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Studies on the production and optimization of PHB (polyhydroxy butyrate) using *Pseudomonas aeruginosa* and *Rhizobium spp.*

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Abstract: In this present study bacterial species were isolated from the soil sample. Among the bacterial isolates *Pseudomonas aeruginosa* and *Rhizobium* spp. were identified based on the cultural, morphology and biochemical characteristics. PHB production of test isolates were screened by sudan black B staining (slide and plate method).The isolated organism *Pseudomonas aeruginosa* and *Rhizobium* spp. was grown in fermented medium. Among this study high amount of PHB obtained from *Rhizobium* spp. compared with *Pseudomonas aeruginosa.* PHB production was optimized by different parameter such as carbon, nitrogen and various pH and temperature. Maximum PHB production was observed at pH 7, temperature 35^0 C, Sucrose as the carbon source and potassium nitrate as the nitrogen source . From our study concluded that high amount of PHB obtained from *Rhizobium* spp. compared with *Pseudomonas aeruginosa.* Among these, *Rhizobium* spp. *highly* recommended for commercial production of PHB.

Key words: PHB, *Rhizobium, Pseudomonas aeruginosa,* Optimization

Introduction

Fast growing *Rhizobia* are able to synthesize a variety of polymers as bacteroids or free living cells. *Rhizobia* mainly excrete high molecular weight polysaccharides. The biosynthesis of different polymer by *Rhizobia* depends on genetic characteristics and cultural condition of the strain (Sutherland, 1985).

The ability of bacteria to form intracellular storage granules composed of PHA in general and PHB in particular has been used as a parameter for the taxonomic classification of the Genus *Pseudomonas* (Palleroni, 1977).

PHB is a carbon storage polymer widely distributed among prokaryotes including *Rhizobium, Bradyrhizobium* and nodule bacteroids. In recent years, PHB And other PHAs have been considered commercially important because of their possible use as biodegradable thermoplastic (Lee, 1996). Poly hydroxyl butyrate a representative compound of the family of PHA, has many potential applications in medicine, Veterinary practice and agricultural due to its biodegradability and biocompatibility (Wang and Yu, 2007). PHB is widely distributed intracellular reserve substance typical of prokaryotes.

PHB exists in the cytoplasmic fluid in the form of crystalline granules about 0.5 µm in diameter and can be isolated as native granules or by solvent extraction (Anderson, 1990; Page, 1992).PHB has been identified in more than 20 bacterial genera including *Azotobacter, Bacillus, Beijerinckia, Alcaligens, Pseudomonas, Rhizobium and Rhodospirillum* (Kato *et al.,* 1992).PHB is produced by microorganisms (like *Alcaligenes eutrophus* or *Bacillus megaterium*) apparently in response to conditions of physiological stress.

Plastic materials have become a integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. Recently problems concerning the global environment and solid waste management have created much interested in the development of biodegradable plastics (Anderson,1990). Poly α- hydroxybutyrate is an alternative source of the plastics which has similar physical properties like polypropylene and it can be easily biodegradable aerobically and anaerobically Hankermeyer (1998).

Petroleum derived plastics are widely used in our daily lives, but they cause environmental pollution because they are persistent for long years. Because of this biodegradable microbial plastics are emphasis to replace the plastics. Hence the present study aimed at poly-α-hydroxy production, which is an important raw material for microbial plastics (Page, 1992).

Materials and Methods

Isolation of bacteria: Soil samples were collected from municipality waste disposal area. Isolation of bacteria was done by serialdilution. Yeast Extract Mannitol Agar (YEMA) medium is specifically used for Rhizobial growth. The YEMA medium were prepared and inoculated with serially diluted soil sample from 10^{-5}, 10^{-6}, 10^{-7} dilutions. The plates were incubated at 37°C for 48-72 hr. Nutrient agar medium was used for *Pseudomonas aeruginosa.*

Identification of bacterial isolates: The isolated bacterial strains were identified based on their cultural morphological and biochemical characteristics were conducted by the following methods as described by Cappuccino and Sherman (1999) to identify the bacteria.

Screening of PHB: The production of PHB by the bacteria can be confirmed by staining with sudan black method. Both slide and plate methods were performed (Kitamara and Doi, 1994).

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Slide method: -- Sudan black B- 0.5 g
-- Ethylene glycol -100 ml.

The culture heat fixed on a slide and immersed in 0.5% (w/v) sudan black B staining with ethylene glycol for 5 min. Then the slide was air dried, the excess amount of stain was destained using xylene several times and blot dries with absorbant paper. Then the counter stain (0.5% w/v safrannin) was added for 5 to 10 seconds. The slide was washed with tap water and dried. The stained cells were observed under oil immersion microscope.

