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Screening, Isolation and Quantification of PHB-Producing Soil Bacteria

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ABSTRACT: *Polyhydroxybutyrates are biodegradable polyesters synthesized by many bacteria Biodegradable polymer plays a predominant role as a biodegradable plastic due to their hydrolysable ester bonds. PHB is produced variety of microorganisms under appropriate conditions such as the presence of nitrogen, calcium, magnesium, iron or essential vitamins. Due to their biological origin it is an advantage of PHB is, they are degraded n aturally and completely to carbon dioxide and water under natural environment by the enzymatic activities of microbes. The present study reports the isolation and screening of soil bacteria and subsequent PHB production under normal conditions. It was observed that red soil was able to produce maximum yield of PHB.*

KEYWORDS: *poly-β-hydroxybutyrates (PHB), PHV, PHA.*

I. INTRODUCTION

Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic, there are a class of bacterial polyesters collectively called polyhydroxyalkanoates (PHAs), accumulated intracellularly as reserve granules by many bacteria in harsh environmental conditions[1]. PHB was first isolated and characterized in 1925 [2]. PHB is primarily a product of carbon assimilation and is used by micro-organisms as a form of energy storage molecule [3]. It can be made into many forms and shapes. PHB & PHV (polyhydroxy valeric acid) are being developed for a ariety of applications[4]. PHB differentiates itself from other biodegradable plastics it has unique properties like insoluble in water, highly resistant to hydrolytic degradation, oxygen permeability, UV resistant, other biodegradable plastics are moisture sensitive and water soluble.PHB is poor resistance to acids and bases, soluble in chloroform and other chlorinated hydrocarbons and biocompatible and hence it is suitable for medical applications.

poly-β-hydroxybutyrate (PHB) is synthesized as an intracellular storage material and accumulates as distinct white granules during unbalanced growth in the cell, these are clearly visible in the cytoplasm of the cell. During the adverse conditions PHB is used by the cell as an internal reserve of carbon and energy. Many bacteria including those in the soil, are capable of PHB production and breakdown [5]. A series of enzymes, synthetases or depolymerases, are implied in the biosynthesis and biodegradation of poly-β-hydroxybutyrates and also of other polyhydroxyalkanoates [6]. These biodegradable polyesters display a special interest due to their possible use as substitutes of common plastics because they are completely degraded by the microoganisms present in the environment and they can be produced from regenerable carbon sources [7,8]. Alicaligens eutrophs H_{16} is a facultative autotroph and can grow rapidly in simple media, for PHB prod it requires anaerobic conditions with Co_2 and N source [9]. Molecular structure of PHB doesn't depend on the features of the strain and conditions of carbon nutrition of microorganisms producing PHB[10]. Most of the bacteria which produce PHB are nitrogen-fixing microorganisms. The Azotobacter species fix the molecular nitrogen and have the capacity to accumulate polyhy-β-droxybutyrates when they are grown on different carbon sources, including sucrose media[11]. The cysts forme during adverse conditions germinate under favourable conditions to give vegetative cells, produce polysachharides. These bacteria utilize atmospheric nitrogen (N2) for their cell protein synthesis

The study focused on the producing of poly-β-hydroxybutyrate (PHB) granules by strains isolated from different soil samples in AP. There were Screening, isolation, and optimization techniques done for the bacteria by using various techniques. The poly-β-hydroxybutyrate (PHB) granules production was tested by using various sources of C & N, C:N ratios, concentrations of C& N used, and the effect of PH.
It was noticed that most of the soils had the PHB producing strains. Maximum density of granules was recorded from the red soil.

II. MATERIAL AND METHODS

Isolation of PHB from different cultures:

The bacteria used in this study was collected from Soil samples like Vegetable soil, Paddy Field soil, Sunflower field soil, Red soil, Hussein sagar lake soil , for screening of best PHB producing bacteria and the below mentioned media is used as nutrient source. Various samples which were collected were serially diluted and the 10^{-5} dilution was plated on nutrient media, with Peptone- 2gm, Yeast extract-2gm, NaCl-1gm, Agar-4gm, Distilled water- 1lt, the media was then autoclaved at 121 ^{0}c for 20mins at 15lbs pressure to avoid contamination. These plates containing soil sample were incubated overnight. Among the four soil samples which were used RED soil gave more number of isolated single colonies. Ten colonies from red soil were again grown in nutrient broth and then again incubated overnight and then preserved for rest of the experiment

Screening for PHB producing bacteria:

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, nutrient agar media supplied with 1% of glucose was autoclaved at 121^{0}c for 20 min at 15lbs 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.

