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ISOLATION AND PRODUCTION OF POLY-β-HYDROXY BUTYRATES

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Abstract - Polyhydroxybutyrates (PHB) are bio-plastics produced by many microorganisms under carbon rich conditions. PHBs are carbon storage compounds also known as Poly hydroxyalkanoates.PHB's are high in molecular weight, polyester is accumulated as a storage carbon in many species of bacteria and is a biodegradable thermoplastic. Several gram-negative bacteria, including pseudomonas aeruginosa, have been employed for the efficient production of PHB. These gram negative bacteria can release endotoxin(pyrogen), which was in the form of lipopolysaccharides, from the cell wall (Raetz, 1993). Since PHB was most efficiently produced by gram-negative bacteria as described, the endotoxin levels present in the purified PHB should be examined can accumulate PHB in the form of multiple granules of 0.2 to0.5µm size. Pseudomonas species contains high PHB accumulating ability. Pseudomonas species are Gram negative, rod-shaped aerobic bacteria Bacteria were isolated from native environment by culturing on nutrient agar and cetrimide agar media. When stained with Sudan Black Dye black colour granules are observed As a positive result. Poly-beta-hydroxybutyrate can be further used for the production of Bio plastics. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetylcoenzyme to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize. The microbial production of copolymers of 3-hydroxybutyrateand 3- hydroxyvalerate, with properties varying according to copolymer composition, must be considered. The production cost of PHB's are very high when compared. to non-degradable plastics hence search for potential strains that accumulates high PHB's

***Keywords*—Bioplastics, *Polyhydroxyalkonates*, Poly-beta-hydroxybutyrate, Sudab black dye**

I. INTRODUCTION

Biodegradable polyesters are candidates for the development of environmental friendly plastics.
Poly-β-hydroxybutyrate (PHB) is a type of polyester from the hydroxyalkanoates family, synthesized by bacteria as an intracellular material and accumulated as granules in the cytoplasm. The aim of this study was to isolate Poly β-hydroxybutyrate over producing bacteria and optimize the production medium.

Biodegradable plastics can be produced from renewable resources. Many microorganisms accumulate PHAs in the form of intracellular granules as carbon supplies, exploitation of which could contribute significantly to maintenance of clean and green environment.

Biodegradable polyesters are used to maintain clean and Green Environment. The objectives are

- To isolate Pseudomonas from native environment and production of poly hydoxy butyrate.
- Extraction of PHB accumulating bacteria.
- Production of biodegradable plastic from Poly hydroxybutyrate
- Recovery process using chloroform, sodium hydroxide and enzyme
- To characterize the recovered PHA
- To develop mass from the recovery process.

II. MATERIALS AND METHODS

Soil sample was collected from municipal dumping yard and is serially diluted

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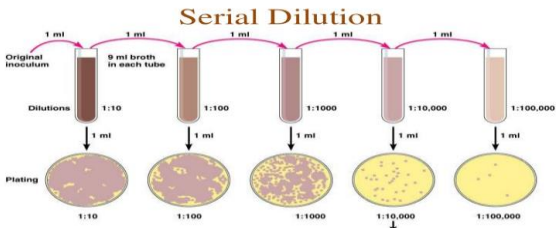

10^{-7} and 10^{-8} dilutions were taken and then inoculated on Nutrient agar plates.
The media was then autoclaved at 121 c for 20mins at 15lbs pressure to avoid contamination and are incubated at 37^0C for 24hrs.
Colonies were found after the incubation.
Large moist colony was taken and is Gram stained

FIG.1 **FIG.2** **FIG.3**

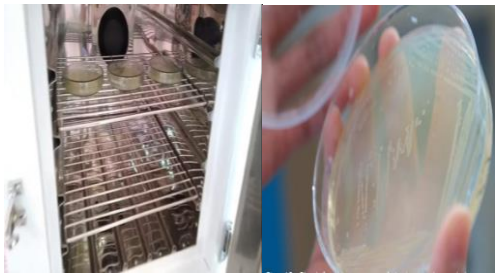

FIG.4 **FIG.5**

GRAM STAINING PROCEDURE
Bacterial smear was prepared on a clean glass slide and heat fixed.
Smear was flooded with crystal violet reagent for 30 sec.
Slide was then flooded with Grams iodine, the mordant for 30 sec.
Slide was then washed with decolorizer 70% ethanol.
Safranine was added to the smear and allowed to stand for 30 sec
The smear was washed under gentle tap water and air dried.
Slide was observed under the microscope.

