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Isolation of Nodule Bacteria from *Aeschynomene indica* and Screening of its Efficiency on PHB Production

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A B S T R A C T

The present research investigation was aimed to isolate nodule associated endophytic bacterium and to screen the PHB production efficiency among different carbon source. The *Aeschynomene indica* root qualitative phytochemical anlaysis reveals presence of all tested phytochemicals except cardiac glycoside.*two bacterial genera namely Rhizobium sp nand Blastbacter sp were* isolated and identified based on biochemical characters. Bot the isolated strains were solublize phosphate, produced Indole acetic acid and failed to produce siderophore. Both the isolated bacterial strains were PHB positive in nature. Among the tested bacterial strains maximum of 80% PHB was recovered from Blastobacter sp under five percentage Jaggery as carbon source. The phylogenetic tree reveals that the isolates was found to be *Blastobacter capsulatus.*

Introduction

Soil is considered as host of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space and plays an important role in soil processes that determine plant productivity (Tilak *et al*., 2005). The success of legumes is largely debted to their symbiotic relationship with specific nitrogen fixing bacteria known as rhizobia, a name that portray root and stem nodulating bacteria. Microorganisms collectively belongs to rhizobia are important plant-associated bacteria which have been studied extensively over the past few decades. Phylogenetic data place the rhizobia and Agrobacteria in the alpha-subdivision of the subclass Proteobacteria,and divide them into seven genera: *Rhizobium*, Agrobacterium, Sino*Rhizobium*, Meso*Rhizobium*, Allo*Rhizobium*, Azo*Rhizobium* and Brady*Rhizobium*. Members of the genera *Rhizobium* and Sino*Rhizobium* are significant because of their ability to nodulate the roots of leguminous plants and carry out nitrogen fxation (Keyser *et al*., 1982; Long, 1989). Most rhizobia are host specific and several different bacterial species are also isolated from a single legume species and it is only from limited hosts which have been examined as far as microsymbionts are concerned (Arora *et al*., 2001). These rhizobia are characterized into

two groups on the basis of growth rate. First group is fast grower rhizobia and second is slow grower rhizobia. The slow growing bacteria have mean generation time greater than 6 h and fast growing bacteria have less than 6 h in selective broth medium (Elkan, 1992). Unlike the genera *Rhizobium*, *SinoRhizobium*, *MesoRhizobium*, *BradyRhizobium*, *AlloRhizobium* and *AzoRhizobium*, members of the genus *Agrobacterium* are unable to nodulating the roots of leguminous plants, but instead are considered to be causing gall disease and root hair disease (Bradbury, 1986). Generally, the endophytic bacteria live inside the plant tissues and do not cause visible damage or morphological change on their hosts. They can benefit the host plants by the production of IAA (indoleacetic acid, phytohormones), siderophores, and antibiotic compounds, through nitrogen fixation, by phosphate solubilization, and with the suppression of phytopathogens through competition. Additionally, they may help the symbiotic rhizobia form nodules with non-specific hosts, further aiding plant growth (Liu *et al*., 2010). The occurrence of *Bacillus* species as endophytes has been reported from different plants such as pigeon pea,wheat, kudzu, and soybean nodules. In addition to plant growth stimulations the root nodule endophytes are also capable to produce Poly Hydroxy butyrate polymer (PHB) due to lack of citric acid cycle inside the host. PHAs being biodegradable and biocompatible have a promising future in medical related fields. However, their industrial production is well established, the cost of production is not competing to meet economic gain of industries. PHAs are among the most fascinating and largest groups of biopolyesters, with over 150 different types of monomer composition that provide different properties and functionalities (Hazer and Steinbüchel, 2007). They are thermoplastic or elastomeric polyesters (polyoxoesters) of R-hydroxyalkanoic acid (HA) monomers that are biosynthesized by wide range of Microorganisms. Poly (3-hydroxybutyrate), is the most abundant PHA in nature. However, the application of P(3HB) is limited by its high crystallinity and brittle nature (Sudesh *et al*., 2000). A variety of PHAs containing 3-hydroxybutyrate (3HB) and a second monomer, such as 3-hydroxypropionate (3HP), 4-hydroxybutyrate (4HB), 5-hydroxyvalerate (5HV), and 3-hydroxyhexanoate (3HHx), have been produced to improve material properties (Chuah *et al*., 2013; Fukui *et al*., 2009).

