A Comparative Analysis of Growth and Polyhydroxybutyrate production in Selected Strains of Bacteria

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Abstract

Polyhydroxybutyrate (PHB) is a class of polyester accumulated intracellularly by many gram positive and gram negative bacteria as carbon and energy reserve granule. The potential of PHB as biodegradable plastic has been long recognized but several factors like time of extraction, method of extraction, carbon sources etc. hampered their commercialization for a wide range of application. So this study was aimed for isolating potential PHB accumulating strains and optimizing time of extraction of PHB from the strains. Time course study of growth and PHB production of bacteria revealed that optimum PHB production was associated with the stationary phase of its growth.

Key words– *Bacteria, growth, PHB production*

1. INTRODUCTION

Living "green" has become a popular trend in the last twenty years and reducing oil consumption remains an important goal for the sustainably-minded today. One of the top uses of crude oil is the production of plastics. The rapid increase in the usage of plastics and its disposal has caused serious threats to the environment. Synthetic plastics possess several negative attributes mainly with regard to their disposal[1]production of toxic substances during incineration[2] and higher waste accumulation in the landfills and marine environments. These economic and ecological drawbacks of petroleum-based plastics have pushed researchers to develop and investigate biodegradable plastics as an environment-friendly alternative, which is possessed of comparable physical and chemical properties paralleled with conventional synthetic plastics [3], [4], [5].

To produce biodegradable plastics resembling conventional plastics, bacteria are employed to make the building blocks for plastic polymers from renewable sources. Some types of the biodegradable plastics being developed include polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and copolymers and blends of starch and polypropylene [6]. PHAs are polyesters accumulated by various bacteria under unbalanced growth conditions when the carbon substrate is in excess of other nutrients such as nitrogen, sulfur, phosphorus or oxygen [7], [8], [9].

Polyhydroxybutyrate(PHB) is a type of polyhydroxyalkanoate[10]. They are commonly composed of (R) – hydroxyl fatty acids, where the R groups varies from methyl (C1) to tridecyl (C 13) [7]. The monomer units are all in D-configuration owing to the steriospecificity of biosynthetic enzyme [11], [12]. PHBs are divided into two groups depending on the number of carbon atoms. The molecular weight of PHA is in the range of $2x10^5$ to $3x\ 10^6$ Da, depending on the producers and growth conditions [13].

Polyhydroxybutyrate production starts from the initial stage and varies in each stage of microbial growth. In some cases prolonged incubation will leads to the utilization of PHB as a stored carbon and energy in the presence of PHB depolymerase. Hence identification of maximum PHB producing growth stage was an unavoidable task. So this work was aimed to screen potential PHB producing microbes and its optimum of PHB producing time.

11. MATERIALS AND METHODS

A. Collection of samples

Samples were collected from different ecological niches, mainly from the surface of waste canals, polluted soil, garden soil and tap water in and around Thiruvananthapuram Districtof Kerala State. The samples were collected in sterile polythene bags at a depth of about 8 cm using a grab. Around 1.0g or 1ml of sample was serially

diluted in sterile distilled water and plated onto nutrient agar plates and incubated at 30^0C for 24 hours. The bacterial colonies with different morphologies were selected and sub cultured 3-4 times on nutrient agar plates.

B. Relation between growth and PHB production

The relation between growth and PHB production by the strains were assessed by measuring cell and PHB dry weight at regular intervals (12 hours) and plotted against the days of incubation

C. Extraction of PHB

PHBs were recovered from the cell mass by sodium hypochlorate method. Selected cultures were grown in 250 ml Erlenmeyer flasks containing 100ml of the medium. A simplified media containing 1.0% (w/v) glucose, 0.5% (w/v) peptone, and 0.25% (w/v) NaCl was used for the production [15]. The cultures were incubated in a shaker (JSR Research INC, Korea) at 37°C for 48hrs at 150rpm. After incubation, the cultures were taken and centrifuged at 4000rpm for 30min. The supernatant was discarded and the pellet was treated with sodium hypochlorate (100%) equal to the original volume of the medium and the mixture was incubated at 37°C for 1hr. The incubation mixture was subjected to centrifugation at 4000rpm for 30min. Then the pellet was washed with distilled water, acetone and alcohol respectively. The pellets were dissolved in 5ml of chloroform which was then evaporated by pouring the mixture on to sterile glass trays [14].