Quantification of PHB production and selection of isolates:

All the Sudan Black B positives isolates were subjected to quantification of PHB production as per the method of Jhon and Ralph (1961). The bacterial cells containing the polymer were centrifuged at 10,000 rpm for 10 min and the pellet was washed with equal volume of acetone and ethanol to remove unwanted materials. The pellet was resuspended into 4% of sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged and the supernatant was discarded. The cell pellet containing PHB was again washed with equal volume of acetone and ethanol. Finally the pellet containing the polymer granules were dissolved in hot chloroform. The chloroform was filtered, and to the filtrate 10ml of concentrated hot sulphuric acid was added. The addition of sulphuric acid converts the pellet into crotonic acid which is brown in color. The solution was cooled and the absorbance was read at 235nm against sulfuric acid as blank.

III. SREENING TECHNIQUES

Gram Staining

Twenty four hours old culture was gram stained and the slide was observed under microscope for gram reaction. IMViC ,oxidase and peroxidase tests were also done

IV. GELATIN LIQUIFICATION

The isolates were inoculated on gelatin agar slants at 370C, for 2 days.

Optimization of Cultural Parameters for maximum PHB production:

Different factors affecting PHB production by the selected bacterial isolates were optimized.

Effect of different carbon sources on PHB production:

The selected bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth with different carbon sources like glucose, fructose, sucrose, maltose and cellulose. The flasks were incubated at 30^{0}C on a rotary shaker (150 rpm) for 48 hours. After incubation, PHB produced by the isolates were quantified spectrophotometerically following the method of John and Ralph (1961).

Effect of different nitrogen sources on PHB production:

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth with the best carbon source, and different 'N' sources were used like ammonium sulphate, ammonium chloride, ammonium nitrate and yeast extract, all at 1.0 g/l concentration. After 48 h, PHB yields were quantified.

Effect of different concentrations of the best N sources on PHB production:

The bacterial isolates were grown in 250 ml conical flasks, containing 100 ml MSM broth having the best carbon source and different concentrations of the best N source i.e. 05, 1.0 and 1.5 g/L. After 48 hr, PHB yields were quantified.

Effect of pH sources on PHB production:

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth containing the best carbon source and the best N source at the optimum concentration. Different pH of media were maintained i.e. 6.0, 7.0 and 8.0 and incubated. After 48 h, PHB produced were quantified.

Effect of different C:N ratios on PHB production:

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth with different C:N ratios i.e. 10:1, 15:1, 20;1 and 25:1 using the best C and N sources and incubated on a rotary shaker (150 rpm) at 300C. After 48 h, PHB yields were quantified

V. RESULTS

Screening for PHB producing bacteria:

Black color colonies were taken as positive result.

Screening for PHB producing bacteria

Table No: 1

S. No.	Sample description	No of bacteria isolated	No. of Sudan black positive isolates
1	Vegetable soil	11	4
2	Paddy Field	20	8
3	Sunflower field	15	7
4	Red soil	10	4
5	Hussein sagar lake	12	2

Quantification of PHB production and selection of isolates:

Selected isolates for PHB production

Table No: 2

S.No	Source	Isolates	Yield (g/100ml)
1	Red Soil	R3	0.180
2	Red Soil	R9	0.160

Screening Test:

Table No: 3

S.No	TEST	Result(R3)	Result(R9)
1.	Gram Staining	Gram negative rods	Gram negative rods
2.	Gelatin Liquification	Negative	Negative
3.	Citrate Test	Positive	Positive
4.	Indole Test	Positive	Positive
5.	Methyl Red	Positive	Positive
6.	Voges-Proskauer	Negative	Negative
7.	Oxidase	Positive	Positive
8.	Peroxidase	Positive	Positive

Colonies of PHB producing bacteria

VI. OPTIMIZATION OF CULTURAL PARAMETERS FOR MAXIMUM PHB PRODUCTION

Effect Of Different Carbon Sources On Phb Yield

Table No: 4

S.N0	Isolates	Glucose	Fructose	Sucrose	Maltose	Cellulose	Mean yield g/100ml
1	R9	0.430	0.290	0.110	0.00	0.00	0.276
2	R3	0.500	0.410	0.160	0.00	0.00	0.356