Plate method: -- Sudan black B- 0.03 g
-- 96% ethanol-100 ml.

Nitrogen limited agar plates were prepared and the test culture was inoculated by using spread plate method. The plates were incubated at 37°C for 48 to72 hr. After incubation the plates were flooded with ethanol solution containing sudan black B for 20 min. Later the solution was drained off and observed for screening of PHB producers.

Extraction of PHB: PHB was extracted from the fermentation medium after incubation by the method of Ramasy *et al.* (1994).After incubation, 10ml of culture was centrifuged at 4000 rpm for 35 min. the supernatant was discarded. The pellet was treated with 10 ml of sodium hypo chloride and the mixture was incubated at 37°C for 2 hr. After incubation, the mixture was centrifuged at 4000 rpm for 20 min and then washed with distilled water, acetone, methanol respectively for washing and extraction and centrifuged. The pellet was resuspended in 5 ml of chloroform and evaporated the chloroform by pouring the solution on sterile tray and kept in hot air oven at 4 °C. After evaporation, the final product obtained as PHB in powder form will be 99% pure.

Assay of PHB: The chloroform containing PHB was treated with 5 ml concentration sulphuric acid and boiled at 100 °C for 10 min. Concentration sulphuric acid will reacted with PHB and form crotonic acid, by the method of Lee *et al.* (1995).

Effect of pH and temperature for PHB production: The production of PHB was analyzed in different pH and Temperature for the each organism. The fermentation medium were prepared at various pH level, (5, 6, 7, 8 and 9) adjusted by HCl and NaOH and test organism were inoculated in each test tubes, and incubated for 4 days. At the same time different temperature used for the production of PHB (25°C, 30°C, 35°C, 40°C and 45°C).

Effect of carbon and nitrogen sources for PHB production: The production of PHB was analyzed in two different carbon and nitrogen sources separately for the each organism. The test organism were inoculated in a fermentation medium at various carbon sources such as sucrose, mannitol and maltose, and nitrogen sources such as ammonium sulfate, potassium nitrate and glycine all medium were incubated at 4 days for PHB production.

Statistical analysis: The results obtained in the present investigation were subject to statistical analysis like Mean($\bar{x}$) and Standard Deviation (SD) by Zar(1984).

Results

Identification of isolated bacteria: In the present study the selected two bacterial strains were identified based on the cultural, morphological and biochemical characteristics. Based on the isolated bacterial strains BC1and BC2 were confirmed as *Pseudomonas aeruginosa* and *Rhizobium spp* respectively (Table1).

Screening of PHB production: The PHB productions were screened in *Pseudomonas aeruginosa* and *Rhizobium spp* by plate and slide methods using Sudan black staining. In plate method blue blackish granules was appeared in pink colour cytoplasm. In slide method cytoplasm appeared blue colored, the blue colored granules observed in the cells.

Assay of PHB production: PHB productions were analyzed in *Pseudomonas aeruginosa* and *Rhizobium* spp. using fermentative

Table – 1: Morphological and biochemical characterization of isolated bacteria

Morphological and biochemical characters	BC1	BC2
Cultural characters	Pyocyanin pigment	Milky white
Gram	Negative	Negative
Staining	Motile	Motile
Motility	Rod	Rod
ShapeIndole production test	-	+
Methyl red test	-	-
Voges-proskauer test	-	+
Citrate utilization test	+	+
Catalase test	-	+
Oxidase test	+	-
Urease test	-	+
Carbohydrate fermentation		
Sucrose	+	-
Mannitol	-	+

\+ postivie - negative

Based on the morphological and biochemical characteristics the isolated organisms were identified as
BC1- confirmed as *Pseudomonas aeruginosa*
BC2- confirmed as *Rhizobium* spp.

Table – 2: Assay of PHB production

Microorganisms	Dry cell weight (g L^{-1})	PHB production (g L^{-1})	Yield of PHB (%)
Pseudomonas aeruginosa	0.17± 0.08	0.043± 0.01	25.29
Rhizobium spp.	0.21± 0.02	0.062± 0.02	29.92

Values are represented as Mean ± Standard Deviation

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Table-3: Effect of pH and temperature for PHB production by *Rhizobium* spp.