C. Cell dry weight meassurement

Selected cultures were grown in 250 ml Erlenmeyer flasks containing 100ml of the medium. A simplified media containing 1.0% (w/v) glucose, 0.5% (w/v) peptone, and 0.25% (w/v) NaCl was used for the production of PHB. The cultures were incubated in a shaker at 37°C for 48hrs at 150rpm. After incubation, these cultures were taken and centrifuged at 4000rpm for 30min. The supernatant was discarded and the pellet was dried until a constant weight [15].

D. Statistical analysis

Relation between growth and PHB production were carried out in triplicates. The results were an average of triplicate experimentsanalyzed by single factor analysis of variance (ANOVA) with replication and statistical analysis was done using the computer software SPSS 11.0 and Microsoft excel 2007.

III. RESULTS AND DISCUSSIONS

Microorganisms were isolated from different regions like polluted water, polluted soil, garden soil and tap water in which high microbial diversity was showed by polluted soil of Chackai and polluted water of Medical College. Origin and description of the samples collected from Thiruvananthapuram District of Kerala State were given in Table: 1.

Sampling stations	**Sampling sites**	**Mode of collection**	**Nile Blue A +ve strains**	**Name of the organism**
Medical College	Waste canal	Grab	KUB 3 & KUB 5	*Azotobacter*and *Ralstonia*
Veli	Waste canal	Grab	Nil	-----
Poovar	Polluted soil	Grab	KUB 15	*Pseudomonas*
Chackai	Polluted soil	Grab	KUB 18 & KUB 20	*Bacillus* and *Streptomyces*
Karyavattom	Garden soil	Grab	Nil	----
Palode	Garden soil	Grab	KUB 27	*Halobacterium*
Kazhakootam	Tap water	Grab	Nil	-------

A. Growth and PHB production by Azotobactersp.

The data clearly showed that the strain*Azotobacter* sp. isolated from Medical college attained maximum growth and PHB production at 48 hours of incubation and was 25% of cell dry weight (Fig: 1). *Azotobacter sp.*showed stationary phase from 48 to 60 hours of growth. Cell dry weight attained during this time was 2.8g/l. At the end of stationary

phase, the organism showed a sharp decrease in PHB production and constant cell growth, suggesting that the PHB accumulated during the early phases was possibly utilized for the growth and spore formation. The strain showed cell growth upto 108 hours and a gradual reduction in cell growth after 60 hours.

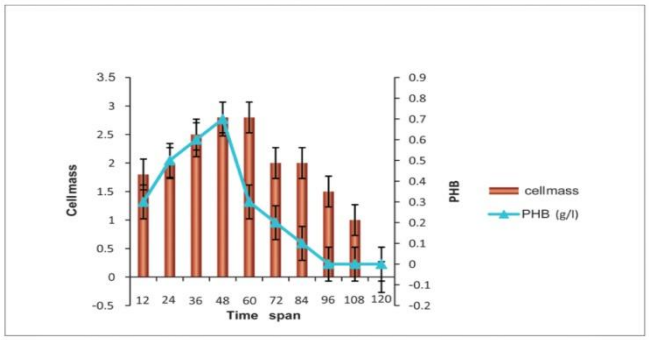

Fig: 1) Growth and PHB production by *Azotobacter* sp.

B. Growth and PHB production by Ralstonia sp.

Ralstonia sp. isolated from Medical college started PHB production from the initial stage of growth and showed upto at 96 hours but cell growth showed till 108 hours. Optimum amount of PHB produced by the organism was 0.8g/l from 2.5g/l of cell mass at the initial stage of stationary phase (Fig: 2). The organism showed a long stationary phase of growth from 48 to72 hours, and utilizes PHB for its sustainable growth during the stationary phase.

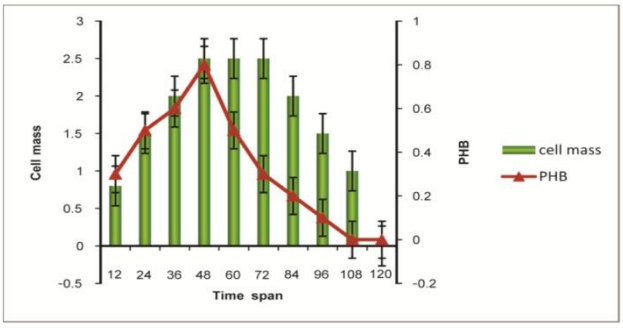

Fig: 2) Growth and PHB production by *Ralstonia*sp.

