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Research Article

Comparative Study of Polyhydroxy Butyrate Granules Production by *AZOTOBACTER CHROCOCCUM* and *Rhizobium SPP.*

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ABSTRACT

Poly-β-hydroxy-butyrate (PHB) is a biodegradable polymer or biocompatible thermoplastic was produced by *Azotobacter chrococcum,Rhizobium spp.*. The accumulation of PHB granule in the bacterial cell was significantly depended on the ratio of carbon source and nitrogen source in medium.

The production was carried out by using crude sugars mannitol and sucrose that showed maximum polyhydroxy butyrate production in most of the references. This amount of production was compared with the production using crude carbon sources *Ficus racemosa* and molasses. Maximum production was observed at pH 7, temperature 30^0C and 48 hours of incubation on *Ficus racemosa* and then on mannitol by *Azotobacter*, and in case of *Rhizobium*, maximum production was observed at pH 5, temperature 30^0C and 48hrs of incubation on *Ficus racemosa* and then on mannitol. The results clearly indicated that crude carbon sources gives better production as compared to production with pure sugars.

Keywords: Poly-β-hydroxy-butyrate (PHB), *Azotobacter chrococcum,Rhizobium spp*.and *Ficus racemosa*, mannitol.

INTRODUCTION

Polyhydroxyalkanoates (PHA) are biological polymers produced by a large selection of bacteria as intracellular inclusions usually accumulated when in starvation of phosphate or nitrogen source and excess of carbon source. PHA compounds are biodegradable when compared to synthetic plastics and are produced from renewable resources. A number of bacteria such as *Azotobacter, Bacillus, Archaebacteria, Methylobacteria, Pseudomonas, Rhizobium japonicum, Rhizobium cicer* have been found to synthesize PHA to varying levels. PHB synthesis depends on a number of conditions including the nature of carbon and nitrogen sources utilized, on their concentration ratio in the medium, on partial oxygen pressure and so on. Nitrogen deficiency or the excess of organic substrates results in PHB accumulation. PHB is the alternative source of the plastics which has

similar physical properties like polypropylene and it can be easily biodegradable aerobically and anaerobically. *Azotobacter chrococcum* is a gram negative free living nitrogen fixing microorganism.

Rhizobium spp. are well known group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. *Ficus racemosa* is also called Indian fig or cluster fig that belongs to *Moraceae* family. Molasses is a thick brown waste substance that is obtained in the process of sugar synthesis from cane juice [1]

MATERIALS AND METHODS

- Isolation of strains

The *Azotobacter chrococcum* was isolated from fields of Udgir region by using Ashby's medium; *Rhizobium spp* was isolated from soybean plant of variety JS335 in fields of Parbhani region on yeast extract mannitol agar with congo red. Both the strains were biochemically characterized according to tests mentioned in Bergey's manual of systematic bacteriology [2]

- **PHB Production**

Medium for PHB production by *Azotobacter chrococcum* includes Ashby's medium at pH7, temperature 30^0C for 48hrs. Medium for PHB production by *Rhizobium spp*. includes Yeast extract mannitol broth at pH5, temperature 30^0C for 48hrs. For production with crude sources, mannitol was replaced by 3% *Ficus racemosa* and molasses separately [3]

- PHB Extraction and Confirmation

After completion of incubation, PHB was extracted using the dispersion method of sodium hypochlorite and chloroform. Cells were collected by centrifugation at 10,000 rpm for 15min at room temperature. Pellet was washed with phosphate buffered saline (pH 7.4).

Cell pellets were air dried for 2 hours and their weights were taken. Chloroform and 4% sodium hypochlorite were added to the cell pellet in a ratio of 12.5µL chloroform to 12.5µL 4% sodium hypochlorite per mg of pellet weight. The mixture was kept at 30^oC overnight. The dispersion was then centrifuged at 8,000 rpm for 10 minutes at room temperature resulting in the formation of different phases.

The bottom phase of chloroform contains PHB. This phase was transferred to another fresh tube and its volume was measured. 5x volumes of a mixture of methanol and water (7:3 v/v) were added to the chloroform solution.

The mixture was centrifuged at 10,000 rpm for 15 minutes resulting in the formation of a precipitate of PHB. The amount of PHB present was quantified by determining the weight of precipitate obtained. (Priya Kumari and Harish Kumar Dhingra). Addition of concentrated sulfuric acid to this pellet converts PHB into crotonic acid which can be detected by UV-VIS spectrophotometer at wavelength 235nm. Further Fourier Transform Infrared Spectroscopy (FTIR) was also performed [4,5]

The product yield was calculated on the basis of amount of PHB obtained with respect to percent carbon source supplied in production medium.

