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Production and Optimization of PHB by *Bacillus megaterium* And *Azospirillum spp*

Kannahi M* and Rajalakshmi M

P.G and Research Department of Microbiology, Sengamala Thayaar Educational Trust Women's College, Mannargudi-614 001, Tiruvarur (Dt), Tamil Nadu, India.

*Corresponding Author: E-Mail: kannahisri79@gmail.com

ABSTRACT

In the present study, bacterial species were isolated from the soil sample. Among the bacterial isolates, *Bacillus megaterium* and *Azospirillum spp* were identified based on the cultural, morphological and biochemical characteristics. PHB production of *Bacillus megaterium* and *Azospirillum spp* were screened by slide and plate method using the sudan black B staining. The isolated organism was grown in fermented medium. Among this study, high amount of PHB obtained from *Azospirillum spp* compared with *Bacillus megaterium*. The bacterial isolates were harvested and bioplastic prepared by using sorbital, gelatin and glycerol compound. In addition, chemical based commercial plastic was prepared and compared with bioplastic for plasticizing and moldable property. The PHB production was optimized using different parameters such as carbon, nitrogen sources, various pH and temperature. Finally concluded that, high amount of PHB was obtained from *Azospirillum spp* compared with *Bacillus megaterium.*

Key words: *Bacillus megaterium, Azospirillum spp,* Sudan black B staining.

1. INTRODUCTION

Bioplastics or organic plastics are a form of plastics derived from renewable biomass sources such as vegetable oil, corn starch, and pea starch unlike fossil-fuel plastics derived from petroleum. Bioplastics provide the twin advantages of conservation of fossil resources and reduction in carbon dioxide emissions, which make them an important innovation of sustainable development. Bacteria serve as an excellent feedstock for plastic production owing to its many advantages such as high yield and the ability to grow in a range of environment. PHB was first identified by Lemoigne in 1926 as a reserve material of *Bacillus megaterium*[1]. PHB is a very common and widespread storage material in many micro-organisms. PHB is an environmentally degradable material [2]. PHB belongs to a family of polyesters called poly hydroxy alkanoates or PHAs. In general, these molecules can be produced with properties resembling their non renewable plastic counterparts, specifically polypropylene. The one striking difference between PHB and petroleum-based plastics is that PHB are biodegradable. When these plastics are disposed of in environments populated by organisms such as bacteria, fungi and algae, PHB are broken down to their essence-carbon dioxide and water recycled by the natural metabolic processes of these microbes[3].

2. MATERIALS AND METHODS

2.1. Sample Collection

Paddy Soil sample were collected from Thiruthuraipoondi, Thiruvarur (Dt), Tamil Nadu Soil was taken from 60 cm depth and stored in the Polythene bag and the atmospheric temperature was maintained.

2.2. Screening of PHB

The production of PHB by the bacteria can be confirmed by staining with sudan black method. Both slide and plate methods were performed [4].

2.2.1. Slide method

The culture heat fixed on a slide and immersed in 0.5% (w/w) sudan black B staining with ethylene glycol for 5min. Then the slide was air dried, the excess amout of stain was destained using xylene several times and blot dried with absorbent paper. Then the counter stain (0.5% w/v saffranin) was added for 5 to 10 seconds. The slide was washed with tap water and dried. The stained cells were observed under oil immersion microscope.

2.2.2. Plate method

Nitrogen limited agar plates were prepared and the test culture was inoculated by using spread plate method. The plates were

incubated at 37ºC for 48 to 72 hrs. After incubation, the plates were flooded with ethanol solution containing sudan black B for 20min. Later, the solution was flooded with ethanol solution containing sudan black B for 20min. Later the solution was drained off and observed for screening of PHB producers.

2.3. Extraction of PHB

PHB was extracted from the fermentation medium. After incubation, 10ml of culture was centrifuged at 4000rpm for 35 min. The supernatant was discarded. The pellet was treated with 10 ml of sodium hypo chloride and the mixture was centrifuged at 4000rpm for 20min and then washed with distilled water, acetone, methanol respectively for washing and extraction. After that, centrifugation process was carried out. The pellet was recompensed in 5 ml of chloroform and evaporated by pouring the solution on sterile tray and kept in hot air oven at 4^{0}C. After evaporation, the final product obtained as PHB in powder form will be 99% pure [5].

2.4. Preparation of Bioplastic

Simple laboratory procedures are available to prepare bioplastis by using simple chemicals. The following combinations produce bioplastic with different properties. They were including under the type of Gelatin- bacteria blend type plastic according to ingredients. Because various types of bioplastic available based on ingredients. The bioplastic ingredients include, Sorbitol 2.25g (3/4cup), Gelatin 2.25g(3/4cup), bacteria 2.25g(3/4 cup), Glycerol solution 180ml (3/4cup) of 1% glycerol solution, Water 240ml (1cup).

All the ingredients were mixed together in the amounts above, and stirred well. It was vortexed without clump formation. The mixture was heated upto 95^{0}C, at the same time it was stirred. The heating was stopped and it was stirred. Excess broth was scooped out using a spoon and make sure are no clumps. The mixture was poured carefully into a dried pan and it was allowed to dry. The time required for separation of plastic from pan is depend on temperature and humidity of the room, it may take several days. The plastic is won't be removed from the plastic sheet easily until it is completely dry. After complete drying, the bioplastic separated from the pan and allowed for further analysis.

2.5. Preparation of commercial plastic

The commercial petrol based plastic was also prepared by the same ingredients followed for preparation of bioplastic without the addition of bacteria. There is no biopolymer was added in the commercial plastic. Hence, the chemical polymer concentration was high. The ingredients for commercial plastic include Sorbitol 3.37g. Gelatin 3.37g. Glycerol solution 180ml and Water 240ml. The commercial chemical plastic also prepared by using the same bioplastic procedures was followed above. The bioplastic and commercial petrol based plastic was compared with one another. The plasticizing capacity, moldable property of bioplastic and commercial plastic was analyzed. The ingredient used in preparation of plastic and bioplastic was compared for biodegradable property and cost required of production.

