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Flowcytometry based analysis for PHB by *Rhizobium* spp isolated from *C.albida*

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ABSTRACT
Rhizobium sp isolated from C.albida root nodule using YEMA with congored. Two different carbon sources were used to find out the activity of Rhizobium spp PHB production. Effect of 2% of carbon and 2% of jaggery with 0.2 mg/L casein were evaluated for PHB production. Glucose (2%) with casein at different concentration showed maximum PHB 66 and 60 % respectively by R.nepotum and Mesorhizobium sp. increasing casein con does not influence the PHB accumulation instead enhanced dry cell weight. Similarly casein (0.2 mg/L) under modified carbon Jaggery (1-5%) shoed maximum of 80 and 74% PHB by R.nepotum and Mesorhizobium sp at 2:0.2 ratio. Increased carbon also found to be less significant on PHB accumulation. Further the maximum PHB producing R.nepotum flowcytometry reveals 56% of parent cells produce PHB under jaggery and 44% under glucose. It confirms both isolated strains capable to utilize glucose and jaggery and stores as intracellular poly-3-hydroxybutyrate. The data concludes jaggery is an ideal cheap carbon for industrial production of PHB.
Keyword: *Biopolymer, Rhizobium, carbon, flowcytometry, PHA*

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INTRODUCTION

Plastic materials obtained from petrochemicals are generally inexpensive and non-degradable nature cause serious environmental impact to all life. For efficient management of used-plastic materials, recycling is one solution. Another solution to reduce plastic residue is the use of biodegradable plastic. Biodegradable plastics offer the best solution to protect the environment from hazards caused by conventional petroleum based plastics as they are 'eco-friendly' in nature. There are many types of biodegradable plastics with different degrees of biodegradability. Polyhydroxyalkanoic acids (PHAs) are common intracellular compounds found in bacteria, archaea, and in few eukaryotes such as yeasts and fungi [1]. PHAs are found to accumulate in varieties of microorganisms synthesized as natural biopolymers and catabolized for carbon source [2].*Alcaligen eslatus, Ralstonia eutropha*, *Azotobacter beijerinckii, Bacillus megaterium,* and *Pseudomonas oleovorans*, were frequently reported as PHB producer under nitrogen depletion[3]. Among them polyhydroxybutyrate (PHBs) are the only 100% biodegradable ones. PHBs are macromolecules synthesized by bacteria and are inclusion bodies accumulated as reserve material when the bacteria grow under different stress conditions [4]. PHB has been the most promising biodegradable alternative to petrochemical plastics. This is due to their molecular weight (2-3 × 10^3 KDa), biocompatibility, biodegradability and eco-friendly substitute for synthetic polymers [5]. *Alcaligenes eutrophus* is the most widely studied bacteria produce PHB under limited nitrogen in the presence of high carbon up to 80% [6]. diazotrophic bacteria specifically associated with legume plants, contributing plant-growth-promotion and occasionally PHB synthesis. *Azospirillum brasilense, Sinorhizobium meliloti, Bradyrhizobium elkanii* were found to synthesis of PHB is favored under oxygen limitation. The biosynthesis of different polymers by rhizobia depends on its genetic characteristics and culture conditions of the strain. Some studies have documented the separate production of EPS or PHB by various

strains, including *R. meliloti* [7]. The alfalfa root nodule symbiotic*Sinorhizobiummeliloti* reported as produce polymer under excess carbon as intracellular as poly-3-hydroxybutyrate (PHB) granules [8]

MATERIAL AND METHODS

Isolation of *Rhizobium*[9]

The healthy and pink nodules from *C.albida* 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 100µL contents were spread on YEMA plate containing 25 µg/ml congo red. All the plates were incubated at 28 ± 2°C for 5 days. Colonies were picked after 5 days of incubation. The cultures were maintained on YEMA slants.

