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ISOLATION AND CHARACTERIZATION OF PHB PRODUCING MICRO-ORGANISMS ISOLATED FROM ROOT NODULES OF LEGUMINOUS PLANTS

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ABSTRACT
Synthetic plastics are highly resistant to be degraded in the environment and pose a number of serious problems. Therefore, there is an urgent need to have a biodegradable alternative to these synthetic polymers.*Rhizobium* spp. is able to produce poly hydroxyl butyrate (PHB) which can be used as biodegradable plastic. But the cost of production is very high because of high cost of media ingredients and chemicals used for their extraction from the cell. In the present study of investigation, attempts have been made to isolate and characterize the *Rhizobium* isolates capable of producing PHB and to analyze the effect of cheap carbon and nitrogen sources on their growth. *Rhizobium* was isolated from the root nodules collected from various leguminous plants. Biochemical and molecular characterization of PHB producing isolates were carried out.To analyze the effect of cheaper carbon and nitrogen sources on PHB production, different concentration of jaggery and soybean meal was used.Different concentration of jaggery and soybean mealranging from 0.2% to 1% was added in the minimal media. It was found that use of 1% jaggery and 0.2% soybean flour in minimal media proved to be the most effective combination for the maximum growth of biomass and production of PHB.

INTRODUCTION

A number of the micro-organisms are capable of producing polyhydroxyalkanoates (PHA's) as storage food reserve under unbalanced growth conditions. The mechanical properties of PHA's are similar to that of synthetic plastics like polyethylene, polypropylene etc. The advantage of using PHA's over synthetic plastic is that PHA's are completely mineralized into carbon dioxide and water through the action of various micro-organisms. Polyhydroxybutyrate (PHB), Polyhydroxybutyrate-co- hydroxyvalerate (PHBV) are the best known representatives of PHA family. These representatives of PHA can be used to produce environment friendly alternative to the synthetic polymers which pose a number of environmental problems. PHB are produced intracellularly by various organisms such as *Bacillus megaterium, Ralstonia eutrophus, Cupriavidus necator, Rhizobium spp., Azotobacter spp., Pseudomonas* spp., etc under physiological stress conditions. These bacteria can accumulate up to 60-80% of their weight as PHB under limiting nitrogen substrate and in the presence of an abundant source of carbon (Anderson and Dawes, 1990). In last few years, various attempts have been made to use PHB for the production of biodegradable plastics (bioplastic) (Mercan, 2002; Mukherjee *et al.,* 2011). The major limitation in the use of biodegradable plastic is their high cost as compared to the synthetic plastic. It is well established that the cost of production of any microbial product can be reduced significantly if these micro-organisms are harvested on low cost media having agro-industrial byproducts since media is the main component that add to cost of production (Dhingra, 2012; Goyal and Dhingra 2011).

Rhizobium species are symbiotically associated with several leguminous plants like *Pisum sativam*, *Glycine max, Alfa alfa* etc. These are Gram negative, motile, non-endospore forming bacteria. These bacteria are generally cultured in Yeast Mannitol Agar medium. *Rhizobium* gives colorless gummy appearance when grown on YEMA medium supplemented with congo red. The gummy appearance is because extracellular polysaccharide production. Most importantly, they are able to accumulate a high amount of PHB intracellularly. In the present study of investigation, attempts have been made to produce bioplastics from *Rhizobium* spp. For selecting the strain producing highest PHB, the appropriate growing time and appropriate source and concentration of carbon and nitrogen were tested for further PHB yield. To reduce the cost of production of PHB, various combinations of production media containing cheap agro-industrial carbon and nitrogen sources were evaluated.

MATERIALS AND METHODS

Isolation of microbial strains

Root nodules from different areas of Shekhawati region, Rajasthan were collected. Pink colored healthy root nodules were selected from the roots of various leguminous plants and sterilized with 0.1%mercuric chloride, crushed and a loopful of crushed material was streaked on YEMA medium

containing congo red. Colorless gummy colonies were selected and were sub cultured to obtain pure culture. Pure culture was maintained in agar slants. *Cupriavidus necator* (MTCC 1472) was used as a positive control which was obtained from IMTECH, Chandigarh.

Morphological examination of the isolates

Gram staining

The overnight grown **c**ultures were stained with gram reagents as per Gram (1884) to determine their Gram reaction. Only Gram negative isolates were selected for further studies.