2.6. Assay of PHB

The chloroform containing PHB was treated with 5 ml concentration sulphuric acid and boiled at 100^{0}C for 10 min. The different concentration of crotonic acid was prepared with concentration sulphuric acid and boiled using water bath. The optical density of crotonic acid was read at 230nm and the standard graph was prepared. The optical density of sample was read at 230 nm using UV spectrophotometer. The concentration of PHB was determined by plotting the OD with the standard graph of crotonic acid [6].

2.7. Effect of pH and temperature for PHB production

The fermentation medium were prepared at various pH level, (5, 6, 7, 8 and 9) adjusted by HCL and NaOH and test organisms were inoculated in each test tubes, and incubated for 4 days. At the same time, different temperature used for the production of PHB was 25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C and 45^{0}C.

2.8. Effect of carbon and nitrogen sources on PHB production

The production of PHB was analyzed in two different carbon and nitrogen sources separately for the each organism. The test organisms were inoculated in a fermentation medium at various carbon sources such as Sucrose, Maltose, and Nitrogen sources such as Ammonium sulfate, potassium nitrite and Glycine. All medium were incubated for 4 days for PHB production.

3. STATISTICAL ANALYSIS

The correlation between cell dry weight (g L $^{-1}$) and PHB production (g L^{-1}) is determined according to Sperman's ρ correlation coefficient test. The ρ value is estimated with the formula [7] .

$$\rho = 1 - \frac{6\sum(X1 - Y1)^2}{n(n^2 - 1)}$$

4. RESULT AND DISCUSSION

PHB productions were screened in *Bacillus megaterium and Azospirillum spp* by plate and slide methods using Sudan black staining. In the plate method blue blackish coloured granules, noted in the cytoplasm. In the slide method, blue coloured granules were observed. The PHB is a biocompatible, biodegradable, thermoplastic and hydrophobic material consisting of building blocks of 3- hydroxybutyrate. Because of the high degree of polymerization, it has a high molecular mass and a high degree of crystallinity (55-75%), exhibits good chemical resistance, and its barrier properties are of interest for practical packaging application. Many of the physical and mechanical characteristics of PHB are similar to those of polypropylene (PP) [8]. High amount of PHB was obtained from *Azospirillum spp* compared with *Bacillus megaterium.* Hence, PHB production of *Azospirillum spp* was optimized using different parameters such as carbon, nitrogen and various pH and temperature. Among this study, maximum PHB production was noted in pH 7 and temperature 35^{0}C compared with other ranges. Maximum PHB production was noted in sucrose incorporated fermentative medium when compared with mannitol and maltose. Highest PHB production was observed in potassium nitrate when compared with ammonium sulfate and glycine.

Bacterial plastic was prepared by using *Bacillus megaterium* and *Azospirillum spp* as biopolymer in 30% concentration, 70% of chemical polymer (sorbitol and gelatin) and glycerol as plasticizer. The commercial plastic and bacterial plastic was prepared by using different ingredients with different concentration. Commercial plastic was prepared by using commercial synthetic polymer such as sorbitol, gelatin and glycerol used as plasticizer. The PHB content of the bacteria was demonstrated by UV spectrophotometer method by crotonic acid conversion[9]. The ultraviolet absorption spectrum of crotonic acid derived from PHB. The PHB purified from the bacteria was compared with the spectrum of commercial crotonic acid, they were found to be identical.

Table-1: PHB production of *Azospirillum spp* on media with different carbon sources

Carbon Sources	Dry cell weight (g/L)	PHB Production (g/L)	Yield of PHB(g/L)
Maltose	0.15 ± 0.04	0.0422 ± 0.01	0.28 ± 0.13
Mannitol	0.14 ± 0.02	0.0414 ± 0.01	0.29 ± 0.57
Sucrose	0.21 ± 0.02	0.0720 ± 0.02	0.34 ± 0.28

The bioplastic and commercial plastic was compared for moldable and plasticizing property. Here the two plastic had good moldable and plasticizing capacity was observed. Bacterial plastic had good moldable property. The synthetic polymers also have good moldable property[10] . Three different carbon sources used for PHB production. Highest PHB production was observed in sucrose (0.0720 g/L) compared than other carbon sources such as maltose (0.0422 g/L) and mannitol (0.0414 g/L).

Table-2 : PHB production of *Azospirillum spp* on media with different Nitrogen sources

Nitrogen Sources	Dry cell weight (g/L)	PHB Production (g/L)	Yield of PHB (g/L)
Ammonium sulfate	0.15 ± 0.03	0.039 ± 0.01	0.26 ± 0.10
Glycine	0.13 ± 0.04	0.022 ± 0.01	0.16 ± 0.76
Potassium nitrate	0.21 ± 0.01	0.52 ± 0.01	0.24 ± 0.76

PHB production was analyzed in various Nitrogen sources such as Ammonium sulfate, potassium nitrate and Glycine. Highest PHB productivity was observed in potassium nitrate (0.052 g/L) compared than other Nitrogen sources such as ammonium sulfate (0.039 g/L) and glycine.

5. CONCLUSION

The present investigation showed that, high amount of PHB was obtained from *Azospirillum spp* compared with *Bacillus megaterium.* The two bacterial isolates are highly recommended for commercial production of PHB. It is worth noting that biodegradable plastics cannot yet compete with traditional plastics because of their higher production cost. Therefore, the reduction of PHB production costs is presently one of the major topics in biopolymer research.

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