Identification of isolates

All the collected samples were processed through different biochemical tests viz, Catalase Test, Indole Production Test Methyl Red Test, VogesProskauer Test, Citrate Utilization Test, Starch hydrolysis Test, Gelatin liquefaction Test and ONPG Test (O-Nitrophenyl-D- Galactopyranoside)

Hofer's alkaline test:

In order to differentiate the *Rhizobium*isolates from the *Agrobacterium*, log phase actively grown isolates were grown in the Hofer's alkaline medium (pH 11) at 28±2^{0}C for 3-7 days. Normally, *Rhizobium* cannot grow in Hofer's medium and help to detect the contamination of *Agrobacterium*.

Sudan black staining

Thin smear was prepared and thoroughly air dried. 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 blue violet and shows negative in yellow-brown.

Effect of Nitrogen source on PHB Production

1000 ml minimal broth contains different concentration of casein (0.1 to 0.5 mg/L) was prepared. 2% glucose used as carbon. The production of PHB was determined qualitative and quantitatively as described above. The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight

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

Effect of Carbon source on PHB Production

The potent isolate was subjected to grown under different ratio of jaggery with standard nitrogen. Casein used as nitrogen.Various concentration of jiggery and glucose (1, 2, 3, 4, 5 % w/v) was prepared in minimal medium with 0.2 mg/l casein. Starter culture were prepared by inoculating the selected strain in 100 ml minimal medium and incubated on a shaker (150 rpm) at 37ºC for 48 hrs

Flow cytometry analysis[10]

The cells were grown in broth medium for 24h, then harvested by centrifugation and cell pellet was incubated in above culture medium minus carbon source for 24h. Inoculum was prepared by centrifugation and cell pellet washed with phosphate buffer saline (pH=7.0) and incubated in culture plus proper carbon source. Samples were centrifuged for 5mins in 5000 rpm, supernatant was discarded and pellet was washed with PBS three times. Ethanol 35% was used for fixation of samples at room temperature for 15mins. Then the sample centrifuged again and with PBS the optical density of cell biomass adjusted to 0.5 according to McFarland turbidity. For staining the cells, 20µl of acridine orange solution (1mg acridine orange per 1ml DMSO) was added to test tube to give a final concentration of 20µg/ml in 1ml cell suspension and incubated for 30mins. This suspension was used for flowcytometry analysis. Side scatter signal Vs FL2 parameter (Emitted fluorescence intensity of stained granules) were used in the analysis

Differential scanning calorimetry (DSC)

3 mg of sample was taken in a tin crucible heated at a rate of 100^{0}C/min. under nitrogen atmosphere to 400^{0}C and by using these parameters the experimental method was implemented and results were obtained using automated system software.

RESULTS AND DISCUSSION

Colonies on YEMA were white mucoid and Gram-negative rods showed presence of PHAs confirmed by sudan black. Based on biochemical characters and growth on YEMA isolates were identified as *Mesorhizobium* sp and *R.nepotum.* Growth of Hofer's alkaline showed negative supports the isolate Formation of black granules followed by sudan black stain were detected under 100x magnification.

Similar report was given by Aseem Rajan *et al* [11]. Strains of *Mesorhizobium* sp and *R.nepotum* were isolated and its PHB production was confirmed by sudan black stain. The concentration of carbon (glucose/jiggery) and nitrogen (casein) was evaluated and the PHB production was recorded. The results on the optimization of carbon and extraction of PHB show maximum PHB film was extracted from jaggery and glucose (image 1). Table 1 reveals the optimum concentration of casein for the production of PHB by *R.nepotum*. It was observed that among the five different nitrogen concentrations maximum PHB production was found at 0.2 mg/L**.** The ratio of glucose casein 2:0.2 showed maximum 66 % of PHB under glucose. The highest cell dry weight (5.2g) was noted in 5:0.2 with low PHB production (28%). The highest PHB yield 80% was achieved by altering 2% glucose with jaggery showed formation of 6.10±03 gdry cell weight (DCW). PHB yield varied and the maximum value reached after 48 h. The cell dry weight of the strain decreased with an increase in carbon concentration. Percentage of PHB yield under jaggery were 25 ,80,30 ,19 ,18 respectively for the ration of 1,2,3,4,5% of carbon.

