

Effect of carbon sources in the production of polyhydroxybutyrate(PHB) by *Bradyrhizobium* and *Rhizobium* sp. from *Aeschynomene indica*

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ABSTRACT: : In this study, poly-beta-hydroxybutyrate (PHB) producing *Bradyrhizobium* and *Rhizobium* sp. were isolated from root nodule of *Aeschynomene indica*. Six different carbon sources at three different concentration were tested and the PHB production was evaluated by submerged fermentation. Glucose, lactose, mannitol, glycerol, jaggery and pyruvate were used as carbon sources and peptone used as nitrogen source at 0.01%. Among them jaggery and pyruvate (2%) has shown maximum production of PHB (74%) in minimal medium whereas 68% on glucose and glycerol in LB media by *Bradyrhizobium* sp. *Rhizobium nepotum* has given maximum production of PHB (80%) at 2% jaggery and 78% by pyruvate in minimal medium MM. In LB media, pyruvate has given 62% of PHB production by *Rhizobium nepotum*. At 72 h, there was a decrease in PHB yields was observed in lactose, glycerol and mannitol. It was confirmed that isolate *Rhizobium nepotum* capable to produce PHB under limited nitrogen with high concentration of jaggery and pyruvate as simple and cheaper carbon source.

Key Words: Poly- β -hydroxybutyrate, *Bradyrhizobium*, *Rhizobium nepotum*, Different carbon and nitrogen sources

INTRODUCTION

In soil ecosystem, microorganism plays vital role in soil structure formation, recycling of minerals, decomposition of complex organic matter, modulating biogeochemical cycle. Thus, the entire organism in the biosphere directly or indirectly depends on microbial activities (Garbeva et al., 2004). Most of the bacteria isolated from root nodules are found to be good polyhydroxyalkanoates (PHA) producers. Polyhydroxybutyrate (PHB) is the most frequently isolated PHA and widely used for the production of bioplastic. Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic, there are a class of bacterial polyesters collectively called polyhydroxyalkanoates (PHAs), accumulated intra cellularly as reserve granules by many bacteria in harsh environmental conditions (Koller et al., 2008). PHB is primarily a product of carbon assimilation and is used by micro-organisms as a form of energy storage molecule. Many studies show that number of microorganisms like *Alcaligenes eutrophus*, *Azotobacter beijerinckia*, *Pseudomonas oleovorans*, *Rhizobium* sp., *Bradyrhizobium* etc., produce PHAs as reserve food material. PHBs can be produced intracellularly by many microbes such as *B. megaterium*, *R. eutrophus*, *Azotobacter* spp. (Khanna, et al., 2005; Kuniko et al., 1988; Anderson et al., 1990). Most of the bacteria which produce PHB are nitrogen-fixing microorganisms. These bacteria can accumulate more than 70% of PHB under nitrogen starvation with high carbon (Philip et al., 2007). Under limiting nitrogen substrate and in the presence of an abundant source of carbon, these bacteria can accumulate upto 60 to 80% of their weight as PHB (Anderson and Dawes, 1990). PHB degrade naturally and completely to CO₂ and H₂O under environment by different microorganisms (Holmes, 1985; Bonartseva et al., 1994; Dahi et al., 1995; Lee, 1996; Yu et al., 2000; ; Das et al., 2005). The higher cost of industrial production of PHB limits the commercial production of bioplastic. It's well understood that the cost of production of microbial product can significantly reduced by the optimization of low cost media (Philip et al., 2007). Therefore, it is necessary to isolate bacteria capable to complete the cost wise production as compared to synthetic polymer. The study focused on the production of poly- β -hydroxybutyrate (PHB) granules by strains isolated from different root nodules of leguminous plant samples. The poly- β -hydroxybutyrate (PHB) granules production was tested by using different sources of C at varying concentrations for synthesis of bioplastics by *Rhizobium* sp. and *Bradyrhizobium*.

