PHA production.

### Growth Of The Microbes

The biomass dry weight was determined as a measure of the growth at every 24 hrs of the incubation period. Media 2 had resulted in maximum biomass for the organisms under study compared to the media 1. In the media 1 (Fig 2) B subtilis had given the maximum biomass (18.33g/L). For *E coli*, the maximum biomass of11.36g/L was observed at 72 hrs. In case of *B subtilis*, maximum dry weight obtained at 72hrs and was observed as 18.33g/L. In the case of *P aeruginosa*, maximum biomass was observed at 96hrs and observed to be 16.8g/L. All the microorganisms had shown a similar growth pattern in the media 1.

While in media 2 (Fig 3), all the organisms had a similar growth pattern with highest biomass observed in *B subtilis*. In the case of *E coli*, the highest biomass was obtained at 72hrs and was observed to be 24g/L. Hence the performance of *E coli* was better in the media 2 compared to the media 1. In the case of *B subtilis*, similar to media 1, the highest biomass was observed as 23.7g/L at 72hrs. Similarly in the case of *P aeruginosa*, highest biomass was observed as 23.1g/L and was at 72hrs. The total biomass is slightly higher for all the organisms in media 2 compared to media1.

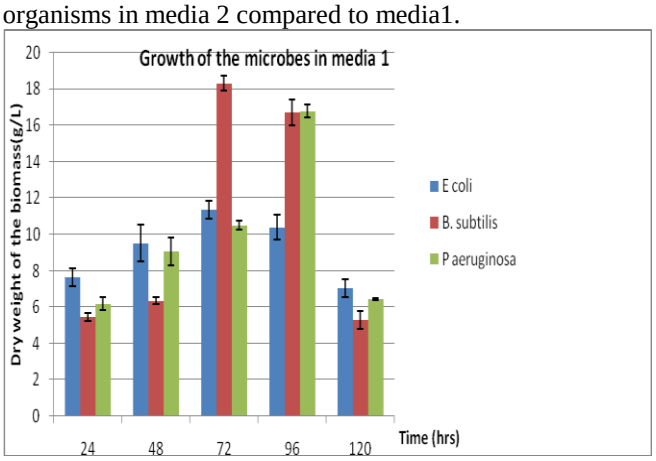


**Fig 2: Growth of the microbes in media 1**

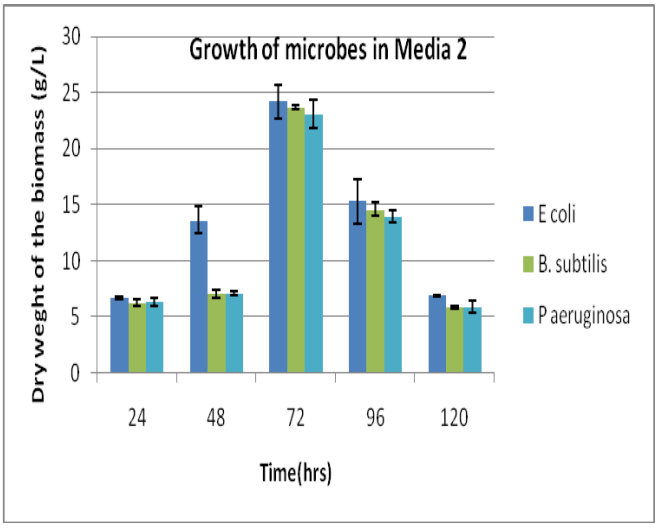


**Fig 3: Growth of the microbes in media 2**

### Estimation Of Pha Production

PHA was extracted and quantified every 24 hrs. While extracting the PHA with sodium hypochlorite, after the cell lysis, aqueous layer containing sodium hypochlorite remained as the upper layer, a bottom layer of chloroform with PHA and an interface layer containing cell debris (Fig 4).