The strain *Mesorhizobium* sp assimilated all the carbon sources tested and gave the maximum cell growth (4.82±06 g/l) and PHB production (3.2±0.028 g/l and 66 %) after 48 hrs cultivation in the medium using 2 % glucose with 0.2 mg casein nitrogen source. The percentage of PHB yield was 22≥60≥22≥19≥23 under glucose with 0.1 ≥0.2 ≥0.3 ≥0.4 ≥0.5 mg casein. Effect of jaggery casein ratio 1:0.2≥2:0.2 ≥3:0.2 ≥4:0.2 ≥5:0.2 showed enhanced PHB yield 20≥74≥30 ≥22 ≥18 %. Maximum PHB was 74% under 2% jaggery. Belal stated that *R. elti* and *P. stutzeri* are capable of accumulating appreciable levels of PHB from glucose, xylose, lactose, whey, molasses, sugar cane bagasse, rice straw hydrolysate when 2% and a maximum of PHB found ratio of C/N that reaches 20:1[12].

Flow cytometry analysis of PHB production

A comparative account of PHB production by *R.nepotum* was studied at the end of24 h by flow cytometry analysis.The value of FSC is correlated to cell size and SSC represents cell density or granularity. Figure 1 reveals the fluorescence-based flow cytometry analysis on PHB accumulation under optimized conditions with *R.nepotum* within 24 h incubation. The dot plots from a sample of *R.nepotum* cells grown under a casein-limiting and glucoseexcess condition that enables PHB accumulated cells.About 41% of cells have been reported by flow cytometric analysis. These data highlighted important differences between PHB accumulation among nitrogen source in *R.nepotum.* cells grown under a casein-limiting and jaggery excess condition that enables PHB accumulation 56% (Fig2).There was a marked rise in the fluorescence intensity as well as granulation by 24h (p3) and extended up to 48h recorded as p2 Population under glucose. Cytometry differentiate between bacterial populations based on their PHB content and fluorescence. Flowcytometry technique is most commonly used in many clinical applications but not yet focused on PHB analysis [13-15]. Hence, this technique was explored to compare PHB producing capability of *Rhizobium* sp by comparing the difference in the median fluorescence intensities over the carbon source on PHB production. The cultures exhibited diverse patterns of PHB accumulation in the selected carbon with hetero population size. Similar work has been reported by Shakeri *et al.*[16]reported formation of two generation with PHB up to 48 h. SSC side scatters on the Y-axis corresponding to the granulation pattern of the cells corresponding to the fluorescence intensity of the sample on the X-axis [17].

TS DSC analysis of extracted PHB

A report on the melting temperature of the extracted PHB sample obtained was determined using DSC. The thermal properties of the polymer such as the melting temperature (T_m) are crucial for polymer processing. The melting temperature of extracted PHB (170 °C) was equal as compared to that was reported in the literature (173–180°C). The melting point near to 170°C showed that the extracted PHB with low molecular weight polymer. DSC analysis was conducted to investigate the melting temperature, glass transition temperature and heat associated with the melting of PHB. The Tm of PHB was 170°C (Figure 3). These results are proved by the previous literature [18].

CONCLUSION

Root nodulated *Rhizobium* spp capable to produce PHB was isolated and maximum PHB production medium was optimized with cheap carbon jaggery and casein at 2:0.2 ratio. Application of this soruces found to be good nutrient choice for PHB production.

Table 1.Quantification of PHB production produced by *R.nepotum*

Percentage of C:N	Dry cell weight(g/l)	PHB	PHB yield (%)
Glucose-Casein			
0.1	4.45±0.6	0.583±0.028	13
0.2	4.8±06	3.2±0.028	66
0.3	4.40±14	1.8±0.018	40
0.4	4.05±2.04	0.50±0.024	12
0.5	5.2±14	1.5±0.018	28
The ratio of jaggery and casein			
1:0.2	4.80±14	1.24±0.024	25
2:0.2	6.10±03	4.9±0.28	80
3:0.2	4.20±14	1.30±0.020	30
4:0.2	3.8±06	0.75±0.024	19
5:0.2	3.8±126	0.720±0.016	18