Materials and Method

Sample Collection and phytochemistry

Root nodule producing leguminous plants *Aeschynomene indica* was collected at Thanjure district and processed. Collected Plant sample herbarium was submitted at Botany department, Saint Joshep College, Tiruchirapalli for identification.

Preparation of extract

The collected plant roots were washed; the bark was peeled off and then dried under shade. The coarse powder of the roots (76.712g) was soaked in one Liter of 50% ethyl alcohol and extracted in the cold for 3 days with occasional shaking. The solvent from the total extract was filtered & concentrated on water bath for 8 hrs. The remaining was used for the analysis of Phytochemical test.

Isolation and Identification of nodulating bacteria (Zhang *et al.*, 1996)

Endophytes and epiphytes were isolated from root nodules of collected plant.

Nodules were taken from freshly uprooted plants. Roots of the plant were thoroughly washed under tap water to remove the mud and soil particles.

Healthy and pink nodules were selected for the isolation of nodule-associated bacteria (NAB). Nodules were safely cut from the root and were washed under running tap water and then for 30 sec in 70% ethanol solution. They were then treated with 0.1% $HgCl_2$ for 2 min and successively washed three times with sterile distilled water under aseptic condition for 1 min each. The nodules were put in 1.5 mL microfuge tubes containing 0.5 mL N-saline. Then the nodules were crushed with the help of sterile forceps and the 100 µL contents were spread on YEMA plate. All the plates were incubated at 28 ± 2°C for 5 days. Colonies were picked after 5 days incubation. The cultures were maintained on YEMA slants with regular subculturing

Morphological Characteristics

All the strains were maintained on Yeast Extract Mannitol Agar (YEMA) as well as Yeast extract glucose agar (YEGA) and the rhizospheric strains were maintained on Luria Bertani (LB) Broth. All the isolates on agar plates were checked for their colony morphology. Similarly using Gram staining technique as described by (Arora, 2003) pink colored Gram negative rods were observed

Biochemical Tests: All the collected samples were processed through different biochemical tests viz, Catalase Test, Indole Production Test Methyl Red Test, Vogas Proskauer Test, Citrate Utilization Test, Starch hydrolysis Test, Gelatin liquefaction Test and Motility Test and ONPG Test (O-Nitrophenyl-D- Galacto-Pyranoside) as described by (Cappuccino, 2007).

Siderophore production test

The chromeazurol (CAS) agar assay was described by Schwyn & Neilands (1987). about 60.5 mg of CAS was dissolved in 50 mL of deionised water, and mixed with 10 mL of a Fe3+solution (1 mmol L^{-1} $FeCl_3$.6H2O,10 mmol L^{-1} HCl). While stirring, this solution was slowly mixed with 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) previously dissolved in 40 mL water. The resulting dark-blue solution was autoclaved, cooled to 50/60°C and mixed with 900 mL sterile MM9 (Silva Stenico *et al*., 2005) containing 15 g L^{-1} agar (also kept at 50/60°C). This medium was allowed to gel on Petri dishes, was subsequently inoculated with bacterial strains and incubated in the dark (28°C for 5 days). Positive results were indicated by the formation of a clear halo around. The colonies, showing a visual change in color from dark-blue to yellow colouration indicates positive.

Indole Acetic Acid (IAA) Production

Bacterial endophytes were tested for their ability to produce IAA under liquid culture. The bacterial cultures were inoculated in tryptone soya broth supplemented with 100 µg ml^{-1} DL-tryptophan and were incubated at 30° C for 72 h. Indole acetic acid was determined in the culture supernatant by adding Salkowski reagent. Two ml of Salkowski reagent was added to 2 ml of culture supernatant, mixed and allowed to stand for 30 min for the development of pink colour.