Factors pH and temperature(°C)	Dry cell weight (g L^{-1})	PHB production (g L^{-1})	Yield of PHB (%)
5	0.11± 0.04	0.034± 0.01	30.90
6	0.23± 0.03	0.072± 0.02	31.30
7	0.13± 0.05	0.041± 0.02	31.53
8	0.14± 0.05	0.043± 0.01	30.71
9	0.12± 0.06	0.031± 0.01	25.83
25°C	0.13± 0.05	0.032± 0.01	24.61
30°C	0.14± 0.02	0.042± 0.01	30.00
35°C	0.21± 0.06	0.071± 0.02	33.80
40°C	0.12± 0.04	0.037± 0.01	30.83
45°C	0.11± 0.06	0.033± 0.01	30.00

Values are represented as Mean ± Standard Deviation

Table – 4: Production of PHB of *Rhizobium* spp. on media with different carbon sources and nitrogen sources

Carbon and nitrogen sources	Dry cell weight (g L^{-1})	PHB production (g L^{-1})	Yield of PHB (%)
Maltose	0.15±0.04	0.0422±0.01	28.13
Mannitol	0.14±0.02	0.0414±0.01	29.57
Sucrose	0.21±0.02	0.0720±0.02	34.28
Ammonium nitrate	0.15±0.03	0.039±0.01	26.00
Glycine	0.13±0.04	0.022±0.01	16.76
Potassium nitrate	0.21±0.01	0.052±0.01	24.76

Values are represented as Mean ± Standard Deviation

medium. The results were presented in Table 2. Among this study maximum PHB production were recorded in *Rhizobium* spp. (0.062 g L^{-1}) when compared than *Pseudomonas aeruginosa* (0.043 g L^{-1}) highest yield efficiency also recorded in *Rhizobium spp.* (29.92 g L^{-1}).

Effect of pH, temperature, carbon and nitrogen sources for PHB production: PHB productivity was estimated in various pH ranges such as 5, 6, 7, 8 and 9. Highest PHB productivity was observed in pH range 7 (31.53 g L^{-1}). Compared than other pH range 6 (31.30 g L^{-1}) lowest PHB recorded in pH 9 (25.83 g L^{-1}) and pH 5, 8 (30.90; 30.71 g L^{-1}) respectively. PHB productivity were analyzed in various temperature such as 25°C , 30°C, 35°C , 40°C and 45°C .Maximum PHB productivity were recorded in temperature 35 °C (0.071 g L^{-1}) compared than other temperature 30 °C, 40 °C, 45 °C (0.042, 0.037 and 0.033 g L^{-1}). At the same time lowest PHB production recorded in temperature 25 °C (0.032 g L^{-1}).The finding results presented in the Table 3.

PHB productivity was analyzed in various carbon sources such as Maltose, Manital and Sucrose. Highest PHB productivity was observed in carbon source sucrose (0.0720 g L^{-1}) compared than other carbon sources maltose (0.0422 g L^{-1}) lowest PHB recorded in manitol (0.0414 g L^{-1}). .The maximum PHB 0.97% was produced at 2% Sucrose. PHB productivity were analyzed in various nitrogen sources such as ammonium sulfate, potassium nitrate and glycine. Highest PHB productivity was observed in nitrogen sources potassium nitrate (0.052 g L^{-1}) compared than other nitrogen sources ammonium sulfate (0.039 g L^{-1}) Lowest PHB recorded in glycine (0.022 g L^{-1}). The finding results presented in the Table 4

Discussion

Many workers have explored various easy available agro-industrial wastes as carbon sources, such as, sugarcane molasses, date syrup, soy molasses for the production of PHA employing *Bacillus* (Gouda *et al.,* 2001; Fullet *et al.,* 2006).

In the study was correlated with the finding of Belma Aslim *et al.* (2002) reported that the amount of PHB in strains of *Rhizobium* was 0.05 g $L^{-1}$and the percentage of PHB in these cells was between 1.38 and 40.0% of dry cell weight. The lowest PHB productivity was found in 1.38%. It was found that a significant relationship existed between dry cell weight and PHB production**.**

Kitamara and Doi (1994) reported the staining of PHB producing bacteria such as *P.oleovorans*, *P.putida* and *Alcaligenes eutrophus* appeared as light white to blue coloured colonies in plate method. Mercan *et al.* (2002) investigated the effect of different nitrogen and carbon sources of PHB production in *Rhizobium* species showed that L- glycine and L- cystine enhanced PHB production comparatively.

Effect of different nitrogen and carbon sources and pH on exopolysaccharide and PHB production in two strains of *R.meliloti* were observed. These two strains showed different growth rates in the medium. They also noted that there was a decrease in PHB content in the medium with an acidic pH in the medium with fructose and yeast extract , the PHB yield was 85% (Tavernier *et al.,* 1997)**.**

The highest level of PHB accumulation was observed in the medium with sucrose as carbon sources in *Bacillus subtilis*

(19.15%), *B. megaterium* (19.49%) by Nur Yuksekdag *et al.* (2004)

PHB accumulating *Bacillus* sp., when grown on the media supplemented with 2% glucose, where as (Rohini *et al.,* 2006) reported 64.10% PHB of CDW from soil bacteria when grown on the media supplemented with glycerol.