Table 2.**Quantification of PHB produced by *Mesorhizobium* sp**

Percentage of C:N	Dry cell weight(g)	PHB (g)	PHB yield (%)
Casein (mg/L)			
0.1	4.4±0.7	0.93±0.028	22
0.2	4.82±06	2.9 ±0.028	60
0.3	3.60±22	0.8±0.018	22
0.4	3.50±2.12	0.68±0.024	19
0.5	4.02±2.12	0.9460±0.024	23
jaggery -casein			
1:0.2	8.2±16	1.64±0.024	20
2 :0.2	7.3±06	5.42±0.28	74
3:0.2	3.9±12	1.2±0.020	30
4 :0.2	4±08	0.9.2±0.024	22
5:0.2	4.8±16	0.90±0.016	18

Figure 1. Flow cytometry analysis of PHB accumulation by *R.nepotum* under glucose

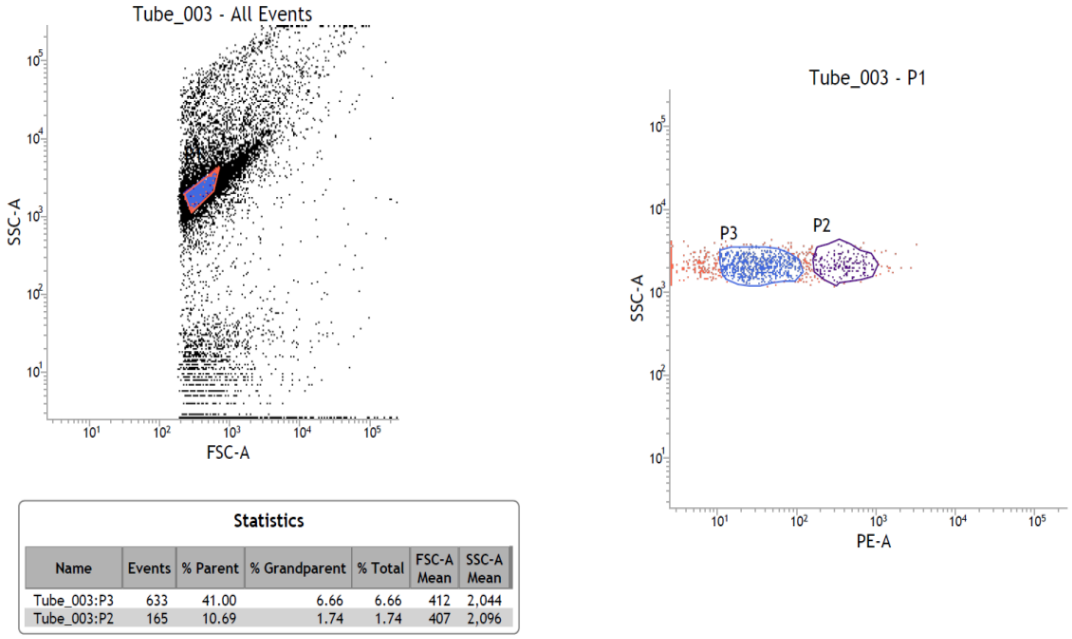
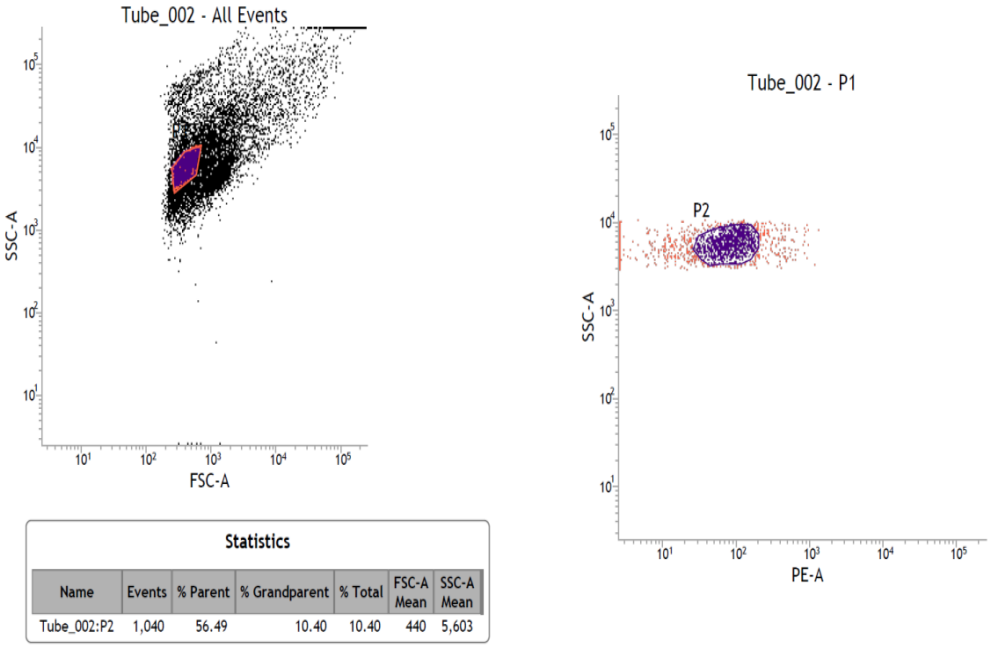