MATERIALS AND METHODS

Isolation of microbial strains (Morita et al.,2001)

Root nodules producing *Aeschynomene indica* from Thanjavure district were collected. Pink colored healthy root nodules were selected from the roots of various leguminous plants and were washed under running tap water and then for 30 sec in 70% ethanol solution. They were treated carefully with 5% hydrogen peroxide for surface sterilization for 2 min and successively washed three times with sterile distilled water and used for serial dilution method. Bacterial colonies were isolated by pour plate method using YEMA plates containing Congo red. Colorless gummy colonies were selected and were sub cultured to obtain pure culture. Pure culture was maintained in agar slants.

Morphological examination of the isolates

Biochemical characters

The overnight grown cultures were identified by Grams staining and IMViC test. Only Gram negative isolates were selected for further studies.

Sudan black B staining

Isolates grown on yeast extract glucose medium and PHB producing bacteria was further confirmed using Sudan black B staining method (Schlegel et al., 1970) with some minor modifications. Smear was prepared followed by 24,36, 48 and 72 h and stained for 10 min with Sudan black solution, rinsed with water and counter stained with 0.5% safranin for 5 min and observed at 1000x magnification.

Media ingredients and culture condition

To determine the growth pattern of various isolates, cultures were grown in yeast extract mannitol media (YEM) broth (Mannitol 10 g/l, yeast extract- 0.5 g/l, K_2HPO_4 - 0.5 g/l, KH_2PO_4 - 0.5 g/l, $MgSO_4 \cdot 7H_2O$ - 0.2 g/l, NaCl- 0.1 g/l, $CaCl_2 \cdot 2H_2O$ - 0.06 g/l) at 30 and 37°C for a period of 66 h in a shaker incubator at 150 rpm. A sample of 1 ml was withdrawn at a constant time interval to check the optical density at 600 nm.

Effect of carbon source on PHB production

The effect of glucose, lactose, mannitol, glycerol, jaggery and pyruvate on production of PHB by the selected isolate were evaluated by incorporating 1,2,5% (w/v) of the sugars in the standard MM and LB at pH 7 on 37° C for 48 h and 150 rpm followed by dry biomass and extracted PHB weight measurements. The fermentation for cultivation was performed at a temperature of 35° C and an agitation rate of 200 rpm under aerobic conditions. The production of PHB was determined qualitatively and quantitatively as described above.

Quantitative analysis of PHB (Mahishi et al.,2003)

The pellet was collected by centrifugation at 10,000 rpm for 15 min, washed with water, acetone, ethanol respectively for washing and extraction. Finally polymer was dissolved in hot chloroform and kept for complete evaporation. Dry weight of extracted PHB was estimated as g/L. The percentage of intracellular PHB accumulation (Arnold et al., 1999).

PHB accumulation (%) = Dry weight of extracted PHB (g/ml) * 100/DCW (g/ml).

SEM and TEM analysis

SEM was used to find out the size and morphology of cells grown under different carbon source. PHB granules were detected using TEM microscopy.

Phylogenetic analysis.

For amplification of the *16SrRNA* genes, the forward primer fd1 (5' AGAGTTTGATCCTGG CTCAG 3') and rP2 (5' ACGGCTACCTTGTTA CGACTT 3') were used amplified near 1500 base pairs of *16SrRNA* from the isolates an initial denaturation at 95 for 3 minutes, 34 cycles of denaturation at 95 for 30 seconds, annealing at 55 for 30seconds, extentsion at 72 for 2 minutes and final extension at 72 for 15 minutes. PCR products were purified for sequencing. The sequence analyses of *16SrRNA* genes were performed by using the algorithm BLASTN and submitted to Genbank.