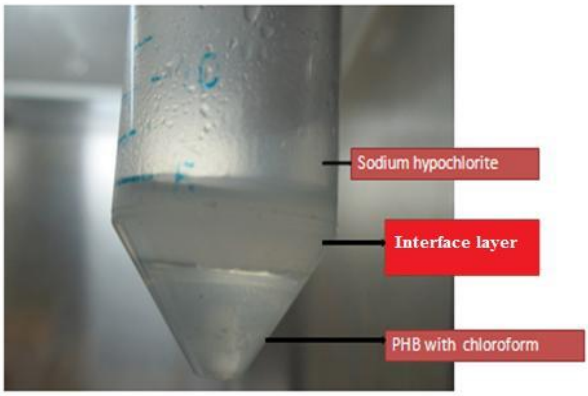


**Fig 4 Sample treated with sodium hypochlorite, acetone, distilled water and chloroform.**

In the quantification of the PHA from the microbes in media 1 (Fig 5), in the case of *E coli*, highest production was observed in 96$^{th}$ hour. In the case of *B subtilis* and *P aeruginosa*, highest production was observed on 72 hrs and specifically in case of *P aeruginosa*, it remained in the highest production state on 96$^{th}$ hour also. The entire three organisms had shown a similar pattern in the production of the product.

In the media 2(Fig 6), all the three organisms had showed a similar pattern in the production with initially less quantity, gradually increased to a highest quantity and then decreasing. For all the organisms, highest production was observed on 72$^{nd}$ hour and among three, *P aeruginosa* produced the highest quantity of PHA.

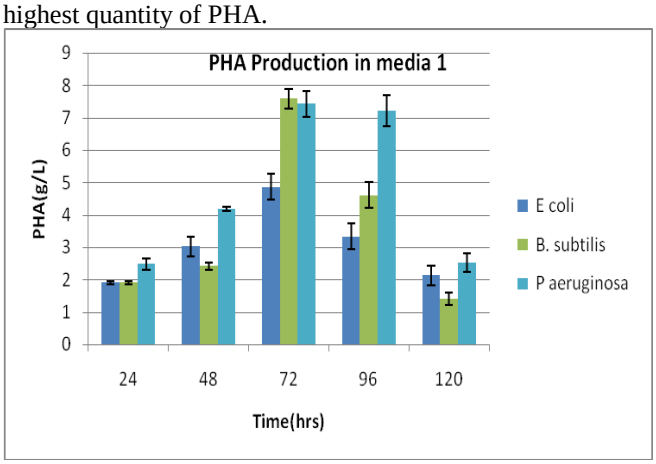


**Fig 5: PHA production in media 1**

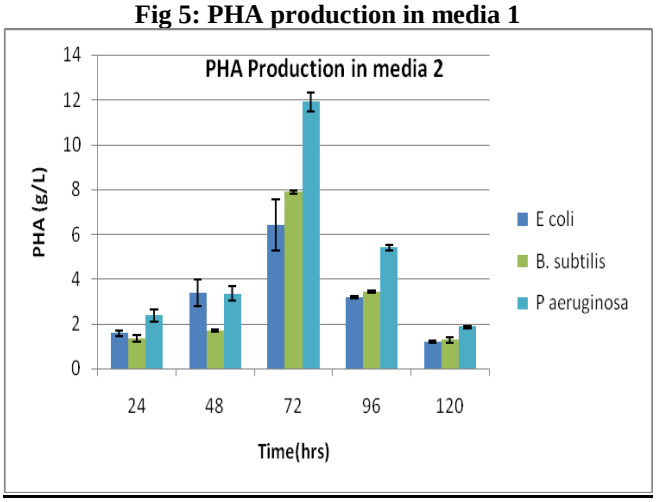


**Fig 6: PHA production in media 2**

**Pha Yield**

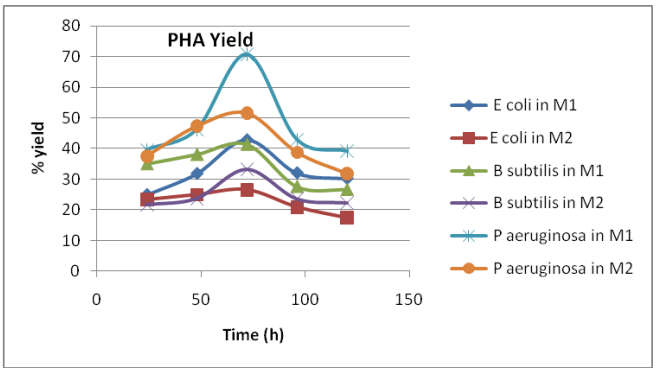


**Fig 7: Yield of PHA in different media by different organisms**

While comparing the yield of PHA (Fig 7) formed in different organism in different media, it is clear that a maximum was observed for *P aeruginosa* in medium 1 followed by *P aeruginosa* in medium 2.