Carbol Fuchsin staining

Carbol fuchsin staining is performed to determine the intracellular production of PHB by the isolate. A thin smear of all the isolates were stained with carbol fuchsin stain for 45 seconds. The isolates capable of producing PHB showed dark colored granules of PHB intracellularly (Aneja, 2001).

Sudan black B staining

PHB producing bacteria was further confirmed using Sudan black B staining method (Schlegel *et al*., 1970) with some minor modifications. Sudan black B stain was prepared as 0.3% solution (w/v) in 60% ethanol. The smear of cultures was prepared on glass slides and heat fixed. The samples were stained for 10 min with Sudan black solution, rinsed with water and counter stained with 0.5% safranin for 5min. 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 10g/L, yeast extract 0.5g/L,$K_2HPO_4$0.5g/L, KH2PO4 0.5g/L MgSO4 0.7H2O 0.2g/L, NaCl 0.1g/L, $CaCl_2$. $2H_2O$ 0. 06g/L)at 30ºC and 37ºC for a period of 66h in a shaker incubator at 150 rpm. A sample of 1mL was withdrawn at a constant time interval to check the optical density at 600nm. The different carbon and nitrogen sources were tested in minimal media (Na_2HPO_4 .$7H_2O$ 30g/L, KH_2PO_4 15g/L, NaCl 2.5g/L, NH_4Cl 5g/L, $MgSO_4$ 2mM, $CaCl_2$ 0.1mM, glucose 0.4%). To analyze the effect of cheaper carbon and nitrogen sources on PHB production, jaggery and soybean meal was used. Soybean meal and jaggery was used at different concentrations varying from 0.2% to 1.0%. The effect of carbon to nitrogen ratio was also analyzed. All the cultures were inoculated with equal volume of inoculums and grown at 28ºC. At an equal time interval, OD at 600nm was checked to study the effect of different carbon and nitrogen sources on the growth assay and PHB production of isolates.

Biochemical tests

To determine the ability of isolates to reduce litmus milk, the cultures were grown in litmus milk at 28ºC for 24 - 48h. Afterwards litmus milk was checked for change in its pH, reduction and peptonization. To examine gelatinase activity of the isolates, plates and deep tubes containing gelatin agar were inoculated and incubated at 28ºC for 4 to 7 days and examined for formation of clear zone after treatment with mercuric chloride (Goyal and Dhingra, 2011).Urease activity was determined on urea broth tubes, incubated at 28ºC for 24 - 48h. These tubes were observed for change in color (from red to pink).

Catalase activity was determined by the method of Graham and Parker (1964). Oxidase activity was determined by the method of Kovaks (1956). Hydrogen sulfide production was determined by inoculating SIM agar deep tubes and incubating at 28ºC for 48h (Hunter *et al.,* 1938).Tubes were observed for appearance of black coloration along the line of stab inoculation. Production of amylase was determined on starch agar medium, incubated at 28ºC for 48h. Iodine was used as an indicator. Plates were observed for the formation of clear zone around the line of growth.

To determine cellulase production, Czapek-mineral salt agar medium was inoculated and incubated at 28ºC for 2-5 days. The plates were flooded with hexadecyltrimethyl ammonium bromide and observed for formation of zone around the growth. For hydrolysis of casein, skim milk agar medium was inoculated and incubated at 28ºC for 24 - 48h. and observed for clear zone around the line of growth. Carbohydrate catabolism was determined by Hugh and Leifson's (Aneja, 2001) medium deep tubes in both aerobic and anaerobic condition, incubated at 28ºC for 24-48h.

16S rDNA sequencing

To carry our molecular identification of the isolates, all the isolates were grown overnight and preceded for DNA isolation. The DNA bands were visualized with the help of Ethidium bromide dye. All the isolates were having DNA of more than 1 Kb size. The purified genomic DNA of these isolates was amplified using 16S rRNA gene amplifying universal primers. 16S rDNA sequencing was performed using commercial service. These sequences were compared using NCBI BLAST (Basic Local Alignment Search Tool) service.

Optimization of growth conditions

The effect of temperature on the growth of *Rhizobium* isolates was estimated by inoculating nine flasks containing 100mL YEM broths. These broths were incubated at different incubation temperature ranging from 22ºC to 37ºC. The samples were withdrawn at regular interval and were used to measure the absorbance at 600nm. To observe the effect of cheap agro-industrial based carbon and nitrogen sources on the growth and PHB production, different combinations of minimal media containing jaggery and soyabean meal was prepared. A total of 0.2 to 1% of jaggery and soyabean meal was added in minimal media to prepare different combinations of production media and the growth pattern in these low cost production media was observed.

