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Studies on the Productivity of Poly-β-hydroxybutyrate by *Alcaligenes eutrophus* and *Rhizobium meliloti* using Waste Substrate

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Poly-β-hydroxybutyrate (PHB) is polymer from microbial origin are considered good substitute for plastics and isomers. Since in properties they are similar to petrochemical plastics, yet are truly biodegradable. The two potential bacteria *Alcaligenes eutrophus* and *Rhizobium meliloti* PHB production ability were analyzed using molasses as substrate at various concentrations in the fermentative medium (10%, 20%, 30% and 40%). Among this study maximum PHB was noted in high concentration of molasses (40%) in both bacteria, compared with *Rhizobium meliloti* and *Alcaligenes eutrophus* produce high amount of PHB in 40%. *Alcaligenes eutrophus* were optimized for two different carbon source (Sucrose and Maltose), nitrogen source (Ammonium chloride and Sodium nitrate) and various pH (5, 6, 7, 8 and 9). Among this maximum PHB was accumulated in maltose as carbon source, ammonium chloride as nitrogen source and 7 as pH. Thus the use of petroleum derived plastics can be minimized and by which we can live in a plastic pollution free earth.

Key words: *Alcaligenes eutrophus*, *Rhizobium meliloti,* Poly-β-hydroxybutyrate (PHB), Biopolymer, Molasses.

Plastic materials have become an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. These non-degradable plastics accumulate in the environment at a rate of millions of ton per year. Recently problems concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics[1]. Poly-ß-hydroxybutyrate (PHB) is an alternative source of the plastics which has similar physical properties like polypropylene and it can be easily biodegradable aerobically and anaerobically[2].

PHB is one of the important storage reservoirs providing energy. It is the cellular inclusion bounded by lipid non-unit membrane separate from cytoplasm. Bete-hydroxy butyrate is connected by easter linking and form PHB[3]. It

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is now well recognized that this lipid inclusion is accumulated by many bacteria as they enter the stationary phase of growth to be used later as an internal reserve of carbon and energy[4]. The aliphatic PHB as a granular component in bacterial cell proceed without any of the controversies, which marked the recognized as a prototypical biodegradable thermoplastic to solve the waste disposal challenge[5]. A major drawback of the commercialization of PHB is their much higher of production cost compared with petrochemical based synthetic plastic materials[6].

The main aim of the work is production of PHB by *Alcaligenes eutrophus* and *Rhizobium meliloti* by using industrial waste as cheap substrate (molasses) to minimize the production cost of the PHB and production of PHB in various parameters such as carbon source, nitrogensource and pH. The two gram negative bacteria and by which making the pollution free environment from non –biodegradable plastic.

MATERIAL AND METHODS

Soil sample collection

Soil sample were collected from municipality waste disposal area of Peravurani at Thanjavur (Dt). Soil was taken from 60 cm deep and stored in the polythene bag and the atmospheric temperature was maintained.

Isolation and identification of bacteria

Bacteria were isolated from soil in serial dilution method, 10^{-6} and 10^{-7} dilution were used for identification. The two major isolates obtained from the above sample were identified by morphological and biochemical characteristics. The morphological characteristic was identified by gram staining. The biochemical characteristics were identified by indole test, methyl red test, voges proskaur test, citrate utilization test, urease hydrolysis test, oxidase test, catalase test, carbohydrate fermentation test and hydrogen sulfide production test.

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[7].

Substrate collection

Molasses was collected from EIT parry sugarmill Pvt. Ltd, kurumpur at Pudukkottai (Dt). The sample was stored in a bottle, and used as a substrate for fermentation medium. Nitrogen Limited minimal medium was prepared. Instead of glucose, different concentration of waste molasses such as 10%, 20%, 30% and 40% was added to above mentioned Nitrogen Limited minimal medium and used for fermentation. Microbes utilize molasses as a carbon source. The test organism was grown in nutrient broth medium was transfer to Nitrogen limited mineral medium and industrial waste based medium as a sole carbon source incubated at 37°C for 72hrs in an incubatory shaker. The fermentative medium was maintained at pH 7 to 7.2.