- **Biodegradation of PHB**

The PHB degradation ability was determined by a soil isolate of *Aspergillus niger* inoculated in Asthana and Hawkers medium supplemented with 0.04 % of PHB produced by and incubated on rotatory shaker for 7 days at 30^0C. (Ramchander Merugu *et al*). And a soil isolate of *Bacillus* was inoculated in minimal medium with 0.5 % PHB, and incubated on rotatory shaker for 7 days at 30^0C [6].

OBSERVATION

Table-1: PHB Production

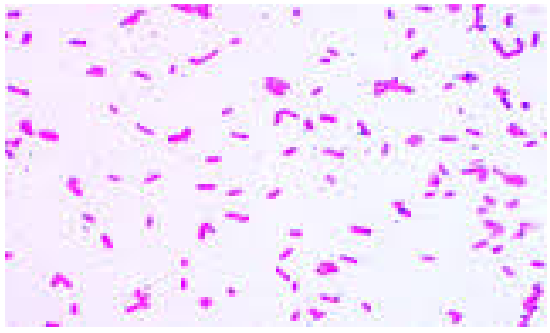
	With pure sugars	With *Ficus racemosa*	With Molasses
PHB produced by *Azotobacter chroccoum* (%)	75	81	78
PHB produced by *Rhizobium spp.* (%)	70	79	76

Table-2: PHB Degradation

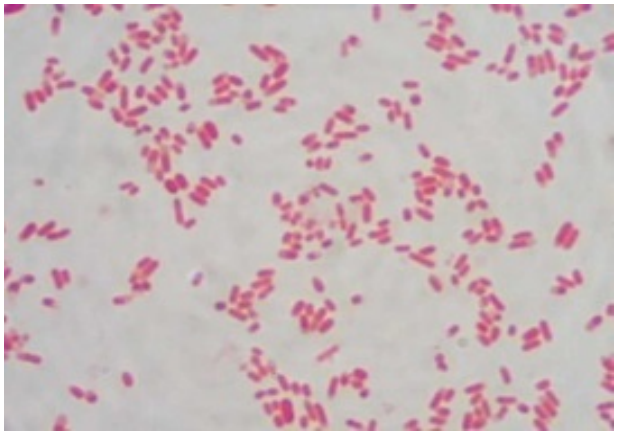
No. of days	O.D (400nm)
1	0
2	0.02
3	0.2
4	0.35
5	0.5
6	0.68
7	1.5