Extraction and quantification of PHB

PHB was extracted using the dispersion method of sodium hypochlorite and chloroform (Chang *et al.,* 1994; Law and Slepecky, 1961; Singh and Parmar, 2011) with minor modifications. 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 1-2h 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 30ºC overnight. The dispersion was then centrifuged at 8,000 rpm for 10 min 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 15min resulting in the formation of a precipitate of PHB. The amount of PHB present was quantified by determining the weight of precipitate obtained. Addition of concentrated sulfuric acid to this pellet converts PHB into crotonic acid which appears as crystals in brown color solution.

RESULTS

A loopful of crushed root nodule was streaked on YEMA plates and incubated at 28ºC for 1-2 days. A total of ten isolates that produced colorless gummy colonies on media plates were collected and preserved on slants with 20% glycerol. All the isolates were stained with Gram's reagents for detection of their Gram reaction. Pink colored rod shaped cells were observed under microscope. All the isolates found to be Gram negative (Fig. 1). To distinguish PHB producers from non-producers, carbol fuchsin staining was carried out. After staining with carbol fuchsin, all the isolates were found to have dark colored granules of PHB with in their cell. Therefore,

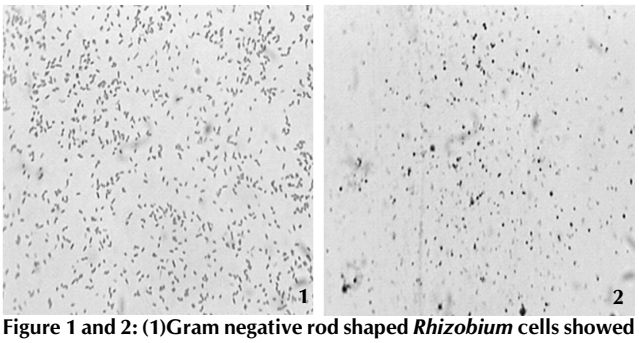

Figure 1 and 2: (1)Gram negative rod shaped *Rhizobium* cells showed at 1000X magnification; (2) Accumulation of PHB in Bacterial culture showed at 1000X magnification. PHB granules are shown as dark purple granules stained with Sudan black B dye

it was assumed that all the isolates were capable of producing PHB. To further confirm PHB production ability of these isolates, they were stained with sudan black B dye (Schlegel *et al.,* 1970). Dark black to purple granules were observed intracellularly with pink background when counterstained with safranin. This confirmed that all isolates were capable to

Table 1: Biochemical Characteristics of different isolates of *Rhizobium*

Biochemical test	Observation				
	M2	M3	M4	M5	Control (*Cupriavidus necator*)
Catalase test	+	+	+	+	+
Oxidase test	-	-	-	-	-
Litmus milk	+	+	+	-	+
Hydrogen Sulfide production test	-	-	-	-	+
Urease test	+	+	+	+	+
Casein hydrolysis	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-
Amylase production	-	+	-	-	-
Cellulase production	-	-	-	-	-
Carbohydrate catabolism	+	+	+	-	+