**Table 1: Effect of different factors on PHA yield**

| Effect | SS | DF | MSS | F | F table(0.05) |
|---|---|---|---|---|---|
| Incubation Time | 1012.535567 | 4 | 253.1339 | 6.601485218 | 3.84 |
| Media | 517.2561633 | 1 | 517.2562 | 13.48953668 | 5.32 |
| Organism | 1749.935527 | 2 | 874.9678 | 22.81830662 | 4.48 |
| Time*Organism | 89.38068667 | 8 | 11.17259 | 0.291370151 | 3.44 |
| Time*media | 25.93349556 | 4 | 6.483374 | 0.169080073 | 3.84 |
| Organism*medium | 2.806449333 | 2 | 1.403225 | 0.036594732 | 4.46 |
| Error | 306.7599285 | 8 | 38.34499 | | |
| Total | 3704.607817 | 29 | | | |

The comparison study followed was for 3 factors such as Incubation time(at 5 levels), Different organism (3 levels) and different media(2 levels). A 3- way ANOVA was performed to validate the statistical significance of the comparison study (Table 1). It resulted that all the factors were statically significant for the production of PHA with F value>F table value. The F values for incubation time, organism and media were obtained as 13.10, 45.30 and 26.78 respectively. Another important observation in the analysis was that none of the interactive effect were significant as its F value was<F-table. The statistical significance of the study supports the result that for the production of PHA is desirable at incubation period of 72 hrs, the most effective organism is *P aeuginosa* and most effective media is Media 1.

The yield of PHA on utilizing wheat bran as a nutrient sources was reported as 38% for a *Bacillus* sp[16]. Similarly, maximum yield of 2.74 g/L [18] was obtained in *Bacllus megaterium* when the media was optimized as glucose (carbon source) and ammonium sulphate (nitrogen source). When cane molasses at 6% and 8% was used as the nutrient source, PHA yield was observed as 54.1 and 47.16% for *B. subtilis* and *E coli* respectively and the incubation period was 96hrs [13].When 15 different agro- industrial wastes were used in the production of PHA by *Bacillus megaterium*, cane molasses showed a maximum PHA accumulation of 19.52g/L after 72hrs of incubation [19]. These yields of PHA are in par with our observation for *B subtilis* and *E coli.*

As in our study, a higher yield was obtained for *P aeruginosa* when water hyacinth was used as the substrate for PHA production with 65% yield at an incubation period of 72hrs [20].In a study on the effect of 5 different carbon sources on biomass production and PHA accumulation by *P aeruginosa*, it showed maximum accumulation of 62% when molasses was used[21]. When wheat bran was combined with nutrient broth for the PHB production, *P aeruginosa* accumulated about 3.8µg/ml while *Pseudomonas alcaligens* accumulated 3.1µg/ml. However, other *Pseudomonas* species accumulated even lesser quantity of PHB[22]. *B.thuringiensis* could not utilize wheat bran for the accumulation of PHA as the yield was low(7.4%) owing to its nitrogen content which favoured the growth phase rather than the production phase [23].

## VII. CONCLUSION

A considerable attention has been given recently to the development of production system of polyhydroxyalkanoates due to its applicability in diverse fields such as medical implants, scaffolds, drug delivery, agriculture field and food packaging. The success of the commercial production depends on the economic feasibility of the process. Utilzation of cheap resources as substrates can considerably reduce the cost of the overall process and the utilization of waste product reduces the constraints related to the waste management system n industries.In the current study, an investigation has been done to determine the effect of three different microorganisms and two different media based on agro-industrial waste on the yield of PHA. It was observed that maximum yield was attained by *P aeruginosa* in cane molasses based media followed by the same organism in wheat bran based medium. Based on the statistical analysis of the data, the production of PHA is desirable at incubation period of 72 hrs with the most effective organism is *P aeuginosa* and most effective media is Media 1.

This study demonstrated capability of different microbes on different substrates to accumulate PHA. This can help in the development of co cultivation of different organism on the complex media to get the desired product. An extensive study needs to be conducted for its applicability. Further focus has to be on the separation and purification of PHA effectively.

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