RESULTS AND DISCUSSION

Aeschynomene indica L collected and the herbarium was submitted at St Joseph College, Tiruchirappalli, Tamilnadu. The accession number of herbarium is SJCBO 2102.. Colonies of isolates were obtained on CRYEM agar medium after incubation at 30°C after 3 days and the CFU was $43X10^{-7}$. The majority of colonies were moderately large (5mm), round colonies as white, translucent mucoid with entire margin. pH of medium found to be changed from 7.0 to 6.0 around some colonies. Isolate were observed to be transient growers as colony become visible after 24 hr incubation produced pink colouration after 48h. Based on the

biochemical characters given in table 1 the genera of isolated strains are identified as *Rhizobium* sp, *Bacillus* sp and *Bradyrhizobium* sp. Microscopic view of isolate S1 shows to be Gram negative rod cells which are positive on catalase, oxidase, MR, ONPG and starch hydrolysis test. Isolated S2 is Gram negative pleomorphic mainly ovoid rods are positive on catalase, indole, VP, citrate and gelatin hydrolysis test. Isolate designated S3 is belongs to Grams variable rod showed positive results on catalase, oxidase, indole, MR, citrate and starch utilization test. Nodulation of *Aeschynomene indica* by *Blastobacter denitrificans* was reported by van Berkum et al. (2002). *Aeschynomene indica* is most frequently nodulated by *Bradyrhizobium japonicum* and *Bradyrhizobium lankii*. The occurrence of *Bacillus* species as endophytes has been reported from different plants such as pigeon pea, wheat, and soybean nodules (Keyser et al., 1982). Occurrence of Gram positive bacteria also reported in many studies

The effect of different carbon sources on the production of PHB by two different organisms was studied. Glucose, Lactose, Mannitol, Glycerol, Jaggery and Pyruvate were added at 1,2 and 5% to the growth medium. The amount of PHB and also the growth rate of the organisms after 48 h were determined. Figure 1 shows the percentage of PHB produced by *Bradyrhizobium* sp (S1) on different carbon sources in Luria broth and minimal media. As shown in Fig.1 the best carbon source for the maximum production of PHB (74%) was found to be minimal medium supplemented with jaggery and pyruvate as carbon source at 2%. In the presence of 5% glycerol, the percentage of PHB was found to be 60%. This was followed by mannitol (38% PHB) and lactose (34% PHB). Whereas in LB media, glucose, glycerol and pyruvate shows the maximum production of PHB (68%). This was followed by jaggery (48%), mannitol (44%) and lactose (40%). Figure 2 shows the percentage of PHB produced by *Rhizobium nepotum* (S2) on different carbon sources. Minimal medium with jaggery has given the maximum PHB production of 80% and this was followed by pyruvate and glucose. The SEM image reveals that the size of Rhizobium cells were 4.52X 1.52 μm long after 48 h with PHB accumulation was observed under TME (plate1) Lactose was found to be the poor carbon source as it produced only 38% of PHB. In LB, pyruvate was found to be the good source as it shows the PHB production and the poorest carbon source was found to be mannitol (32%). The sequence analysis of 16SrRNA gene of isolate S2 revealed that the isolate was 98 % similarity with *Rhizobium nepotum* (fig 3). It was noted that jaggery and pyruvate with peptone was best source for PHB accumulation and peptone in LB medium is not suitable for PHB accumulation. Page (1992) tested PHB production in a variety of commercially available complex nitrogen sources fish peptone, protease peptone, yeast extract, casitone, phytone and tryptone). It was found that complex nitrogen sources increased the yield of PHB produced by *Azotobacter vinelandii*. Mercan et al. (2002) reported the strains produced less PHB in yeast extract mannitol broth media with glucose, sucrose, arabinose are inefficient carbon for *Rhizobium* sp. It was observed that depending upon the sources of carbon utilized and limiting nitrogen influence selectively induced PHB accumulation in Rhizobial strains.

CONCLUSION

Low amount of PHB (34- 50%, w/w) was accumulated during fermentation with lactose and mannitol moderately among glycerol as the sole carbon source. Significantly more PHB ($\geq 70\%$, w/g) was produced with glucose, and pyruvate as sole carbon source.