Phosphate Solubilization (Ponmurugan and Gopi, 2006): Phosphate solubilization ability of the endophytes was determined by spotting of cultures on Pikovskaya's agar plates. Plates were incubated at 30° C for one week and the appearance of clear halo

zone around the colonies indicated solubilization of inorganic phosphate by bacteria. The halo size produced by the respective bacteria was calculated according to the formula:

Solubilization Index= zone diameter (cm) - colony diameter (cm)/ colony diameter (cm)

Screening for PHB producing bacteria

Preliminary screening by indirect staining

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black Dye (Jeran *et al*., 1998). For this screening of PHB producers, YEMA media supplied with 1% of glucose was autoclaved at 121^0 C for 20 min at 15 lbs pressure. This media was poured into sterile Petri plates and allowed for solidification. The plates were divided into 5 equal parts and bacterial isolates were spotted. These plates were incubated for 24 hours. Now ethanolic solution of 0.02% Sudan Black B was spread over the Petri plates containing colonies and was kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from colonies.

Secondary screening by direct staining

Sudan black staining

Thin smear was prepared and thoroughly air dry. Stain with Sudan black B solution and let it stand for 10-15 minutes. Add more stain if the slide starts to dry out. Wash the slide with xylene and counter stain with safranin for 10 seconds.

Wash with distilled water and blot dry with tissue paper. Examine the slide under oil immersion microscope for PHB granules. Organism shows positive in blueviolet and shows negative in yellow-brown

Effect of Carbon and Nitrogen Types on PHB Production

For the growth and production of PHB the simplified minimal medium (NaCl – 0.01g, NH_3HPO_4 -6 g; KH_2PO4- 3g, $MgSO_4$ - 0.02g CaCl- 0.001g, $Fecl_3$ -10Mm, $CaCO_3$ 0.005 g/L and pH at 6.8) and modified Luria-Bertani (LB: casein 10 g; NaCl 10 g, yeat extract 1g/ L) broth was used. All the medium was supplemented with 5 percentage of glucose, lactose, mannitol, glycerol and jaggery as sole carbon source.

The fermentation medium was prepared and sterilized at 121°C and 1% inoculum was added to each flask to carry out fermentation. The flasks were incubated at 37°C under 200 rpm for 78hrs. Samples were collected at 48 hrs and the cell biomass was measured at 600nm using spectrophotometer.

Quantitative analysis of PHB

After 72 hrs Fermentation on broth at 37° C, culture was collected and centrifuged at 10,000 rpm for 15min and lyophilized. The lyophilized pellet was digested with 4% sodium hypochlorite solution for 20min.

Then pellet was collected by centrifugation at 10,000 rpm for 15min, washed with water, acetone, ethanol respectively for washing and extraction. Finally polymer was dissolved in chloroform and kept for complete evaporation. Dry weight of extracted PHB was estimated as g/L. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHB.

The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight (Arnold *et al*., 1999).

Residual biomass (g/ml) = DCW (g/ml) – Dry weight of extracted PHB (g/ml)
PHB accumulation (%) = Dry weight of extracted PHB (g/ml) × 100 / DCW (g/ml)

Results and Discussion

The collected plant sample was identified as *Aeschynomene indica* and the accession number is SJCBT2102. Phytochemical study reveals the presence of carbohydrate, saponins, phenol and tannins, steroid glycoside, flavanoid and alkaloids (table 1). Aside from their ability to form nitrogen-fixing nodules on both the roots and stems of tropical aquatic legumes of the genus *Aeschynomene*, the photosynthetic bradyrhizobia exhibit several other remarkable features. First, their photosynthetic character, which is a rare property among rhizobia, contributes to symbiotic efficiency by providing energy to the bacteria that can be used for nitrogen fixation. Second, they are able to fix nitrogen in the free-living state (Alazard, 1990).

Totally 6X 10^{-6} colonies were isolated and identified as Gram negative based on mucoidal and pink colonies, cell wall by Grams stain found as rod and pleomorphic cells (plate 1 a) and Bichemical characters (Table 2) confirms that the isolates are belongs to *Rhizbium* sp and *Blatobacter* sp. Most of the colonies were glistering mucoid in nature and few colonies were pale pink raised in nature. Both the isolates were siderophore negative but solublize the phosphate.

It was observed that the phosphate solublization index of Rhzobium sp was 62% and 55% for *Blatobacter* sp. Test on indole acetic acid shows positive result only on *Rhizobium* sp. Isolate belongs to *Blatobacter* (S2/2) showed positive test on all biochemical except utilization of citrate. This isolate differ from *Rhizobium* sp on indole, gelatin and starch utilization which was positive by *Blatobacter* and negative in *Rhizobium* sp.