C. Growth and PHB production by Pseudomonas sp.

It was observed that *Pseudomonas sp.*, isolated from Poovargrown in minimal medium and started accumulating PHB at 12 hours and optimum amount of PHB accumulatedduring the early stationary phase and after 48 hours there was a gradual reduction in the amount of PHB (Fig: 3). Optimum amount of PHB produced by the organism was 0.3g/l from 1.8g/lof cell mass at 48 hours. At 60 and 72 hours *Pseudomonas sp.* showed same quantity (1.5g/l) of cell dry weight but the quantity of PHB produced shows reduction. During this period the organism utilizes PHB for its growth and spore formation. The organism shows PHB production upto 96 hours and cell growth upto108 hours.

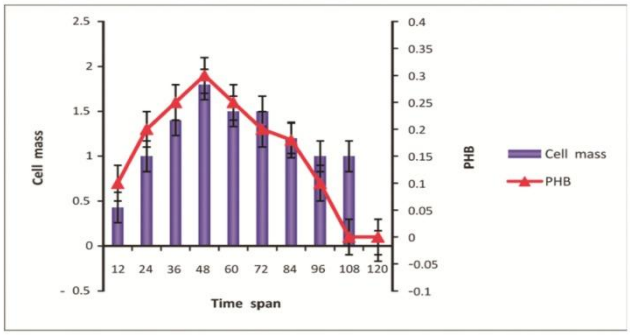

Fig: 3) Growth and PHB production by *Pseudomonas* sp.

D. Growth and PHB production by Bacillus sp.

The strain *Bacillus* sp. isolated from Chackai showed the production of PHB from 12 hours to 96 hours of incubation time (Fig: 4). Although the dry cell weight increased from 12 hours (1.2 g/l) and attained stationaryperiod at 48 hours (3.5 g/l) (Fig: 5). The organism showed a long stationary phase from 48hours to 72hours and maximum PHB production was showed only during the early stage of stationary phase. This indicates that the bacteria started the utilization of PHB as a source of carbon and nitrogen during the stationary stage. After 72 hours *Bacillus sp* showed a gradual reduction in cell growth but the organism showed cell growth upto 108 hours.

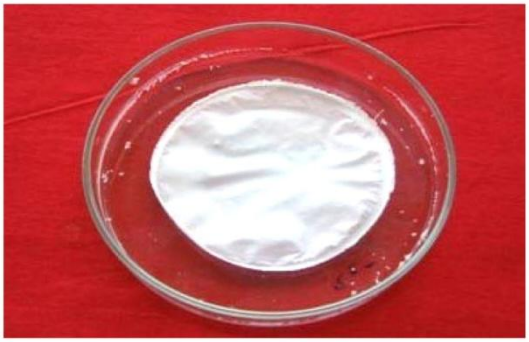

Fig: 4) PHB extracted from *Bacillus*sp

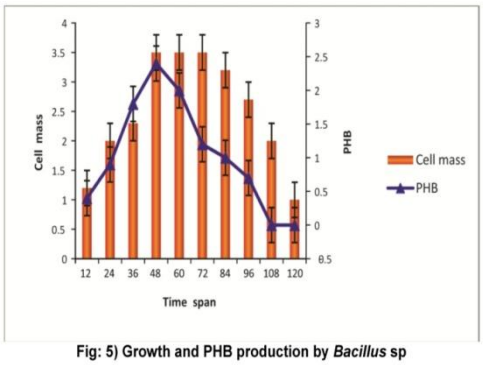

Fig: 5) Growth and PHB production by *Bacillus* sp

E. Growth and PHB production by Streptomyces sp.

The production of PHB in *Streptomyces* isolated from Chackai was observed to be maximum at48hours (0.7g/l) and further cultivation showed reduction in the PHB production (Fig:6).The organism showed PHB production upto 84 hours and cell growth upto108 hours. *Streptomyces sp.* showed stationary phase from 60hours to 72hours.

During the early stage of stationary phase, there was a gradual reduction in the amount of PHB production. Optimum biomass content was 2.8g/l. At 60 and 72 hours showed same quantity (2.6g/l) of cell dry weight and after 72 hours cell growth was reduced.

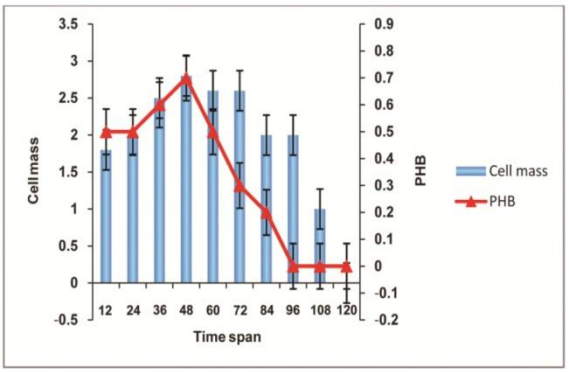

Fig: 6) Growth and PHB production by *Streptomyces* sp.