From our study finally concluded that high amount of PHB production was obtained from *Rhizobium* .Now-a- days plastics and synthetic polymers are mainly produced from hydrocarbons, which don't decompose, thus resulting environmental pollution. How ever the production of PHB will produce a biodegradable plastic. In our future study we are mainly concentrate on recycling agro-industrial wastes can reduce cost of production of PHB and minimize the environmental pollution.

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References

Anderson, A. J. and Dawes, E.A.: Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev*., **54:** 450 -472 (1990).

Belma Aslim., Yuksekdag, Z. and Beyalti.: Production of poly-â-hydroxybutyrate (PHB) by some *Rhizobium Bacteria. Turk. J. Biol.,* **26**: 215-219 (2002).

Cappuccino, G.G. and Sherman, N.: Microbiology a Laboratory Manual Rockland Aommunity College, Suffern: New York. 149-214(1999).

Fullet, T.D., Jung,D.O., Madigan, M.T.: Production of poly-a-hydroxyl alkanoates from soy molasses oligosaccharides by new, rapidly growing Bacillus species. *Lett. Appli. Microbiol.,* **43**: 377-384 (2006).

Gouda, M.K., Azza, E., Swellam, A.E., Sanaa Omer, H.: Production of PHB by *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. *Microbiol. Res.,* **156**: 201-207 (2001).

Hankermeyer, C.R. and Jieeradema, R.S.: PHB plastics degrarded by microorganisms. *Appl. Environ. Microbial.,* **64**: 2859-2869 (1998).

Kato, N., Konishi, H., Shimao, M and Sankazawa, C.: Production of 3 hydroxybutyric acid trimer by *Bacillus magaterium. J. Ferm and Bioerg.,* **73**: 246-247 (1992).

Kitamara. S. and Doi, Y.: Staining methyl of PHA producing bacterial by nile blue. *Biotechnol. Techniques.,* **8**: 345-350 (1994).

Lee,S.Y., Chang, H.N and Park,Y.H.: A simple method for recovery of microbial PHB by alkaline solution treatment. *J. Microbial. Biotechnol*., **82**: 431-438 (1995).

Lee, S.Y.: Bacterial poly hydroxyalkanoates. *Biotech. Bioengineering*, **49**: 1-14 (1996).

Mercan, N., Aslim, B., Yuksekdag, Z.N and Beyati.: Production of Poly- â-hydroxybutyrate (PHB) by some *Rhizobium* bacteria. *Turk.J. Biol*., **26 :** 215-219 (2002).

Nur Yusekdag, Z., Belma Aslim., Yavuz Beyatli and Nazime Mercan: Effect of carbon and nitrogen sources and incubation times on poly – B-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* and *Bacillus megaterium. African J. Biotechnology,* **3**: 63-66 (2004).

Page, W.J.: Production of poly- α- hydroxybutyrate by Azotobacter vinelandii UWD in media containing sugars and complex nitrogen sources. *Appl. Microbiol. Biotechnol*., **38**: 117-121 (1992).

Palleroni, N.J.: *Pseudomonas. In*: A.I. Laskin, H.A. Lechevalier (Ed.) CRC Handbook of Microbiology. Viol. 1. CRC press, Inc, Cleveland . pp. 246-258 (1977).

Ramsay, R., Berger, E and Chaverie, A.: Extraction of PHB using chlorinated solvents. *Biotechnology Techniques*, **8**: 589-594 (1994).

Rohini, D., Phadnis, S., and Rawal, S.K.: Sythesis and characterization of Poly A- hydroxybutyrate from *Bacillus thuringiensis* R1. *Indian J. Biotechnol*., **5**: 276-283 (2006).

Sutherland, I.W.: Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. *Annu. Rev. Microbiol*., **39**: 243-270 (1985).

Tavernier,P., Portais, J.C., Sacvedo, J.E.N., Courtois,J., B and Barbotin, J.N.: Exopolysaccharide and poly-α- hydroxyl butyrate coproduction in two *Rhizobium meliloti* strains. *Appl. Environ. Microbiol.,* **63**: 21-26 (1997).

Wang ,J. and Yu , H.Q.: Biosynthesis of polyhydroxy butyrate and extracellur polymeric substances(EPS) by *Ralstonia eutropha* ATCC 17699 in batch cultures . *Appl. Microbiol. Biotechnol.,* **75** : 871-878 (2007).

Zar, J.H.: Biostatistical Analysis. *In*: N.J. Englewood Cliffes (Ed.), Prentice hall, Inc (1984).

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