Eventhough the *Rhizobium* is the most common nodulation bacteria rare isolates like *Blatobacter* spp. which are freshwater bacteria that form rosette structures by cellular attachment to a common base was also isolated and reported by Peter van Berkum, and Eardly during 2002.

Both the isolated strins showed positive result on PHB sudan black staining (plate 1 b). Among the tested medium LB broth with nitrogen source casein showed maximum growth of both strains. LB with 5% glucose and glycerol showed 68% of PHB polymer. Whereas minimal medium with ammonium as nitrogen source showed moderate growth rate but the occumulation of PHB is high. Among the five different carbon sources MM with jaggery showed maximum PHB accumulation followed by Glycerol. Isolate belongs to *Rhizobium* showed 70% of PHB on MM with Jaggery and a minimum of 68% on LB broth with glucose.

The maximum PHB occumulation 80% was observed in *Blatobacter* sp which was grown in MM with Jaggery and minimum of 74% with glycerol. Production of PHB on LB broth was less significant than minimal medium by these isolates. However LB with glucose and Glycerol showed moderate PHB production (44%) but lees significant on Lactose and mannitol (figure 1 and 2). Figure 3 shows the 16S rDNA relatedness of *Blatobacter* sp reveals that it closely related to *Blatobacter capsulatus* with 99% similarity and the Genbank accession number is KU713053.

Table.1 Qualitative analysis of Phytochemical of root of *Aeschynomene indica* L

S.No	Phyto chemical test	*interference*	*Aeschynomene indica*
1.	Carbohydrate test		
	b) Fehling's test	Brick red- positive	positive
	c) Iodine test	Purple- positive	positive
2.	Saponins test	Stable foam- positive	positive
3.	Phenols and tannins test	Black colour- positive	positive
4.	Glycoside test Salkowski's test (steroid	Reddish brown- positive	positive
	a) Keller Kilani test (cardiac glycoside)	Brown ring- positive	negative
5.	Flavonoid test- Shinoda test	Pink colour- positive	positive
6.	Alkaloids test- Wagners test	Precipitation- positive	positive
7.	Quinones test	Red colour- positive	positive
8.	Terpenoids test	Red brown- posiive	positive

Table.2 Biochemical characters of isolated bacterial strain

Sample code	Colony morphology	Gram staining	Cat	Oxid	Indole	MR	VP	citrate	ONPG	Gelatin	starch	PHB	Phosphate solublizaion
S-2	***Aeschynomene indica***												
1	Large, White mucoid, glistering	negative rod	+	+	-	+	+	-	+	-	-	+	60
2	Pale pink, raised, opaque	negative, pleomorphic	+	+	+	+	+	-	+	+	+	+	55