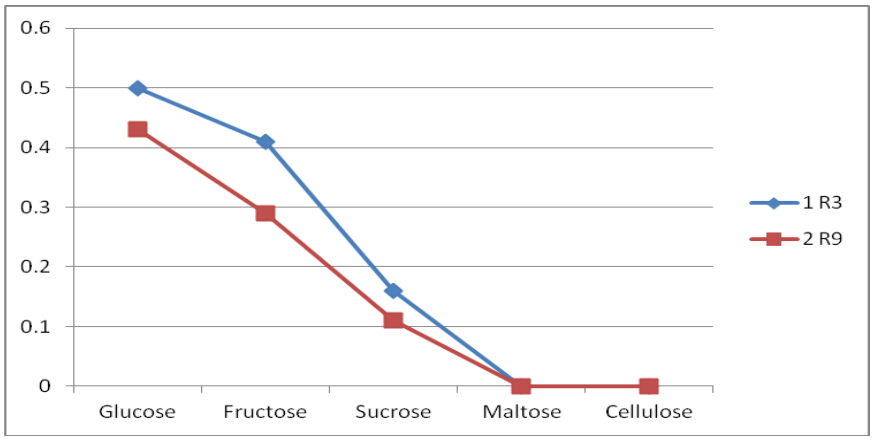


Fig No:1

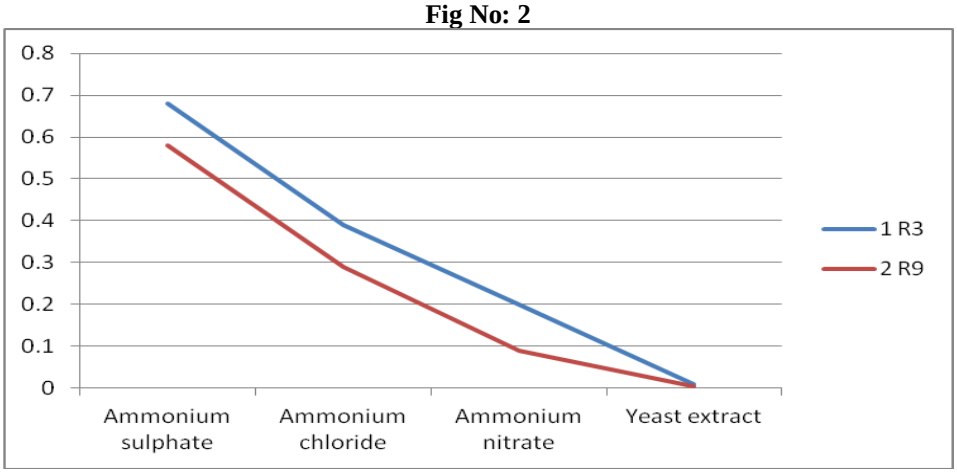
Maximum yield was seen when glucose was supplied as carbon source in both R3 and R9.

VII. EFFECT OF DIFFERENT N SOURCES ON PHB YIELD BY THE SELECTED ISOLATES

Table No:-5

S.No	Isolates	Ammonium sulphate	Ammonium chloride	Ammonium nitrate	Yeast Extract	Mean yield in gm/100ml
1	R3	0.680	0.390	0.200	0.008	0.241
2	R9	0.580	0.290	0.090	0.004	0.320

Fig No: 2



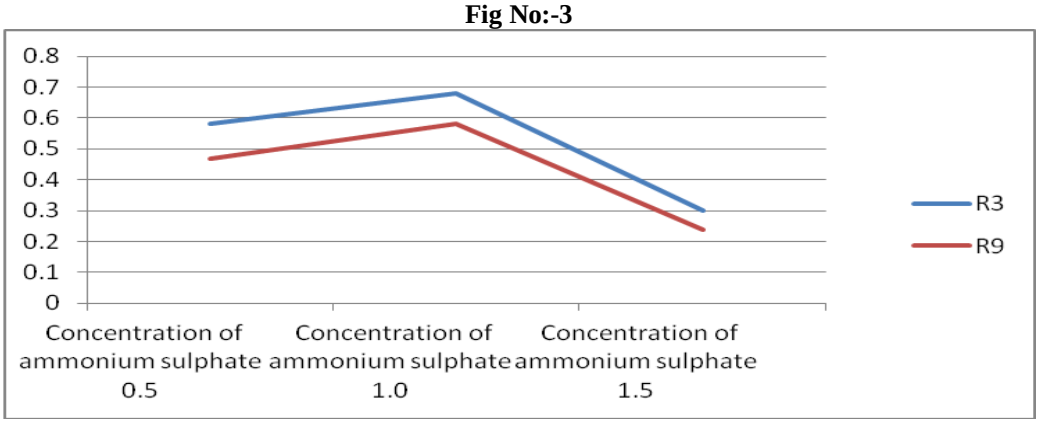
Maximum yield of the bacteria was seen when ammonium sulphate was used as nitrogen source.