Extraction of PHB[8]

10ml of fermented sample was taken and centrifuged at 4000 rpm for 35 min. The supernatant was discarded and the pellet was treated with 10ml of sodium hypochloride and the mixture was incubated at 37°C for 2 hrs. The mixture was centrifuged at 4000 rpm for 20 min and then washed with distilled water, acetone and diethyl ether respectively. The pellets was dissolved in 5ml of boiling chloroform and then centrifuged at 1500 rpm for 10 min. the bottom phase containing PHB with chloroform collected and was used for assay of PHB.

Assay of PHB

The chloroform containing PHB was treated with 2 ml concentration sulphuric acid and boiled at 100°C for 10 min. The different concentration of crotonic acid was prepared with concentration sulphuric acid and boiled using water bath. The optical density of crotonic acid was read at 230nm using UV spectrophotometer and the standard graph was prepared[9]. The concentration of PHB was determined by plotting the OD with the standard graph of crotonic acid[10].

The PHB production was analyzed in two different carbon and nitrogen source and various pH such as 5, 6, 7, 8 and 9). As a carbon source 2% of sucrose and maltose were used. Similarly for nitrogen source 2% of ammonium chloride, sodium nitrate and various pH level range from pH 5 to 9. The pH adjust by adding 1N NaOH and 1N Hcl solution. Nitrogen Limited minimal medium was separately used for each parameter.

Purification of PHB

The pure form of PHB was collected

using the standard method. 10ml of fermented culture was centrifuged at 4000rpm for 35 min. the supernatant was discarded. The pellet was treated with 10 ml of sodium hypochloride and the mixture was incubated at 37°C for 2hrs. The mixture was centrifuged at 4000rpm for 20min and then washed with distilled water, acetone and methanol respectively for washing and extraction. The pellet was resuspened 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.

Statistical analysis

Mean and Standard deviation were calculated to facilitate the comparison of the data The formula for calculating Mean is

$$\overline{X} = \frac{\Sigma X}{N}$$

Where

Σx = Sum of variable

N = Total number of frequency

The formula for calculating standard deviation (ó) is

$$\sigma = \sqrt{\frac{\Sigma\left(X - \overline{X}\right)^2}{N}}$$

RESULTS AND DISCUSSION

The bacterial isolate were identified based on the cultural, morphological and biochemical characteristics. The isolated bacterial colonies confirmed as *Alcaligenes eutrophus* and *Rhizobium meliloti* (Table 1).

PHB production of *Alcaligenes eutrophus* and *Rhizobium meliloti* were screened by sudan black staining methods. Blue colour granule was observed with in the cell around the pink colour cytoplasm were noted. The thin layer of PHB extracted from test organisms grown in synthetic medium, industrial waste, and various carbon, nitrogen and pH based medium was estimated. The PHB content was measured at 230nm using UV spectrophotometer. The OD was plotted using crotonic acid stranded graph. The PHB content was expressed in gram (g/l) of culture. *Rhizobium meliloti* grown in synthetic medium (Nitrogen limited mineral medium) produced 0.435 g/l of PHB in culuture. In the molasses, maximum PHB production was seen in 40% substrate concentration (0.387 g/l). *Alcaligenes eutrophus* grown in synthetic medium (Nitrogen limited mineral medium) produced 0.530 g/l of PHB in culture. In the molasses, maximum PHB production 0.594 g/l was seen in 40% substrate concentration. (Table-2a and 2b).

Table 1. of isolated colonies Identification

S. No.	Morphological and Biochemical Characteristics	Sample-1	Sample-2
1	Gram staining	-	-
2	Shape	Rod	Circle
3	Indole test	-	+
4	Methyl red test	-	-
5	Voges proskaeru test	-	-
6	Citrate utilization test	-	+
7	Urease test	-	+
8	Catalase test	-	+
9	H_2S production test	+	+
10	Oxidase test	+	-
11	Carbohydrate fermentation test		
	Glucose	-	+
	Fructose	+	+
	Sucrose	-	+

+: Positive, -: Negative.