Fig.1 Effect of carbon source on PHB accumulation by *Rhizobium* sp

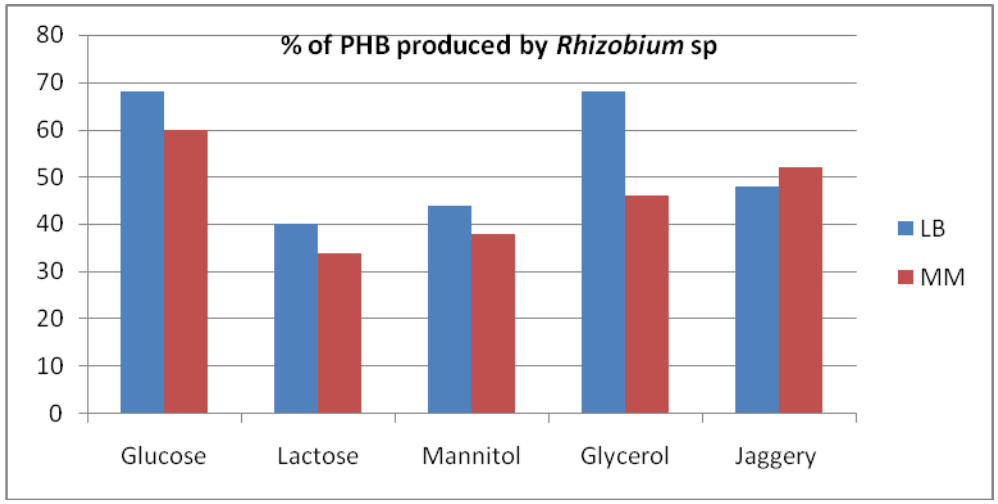


Fig.2 Effect of carbon source on PHB accumulation by *Blatobacter* sp

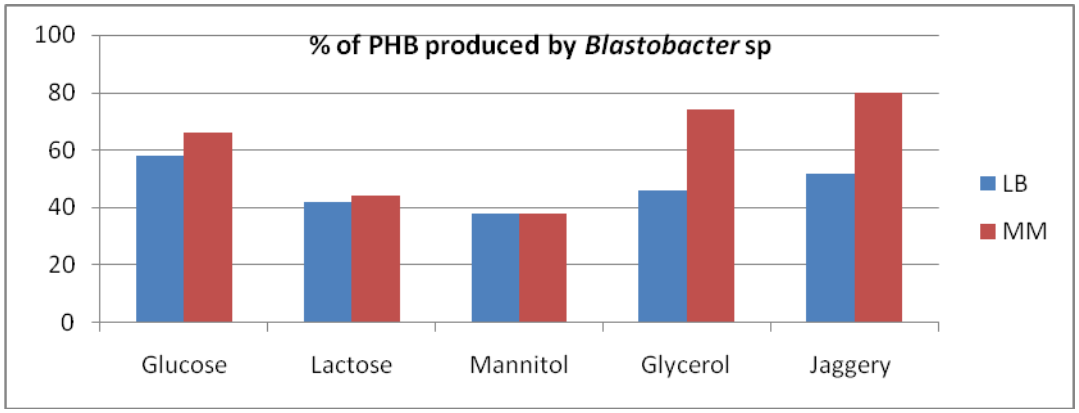


Fig.3 Phyogenetic relatedness of *Blatobacter* strain KU713053

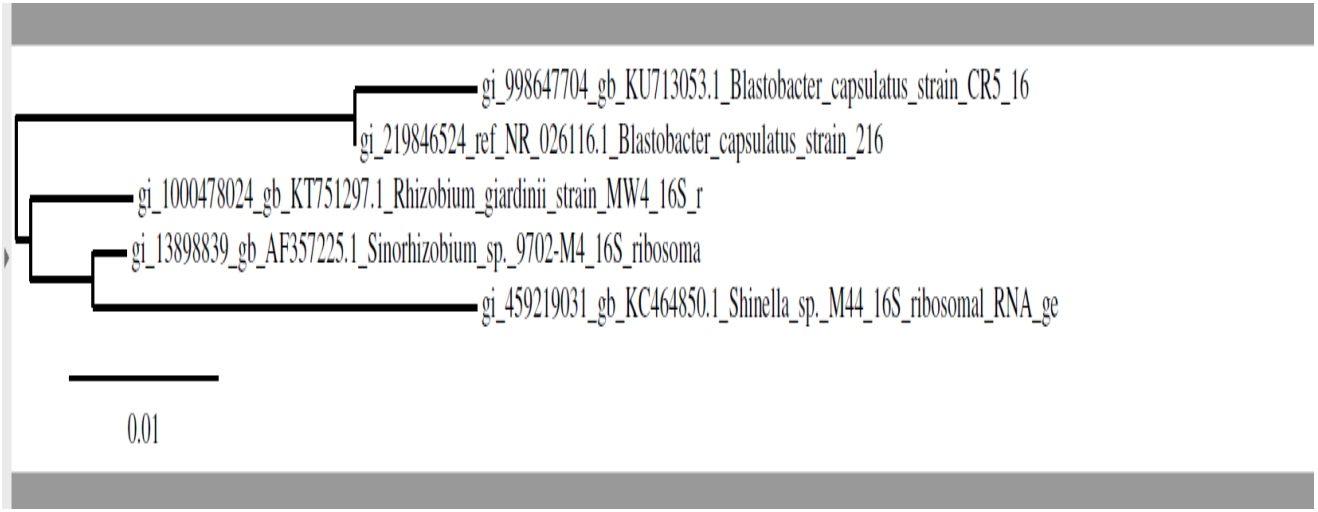
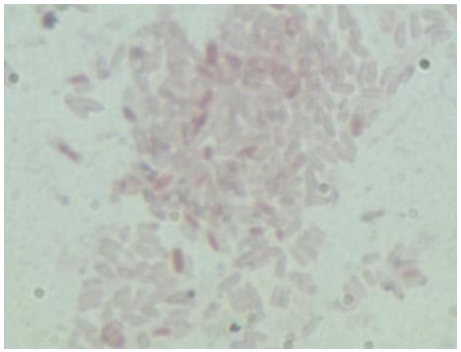
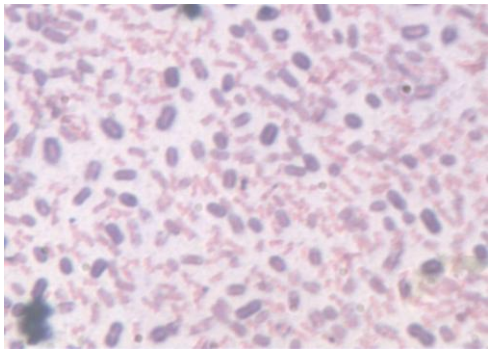