F. Growth and PHB production by Halobacterium sp.

*Halobacterium*isolated from Palodeshowed a very short life span. PHB production started from the early stage of growth (Fig: 7) and maximum PHB content (0.7g/l) from 2.8g/l of biomass was observed at 36 hours (4.3%) of incubation, further cultivation stopped PHB production. At 12 and 24 hours PHB production was constant but biomass production showed a slight increase till 36 hours and stopped at the 48 hours. Incubation after 36 hours will lead to the utilization of PHB for the growth of the organism so PHB production after 36 hours was reduced.

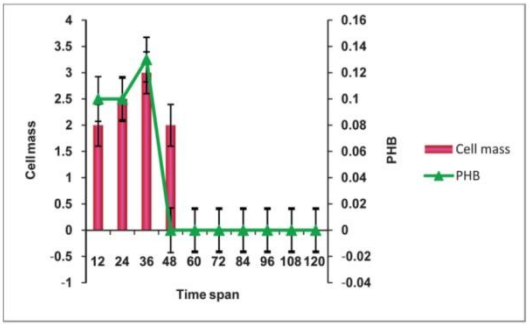

Fig: 7) Growth and PHB production by *Halobacterium* sp.

Time of PHB extraction is an important factor in industrial production of PHAs. Incubation period for PHB production depends on the characteristics of the strain and growth rate. The PHB production in the present study commenced from the initial stage of incubation and reached maximum at 48th hour except *Halobacterium* sp. In this study, yield increases with time dependent manner and highest yield of the selected five strains (*Azotobacter*, *Ralstonia*, *Pseudomonas*, *Bacillus* and *Halobacterium*) was its initial stage of stationary phase except *Streptomyces*. Incubation after 48 hours increases the unfavorable conditions like viscosity of the medium accompany the production of exopolysaccharides resulting in oxygen transfer limitation, caused the decrease in PHB synthesis and utilization of PHB by the bacteria[16], [17],[21]. It was reported that the maximum accumulation of PHAs was during exponential phase in *Rhodopseudomonaspalustris* KU003 [22], *Rhodobactersphaeroides* ES 16 [23]. In *Ralstoniaeutropha* maximum PHB accumulation was occur at the stationary phase [7]. The incubation period up to 28 hours favored PHA accumulation in *Bacillus sphaericus* NCIM 5149, and after that the cell started to utilize the accumulated PHAs [24].

*Bacillus megaterium*strain isolated from municipal sewage sludge yielded a maximum of

62.43% of cell dry weight of polymer in the medium with glycerol as carbon source [25]. In this organism the cell mass increased steadily, attained a maximum yield in the 24 hours of cultivation and then declined slowly, following a short stationary phase. On comparing the PHB yield and growth for five consecutive days, it was observed that time of incubation influence both PHB production and growth. All the bacterial strains, except *Streptomyces*, could be categorized in a single group based on stage of PHB content production. All the strains showed reduction in the accumulation of PHB content on prolonged incubation. The isolates showed maximum amount of PHB production at 48 hours of incubation. Further increase in the incubation time reduces the PHB content in the cell suggesting that the accumulated PHB was possible utilized for growth and spore formation [26]. These findings were also consistent with the previous report[27]. They observed maximal production of PHA at 24 hours (48 in case of *Bacillus megatherium*) and the amount of accumulated PHA decreased on further incubation in fermentation employing various *Bacillus* sp. It is therefore concluded that, PHB production probably does not only serve as a storage material, but also as a mechanism to cope up with stressed and nutrient environmental conditions.

IV. CONCLUSION

Quantitative evaluation of PHB production of the six strains (*Azotobacter*, *Ralstonia*, *Pseudomonas*, *Bacillus*, *Streptomyces* and *Halobacterium*) showed that the maximum accumulation of PHB was at its stationary phase of growth except *Streptomyces*. The *Bacillus sp*isolated from polluted soil of Chackai showed 68% yield at the end of 48 hours. High cell density and PHB production of *Bacillus sp*indicated that, was the most suitable strain for large scale production of PHB from amongst the six strains.

ACKNOWLEDGEMENT

Financial assistance from DST - PURSE programme, Government of India, New Delhi is gratefully acknowledged.

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