Table 1: Colony and Biochemical properties of isolates from *Aeschynomene indica*

Colony morphology	Gram's Stain	Catalase	Oxidase	Indole	MR	VP	Citrate	ONPG	Gelatin	Starch
Whitish, semi-translucent, irregular form, raised with	negative rod	+	+	-	+	-	-	+	-	+
Pale pink, raised, opaque	negative, pleomorphic	+	-	+	-	+	+	-	+	-
Dark yellow	Variable, slender rod	+	+	+	+	-	+	-	-	+

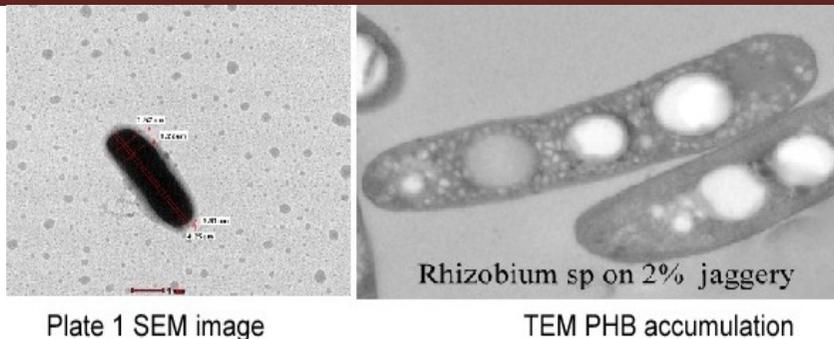


Fig 1 Percentage of PHB produced by *Bradyrhizobium* sp at 5% carbon sources

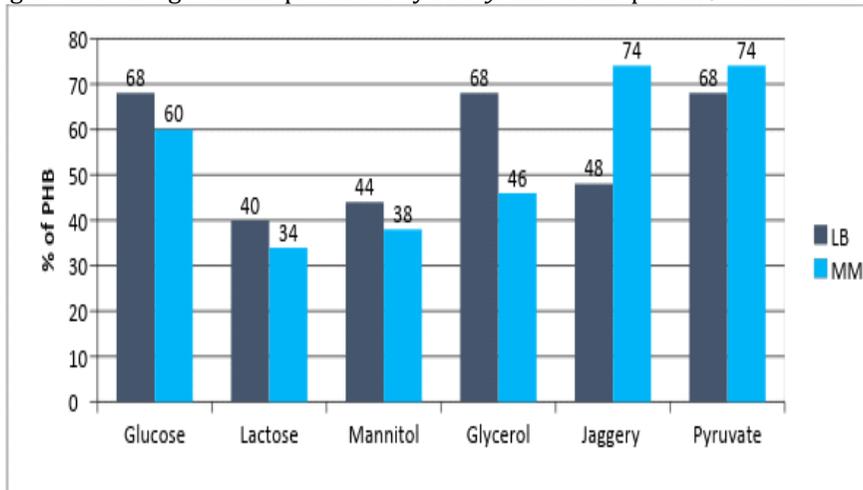
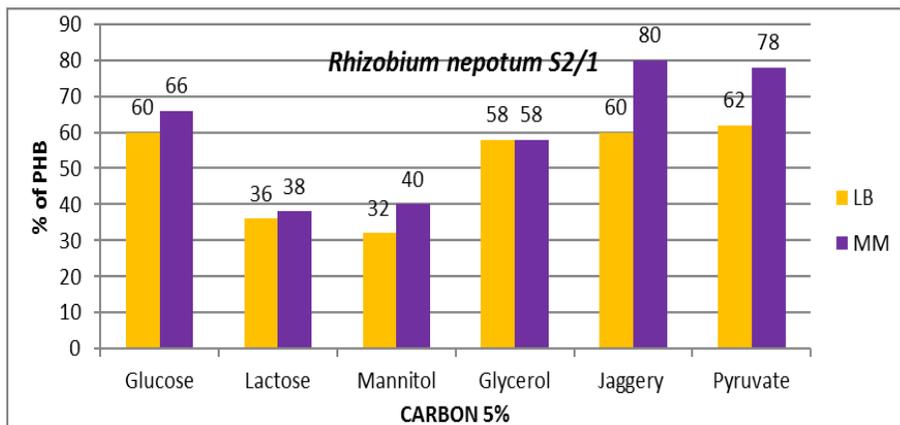


Fig.2 Percentage of PHB produced by *Rhizobium nepotum* at 5% carbon source



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