Sequences producing significant alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NR 036938.1	Rhizobium leguminosarum strain 3Hoql8 16S ribosomal RNA, complete sequence	2724	2724	100%	0.0	100%	
NR 044112.1	Rhizobium phaseoli strain ATCC 14482 16S ribosomal RNA, complete sequence	2700	2700	100%	0.0	99%	
NR 029184.1	Rhizobium etli CFN 42 16S ribosomal RNA, partial sequence	2645	2645	98%	0.0	99%	
NR 036785.1	Rhizobium gallicum strain R602sp 16S ribosomal RNA, complete sequence	2566	2566	99%	0.0	98%	
NR 043150.1	Rhizobium lusitanum strain PI-7 16S ribosomal RNA, complete sequence	2555	2555	99%	0.0	98%	
NR 044774.1	Rhizobium leguminosarum bv. viciae USDA 2370 strain USDA 2370; ATCC	2549	2549	96%	0.0	99%	
NR 043398.1	Agrobacterium rhizogenes strain IFO 13257 16S ribosomal RNA, complete	2531	2531	99%	0.0	98%	
NR 042253.1	Arthrobacter viscosus strain : LMG 16473 16S ribosomal RNA, partial sequence	2492	2492	96%	0.0	98%	
NR 026067.1	Rhizobium tropici CIAT 899 strain USDA 9030 16S ribosomal RNA, partial sequence	2483	2483	99%	0.0	97%	
NR 025157.1	Rhizobium indigoferae strain CCBAU 71042 16S ribosomal RNA, partial sequence	2470	2470	95%	0.0	98%	
NR 026066.1	Rhizobium mongolense strain USDA 1844 16S ribosomal RNA, partial sequence	2462	2462	96%	0.0	98%	
NR 042687.1	Rhizobium alamii strain :GBV016 16S ribosomal RNA, partial sequence	2446	2446	93%	0.0	99%	
NR 029330.1	Rhizobium sullae strain IS 123 16S ribosomal RNA, partial sequence	2409	2409	92%	0.0	99%	
NR 028819.1	Rhizobium loessense strain CCBAU 7190B 16S ribosomal RNA, partial sequence	2409	2409	93%	0.0	98%	
NR 043399.1	Sinorhizobium meliloti strain IAM 12611 16S ribosomal RNA, complete sequence	2398	2398	99%	0.0	96%	
NR 026059.1	Rhizobium giardinii strain H152 16S ribosomal RNA, complete sequence	2398	2398	99%	0.0	96%	
NR 042911.1	Ochrobactrum lupini strain LUP21 16S ribosomal RNA, complete sequence	2394	2394	100%	0.0	96%	
NR 024863.1	Rhizobium huautlense strain S02 16S ribosomal RNA, partial sequence	2394	2394	99%	0.0	96%	
NR 043184.1	Ochrobactrum cytisi strain ESC1 16S ribosomal RNA, complete sequence	2392	2392	99%	0.0	96%	
NR 043548.1	Rhizobium mesosinicum strain CCBAU 25010 16S ribosomal RNA, partial sequence	2388	2388	91%	0.0	98%	
NR 028663.1	Rhizobium yanglingense strain SH22623 16S ribosomal RNA, partial sequence	2379	2379	92%	0.0	98%	
NR 037001.1	Sinorhizobium arboris strain TTR 38 16S ribosomal RNA, partial sequence	2346	2346	97%	0.0	96%	
NR 025828.1	Rhizobium qaleqae strain qal 1261 16S ribosomal RNA, partial sequence	2342	2342	99%	0.0	95%	

Figure 3: BLAST result of 16S rDNA Sequence showing similarity with *Rhizobium leguminosarum*

Table 2: Production of *Rhizobium* cells and PHB with different combinations of carbon and nitrogen sources

Concentration of agro-industrial C and N sources in Minimal media	Dry cell weight (g/L)	PHB yield (%)
0.2% jaggery + 0.2% soyabean meal	0.71±0.18	12.10%
0.4% jaggery + 0.2% soyabean meal	0.99±0.04	17.30%
0.6% jaggery + 0.2% soyabean meal	1.27±0.11	25.80%
0.8% jaggery + 0.2% soyabean meal	1.60±0.16	32.00%
1.0% jaggery + 0.2% soyabean meal	1.88±0.10	54.21%
1.2% jaggery + 0.2% soyabean meal	1.64±0.44	46.10%