Compared with *Rhizobium meliloti* and *Alcaligenes eutrophus* produced in high amount of PHB in both medium. It was optimized for various parameters such as carbon source, nitrogen source and pH. Maltose and Sucrose used as different carbon source for PHB production. Highest PHB production (0.524 g/l) was noted in maltose when compared than sucrose (0.493 g/l, Table 3).

Maximum PHB production was observed in ammonium chloride incorporated (0.572 g/l) when compared with sodium nitrate (0.532 g/l, table-4). PHB production also studied in different pH range. Maximum PHB production was noted at pH 7 (0.492 g/l) when compared with various pH range (Table 5).

Table 2a. Production of PHB in Synthetic Medium

S. No.	Name of the organisms	PHB Concentration (g/l) (M±SD) (n=3)
1.	*Alcaligenes eutrophus*	0.530±0.02
2.	*Rhizobium meliloti*	0.435±0.03

Table 2b. PHB Production in Molasses Medium

S. No	Name of the organisms	Amount of PHB production in different concentration of molasses (g/l) (M±SD) (n =3)			
		10%	20%	30%	40%
1.	*Alcaligenes eutrophus*	0.420±0.002	0.447±0.003	0.567±0.006	0.594±0.004
2.	*Rhizobium meliloti*	0.287±0.003	0.322±0.004	0.355±0.001	0.387±0.001

Table 3. PHB production in different carbon sources

S. No	Carbon source	*Alcaligenes eutrophus* PHB production (g/l) (M±SD) (n=3)
1	Maltose	0.524 ± 0.002
2	Sucrose	0.493 ± 0.004

Table 4. PHB production in different nitrogen sources

S. No	Nitrogen source	*Alcaligenes eutrophus* PHB production (g/l) (M±SD) (n=3)
1	Ammonium chloride	0.572 ± 0.003
2	Sodium nitrate	0.532± 0.002

Table 5. PHB production in different pH

S. No	Various pH	*Alcaligenes eutrophus* PHB production (g/l) (M±SD) (n =3)
1	5	0.295 ± 0.003
2	6	0.397 ± 0.002
3	7	0.492 ± 0.004
4	8	0.483 ± 0.003
5	9	0.318 ± 0.005

The PHB produced from *Rhizobium meliloti, Alcaligenes eutrophus* were purified in powered form. Yellowish white PHB was obtained through the purification strategy. Each extract was tested in triplicated for calculation of mean and standard deviation by[11].

In present studies staining of PHB production of two organisms were observed in slide and plate method. After staining, blue-back colour granule was observed within the cell around the pink colour cytoplasm by slide method. The similar results on staining of PHB producing bacteria and it's appeared as light white to blue colour colonies in plate method[7].

Alcaligenes eutrophus and *Rhizobium meliloti* PHB production was analysed using molasses waste substrate at different concentration level in the fermentation medium (0.594 g/l), when *Alcaligenes eutrophus* was developed in different substrate, high concentrations of PHB 121g/l and total cells 164g/1 was obtained[13]. *Alcaligenes eutrophus* is the most widely used organism for the production of PHB because it is easy to grow, it accumulates large amounts of PHB

up to 80% g dry cell weight in a simple medium, and its physiology and biochemistry leading to PHB synthesis. PHB production by *Bacillus megaterium* grown in cane molasses and corn steep liquor, two of the cheapest substrates available in Egypt[14]. In this present study two different carbon source used for PHB production. Among this two maximum PHB production was noted in maltose compared than sucrose. PHB content in *Bacillus megaterium* has reached a maximum level with glucose. The maximum PHB 0.97% was produced at 2% glucose[15].

In nitrogen sources the highest PHB production was noted in ammonium chloride when compared than sodium sulphate. But the PHB amount was lower in nitrogen sources when compared with carbon sources. Investigated the effect of different nitrogen and carbon sources on PHB production in *Rhizobium sp.,* showed that L-glycine and L-cystine enhanced PHB production comparatively[16]. PHB poduction in a variety of commercially available complex nitrogen sources such as peptone, casitone and phyton. It was found that complex nitrogen sources increased the yield of PHB produced by *Azotobacter vinelandi*[4].

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