Figure 2. Flow cytometry analysis of PHB by *R.nepotum* under jaggery



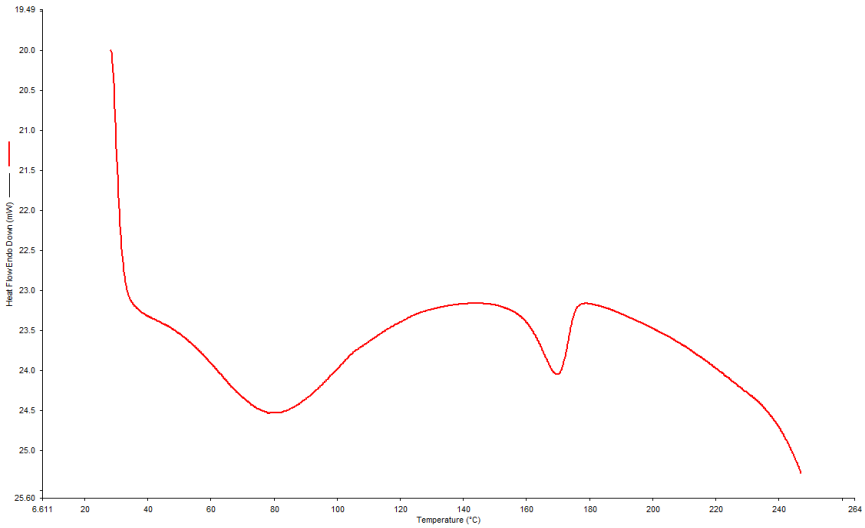


Figure 3. TS DSC analysis of extracted PHB

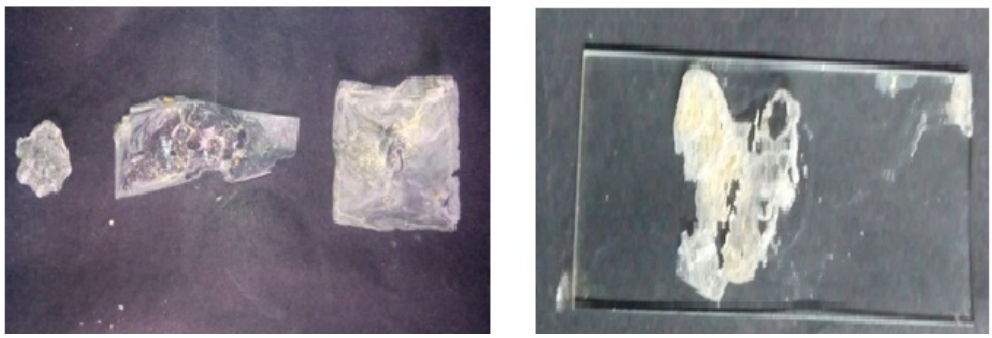


Image 1. Jaggery and glucose based PHB

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