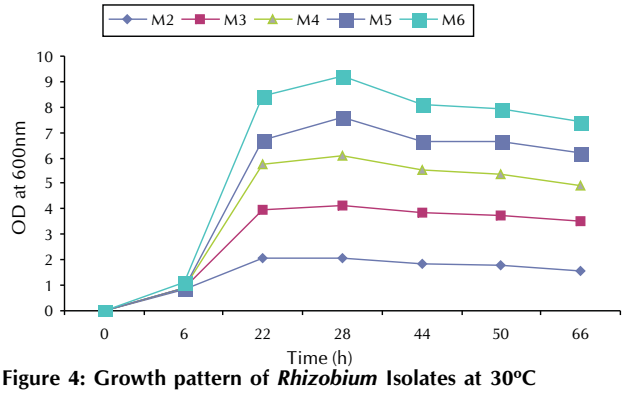

Figure 4: Growth pattern of *Rhizobium* Isolates at 30ºC

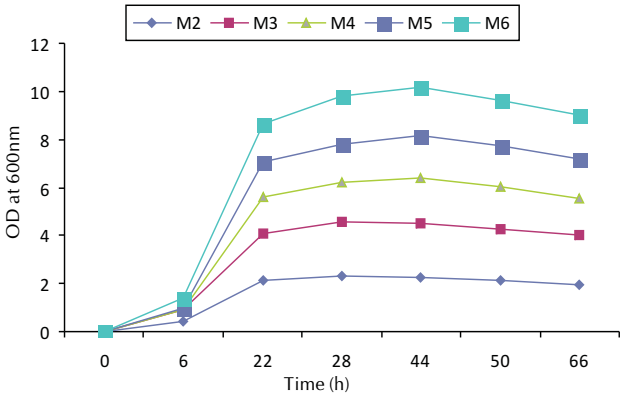

Figure 5: Growth pattern of *Rhizobium* Isolates at 37ºC

accumulate PHB intracellularly (Fig 2). To identify these isolates biochemically, various biochemical tests like catalase, oxidase, litmus milk, hydrogen sulfide production, urease, casein hydrolysis, gelatin hydrolysis, carbohydrate catabolism were carried out. The results of various biochemical tests are summarized in Table 1. Most of the biochemical tests were giving same results as reported for *Rhizobium* spp in literature. Genomic DNA of various isolates was amplified using universal primers for amplifying 16S rDNA gene. 16S rDNA sequencing was performed by commercial service, Chromus Biotech, New Delhi. The results of sequencing showed that all the isolates have 100% similarity with *Rhizobium leguminosarum* (Fig. 3).

The effect of temperature on the growth of *Rhizobium* isolates was determined by inoculating YEM broth and incubating at different temperature ranging from 22ºC to 37ºC. It was found that maximum growth was achieved at 30ºC in most of the isolates (Fig. 4 and 5). To minimize the cost of production media, different low cost C and N substitutes were evaluated for the growth of isolates and their intracellular PHB production. It was found that use of 1% jaggery and 0.2% soybean flour in minimal media proved to be the most effective combination for the maximum growth and production of PHB (Table 2).

DISCUSSION

PHB and other PHAs have been considered commercially important because of possible use as biodegradable thermoplastics (Lee, 1996). Although, previous research has shown that a large number of bacterial species, both Gram positive and Gram negative, produce PHBs (Verlinden *et al.*, 2007), however little work has been done with *Rhizobium*. In the present study, attempts were made to produce PHB from *Rhizobium* species. Hartman (1940) and Burdon *et al*. (1942) used Sudan Black B to determine the accumulation of PHB by bacteria. In the present work, 0.3% solution of sudan black B in 60% ethanol (w/v) was used and PHB was observed as dark black to purple granules against pink background when counterstained with safranin. Sadowsky *et al*. (1983) examined various fast and slow growing Rhizobia for their biochemical relatedness. They showed that almost all the members of both groups are catalase, oxidase, urease positive and H_2S negative. According to them, only fast growing *Rhizobium* gave positive result in case of gelatinase production test. In the present study it was found that the biochemical tests gave results in accordance with the previous research, except oxidase test. Allison *et al*. (1940) showed that the maximum growth rate for *R. trifolii, R. phaseoli and R. leguminosarum* occurs at approximately 29ºC to 30ºC.; with *Rhizobium* from Dalea at about 30ºC to 33ºC. and with *R. meliloti* at 35ºC to 39ºC. Some investigators (Buchanan and Fulmer, 1930) have emphasized that the optimum temperature varies with the composition of the medium. In the present study it was observed that when *Rhizobium* is grown in YEM broth, maximum growth is obtained at 30ºC. In solid media (YEMA), maximum growth is obtained at 28ºC. The high cost of the substrate required for the growth and downstream processing are the limiting factors for PHB production (Mukherjee *et al.*, 2011). In previous research, attempts have been made to cut down the cost of production of bio plastics so that its use can be enhanced. Various low cost carbon and nitrogen sources such as molasses and corn steep liquor (Chaijamrus *et al.*, 2008), whey (Khanafari *et al.*, 2006), banana pseudostem (Kalia *et al*., 2000), damaged food grains, pea shells, apple pomace and palm oil mill effluent (Kalia *et al*., 1992), starch (Lillo and Valera, 1990) and dairy wastes like cheese whey (Yellore and Desai, 1998) has been used for PHB production. In the present study, jaggery and soybean flour was used as low cost substitute of carbon and nitrogen. It was observed that the use of jaggery (1%) and soybean flour (0.2%) in minimal media proved to be the most effective combination for the maximum production of PHB. In this piece of work, since attempt has been made to reduce the cost of production of PHB, the use of jaggery and soybean flour looks promising in future work.

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