VIII. INFLUENCE OF DIFFERENT CONCENTRATIONS OF AMMONIUM SULPHATE (THE BEST N SOURCE) ON PHB YIELD BY THE SELECTED ISOLATES

Table No:- 6

S.no	Isolates	Concentration of Ammonium Sulphate			Mean yield in gm/100ml
		0.5	1.0	1.5	
1	R3	0.580	0.680	0.300	0.52
2	R9	0.470	0.580	0.24	0.43

Fig No:-3



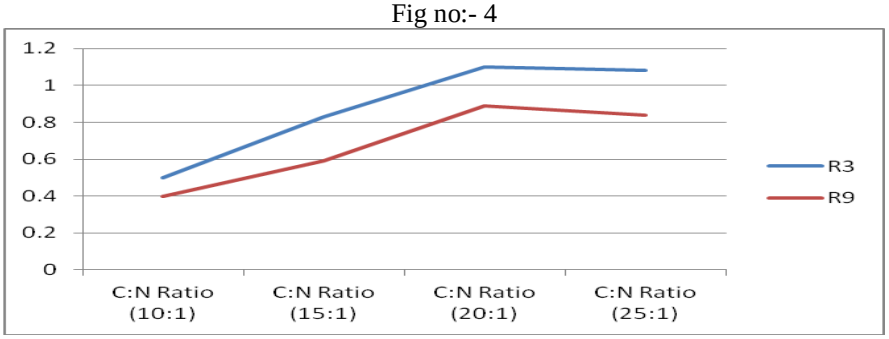
When the concentration of ammonium sulphate was 1.0 the PHB synthesizing bacteria gave maximum yield.

IX. EFFECT OF DIFFERENT C : N RATIOS ON PHB YIELD BY THE SELECTED ISOLATES

Table no:-7

S.No	Isolates	C:N-	C:N-	C:N-	C:N-	Mean yield gm/100ml
		10:1	15:1	20:1	25:1	
1	R3	0.500	0.827	1.100	1.080	0.877
2	R9	0.400	0.590	0.890	0.840	0.680

Fig no:- 4



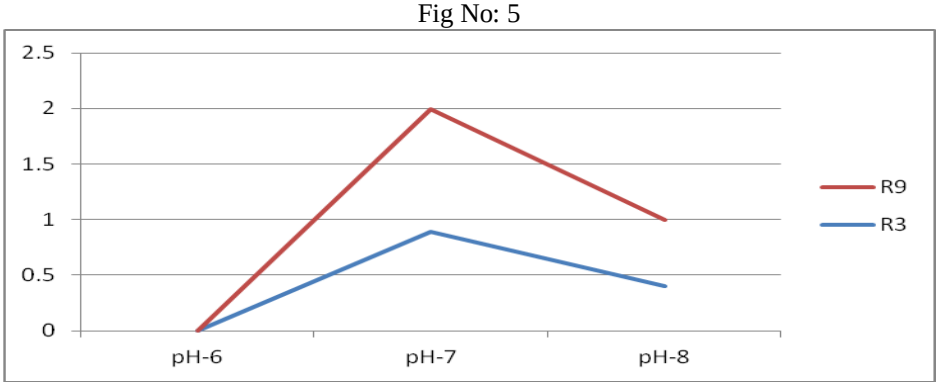
Maximum production of bacteria was seen when C:N ratio is 20:1.

X. EFFECT OF DIFFERENT PH LEVELS ON PHB YIELD

Table no:- 8

S.No	Isolates	pH-6	pH-7	pH-8	Mean yield in gm/100ml
1.	R-3	0.00	0.89	0.40	0.430
2	R-9	0.00	1.10	0.60	0.567

Fig No: 5



Maximum yield of bacteria was seen when the pH is 7.

XI. CONCLUSION

Among the 5 soil samples which were taken Red soil gave the maximum result which was tested using quantitative test ,10 best colonies were isolated among them 6 colonies gave a positive result to PHB producing bacteria and R3 and R9 gave the maximum result. The physical and chemical parameters of R3 and R9 were altered and checked for the maximum yield.

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