No. of days	Wt.of fungal biomass (gm)
1	0
2	0
3	0.12
4	0.4
5	0.56
6	0.64
7	0.8

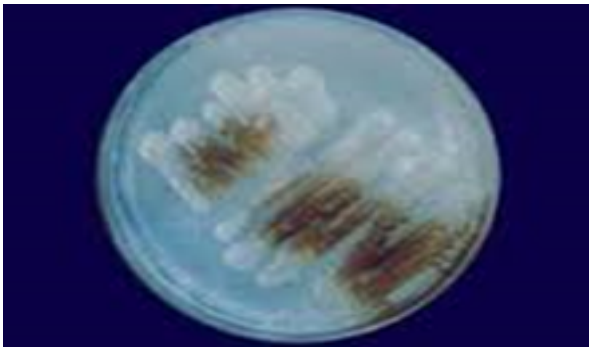
PHOTOGRAPHS



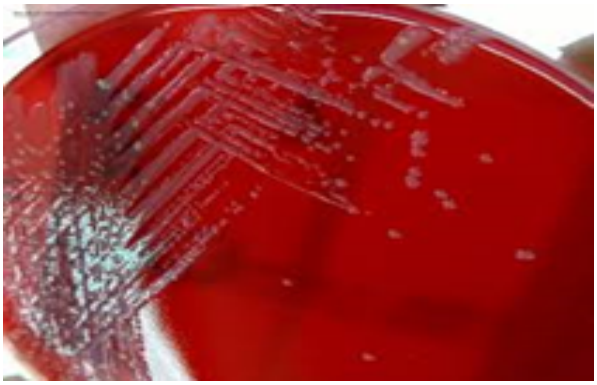
Azotobacter Chrococcum



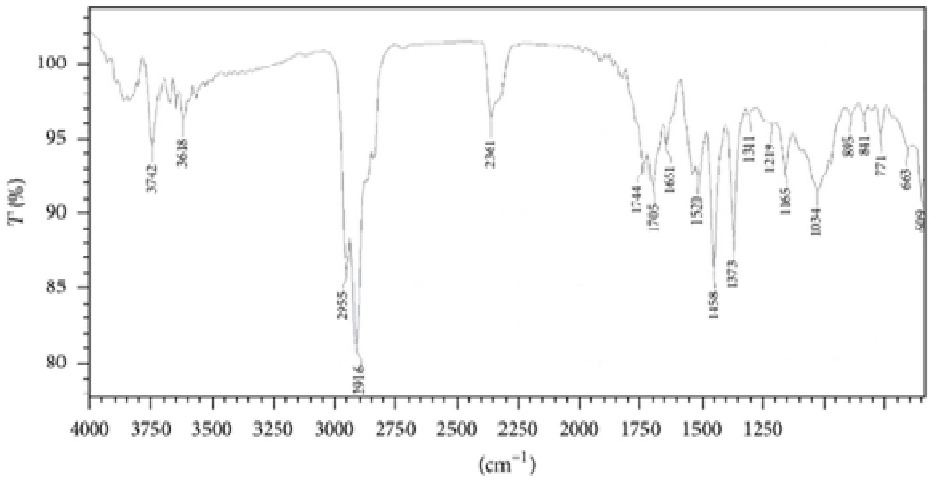
Rhizobium spp.



Azotobacter Chrococcum



Rhizobium spp.



FTIRAnalysis

RESULTS AND DISCUSSION

From the biochemical analysis it was confirmed that the isolates were *Azotobacter chrococcum* and *Rhizobium spp*. The *Azotobacter chrococcum* was cultivated in Ashbey's medium and modified Ashbey's medium(3%*Ficus racemosa* and Molasses instead of mannitol) at 30^0C, pH7 for 48 hrs. The *Rhizobium spp*. was cultivated on yeast extract mannitol broth and modified Yeast extract mannitol broth (3%*Ficus racemosa* and Molasses instead of mannitol) at 30^0C, pH5 for 48hrs. After incubation, the PHB was extracted. Maximum production was observed by *Azotobacter chrococcum* that is 75% with pure mannitol sugar, 81% with *Ficus racemosa* and 78% with Molasses. *Rhizobium* secures the second position with production value 70% with pure mannitol sugar, 79% with *Ficus racemosa* and 76% with Molasses. The produced PHB was confirmed with FTIR analysis. The FTIR(Fourier Transform Infrared Spectroscopy) analysis of isolated polymer was performed in the range 4000–600 cm^{-1}. IR spectra showed two intense absorption band at 1705 and 1034 cm^{-1}, specific for C=O and C–O stretching vibrations, respectively. The absorption bands at 2916 and 2955 cm^{-1} are due to C–H stretching vibrations of methyl, methylene groups. These prominent absorption bands confirm the structure of poly-β-hydroxybutyrate [6].

The biodegradation study has also been carried out by using a fungal strain *Aspergillus niger* and a bacterial strain *Bacillus* isolated from soil. For growth of fungus, a carbon source is essentially required which supports in its growth in the form of fungal biomass production. The medium used here, Asthana and Hawkers medium lacks in any substance which could be used as carbon source. When this medium was supplemented with 0.04% PHB, it was the sole carbon source in the medium. In fungal degradation, a gradual increase in fungal biomass was observed, which clearly indicates utilization of PHB as carbon source. In case of bacterial degradation, a continuous increase in turbidity of medium is observed which reflects growth of *Bacillus* in the medium by utilizing PHB as carbon source, as minimal medium does not contain any carbon source. Both results indicate biodegradation of PHB as it was the sole carbon source in the medium. This simply proves that the produced polymer is completely biodegradable in nature (6).

SUMMARY AND CONCLUSION

The main aim of present study is to make an attempt to popularize the use of *Azotobacter chroccum* and *Rhizobium* strains for PHB production, which is generally ignored in front of *Alcaligens eutrophus, Bacillus subtilis, Bacillus megaterium* and *Azotobacter vinelandii*. The present study has successfully demonstrated that crude carbon serve best for PHB production because it will not only increase the overall yield of the product, but also reduce the cost of production upto considerable level. The study also experimentally proves that the produced polymer is completely biodegradable in nature by both bacteria as well as fungi.

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