Plate 1 a) Gram's Staining

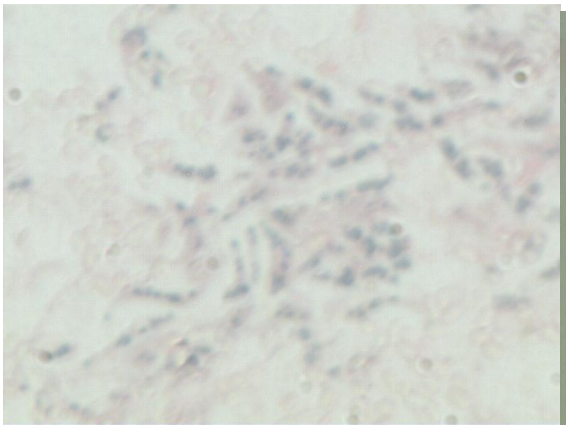


S2/ *Rhizobium* spp

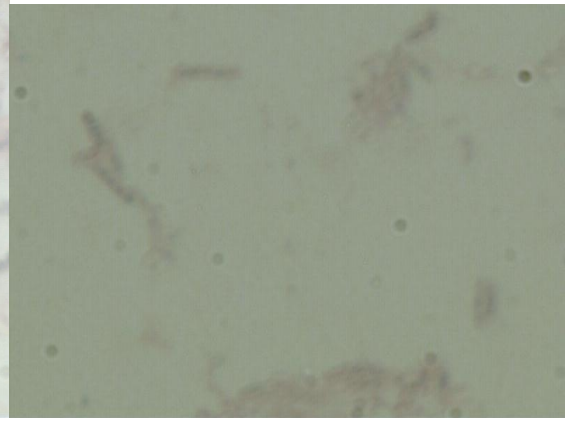


S2/2 *Blatobacter* spp

b) PHB staining



S2/ *Rhizobium* spp



S2/2 *Blatobacter* spp

Despite these advantages, production costs of bioplastics remain higher than those of conventional plastics and will need to be lowered for bioplastics to compete in the marketplace (Choi and Lee, 2000). The overall cost of polymer production could be decreased by the efficient use of inexpensive carbon feedstocks (Nikel *et al*., 2006). Several studies have investigated the production of bioplastics from industrial waste streams. In this study five different carbon sources were tested and it was observed that the cheapest carbon source jaggery was given a luxuriant PHB production. Frequency of production of PHB% by *Rhizobium* sp (Fig 1) was 68>40>44>68>48 respectively for glucose, lactose, mannitol, glycerol and jaggery on LB and 58>42>38>46>52 for *Blatobacter* sp (Fig 2). Similarly growth on Minimal medium, *Rhizobium* sp showed 60>34>38>54>60% and 66>44>38>74>80% by *Blatobacter* sp., The fermentation of these substrates yields favorable amounts of cell mass and bioplastic with few statistical approaches like agitation have been taken to enhance the production of the desired polymer products. Cell dry weight and PHB accumulation was increased significantly under increasing concentration of Jaggery 1>2>4>5 % against 0.6% nitrogen source. Further characterization of PHB like IR and NMR are required to find out the chemical nature of isolated PHB.

In conclusion, PHB producing *Rhizobium* sp and *Blatobacter* sp were isolated from root nodule of *Aeschynomene indica*. This study also concludes jiggery was found to be best and cheapest carbon substitute for the production of PHB.

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