FIG.6 **FIG.7** **FIG.8**
Bacterial smear was prepared

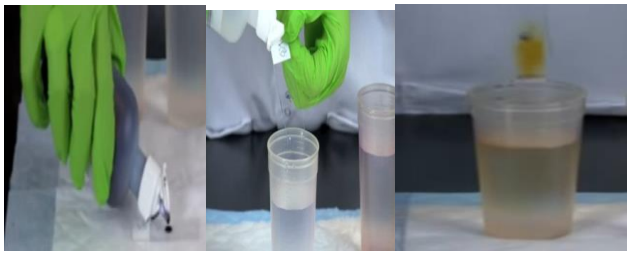

FIG.9 **FIG.10** **FIG.11**
Smear was flooded with Crystal violet **slide was washed With Grams iodine** **slide was flooded**

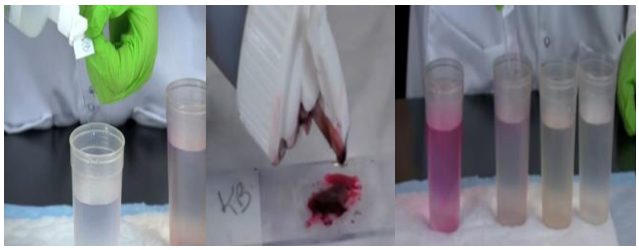

FIG.12 **FIG.13** **FIG.14**
slide was washed with decolorizer **Safranine was added** **smear was washed under gentle tap water**

III. RESULTS AND DISCUSSION

OBSERVATION

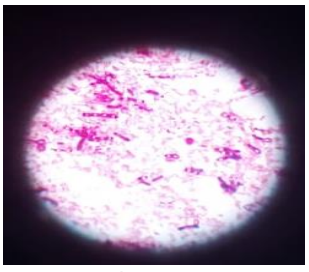

FIG.15
Pink coloured rods were observed under microscope.

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SUB CULTURING

- The isolated bacterial colony was inoculated in nutrient agar for sub culturing and the plate was incubated at37^0C for 24 hrs.
- After incubation the plate was used conduct bio chemical tests as follows.

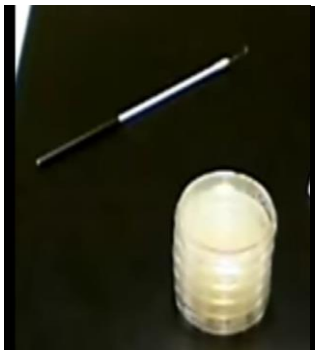

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FIG.16 **FIG.17**

Isolated Bacterial colony was inoculated in nutrient agar for sub culturing

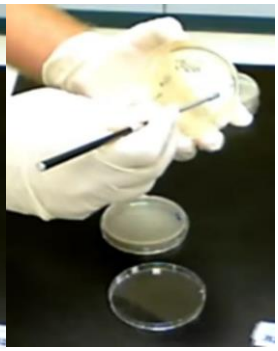

FIG.18 **FIG.19**

BIOCHEMICAL TESTS

INDOLE TEST

PROCEDURE: Indole broth tube was inoculated with culture and was incubated at room temperature for 24hrs then 0.5ml of Kovac's reagent was added to it.

OBSERVATION:

No red color was formed

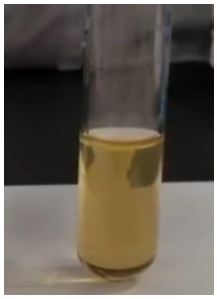

FIG.20

Result: No red colour was found indicating that the organism is Indole Negative.

METHYL RED TEST

PROCEDURE: Glucose phosphate peptone water tube was inoculated with culture and incubated for 24hrs.then 5 drops of Methyl red indicator was added to it.

OBSERVATION: No red color was formed

FIG.21

Result: No red colour was found
indicating the Methyl red test was Negative

VOGES PROSKAUER TEST

PROCEDURE: Glucose phosphate peptone water tube was inoculated with culture and incubated for 24hrs and then add 5drops of Barrit's reagent is added to it.

OBSERVATION: No red colour was observed

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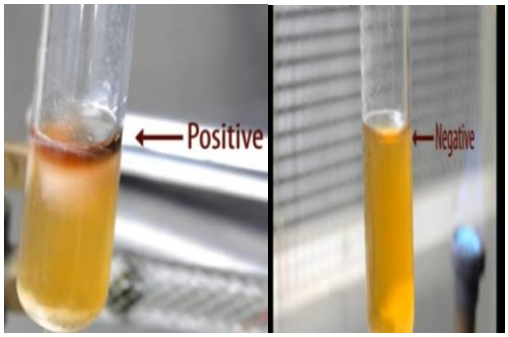

FIG.22

Result: No pink colour was found indicating Voges Proskauer test Negative

CITRATE UTILIZATION TEST
PROCEDURE: Simmon's citrate agar slant was inoculated with the culture and incubated for 24hrs at 37^0C.
OBSERVATION: After incubation the color change of slant from Green to Blue was observed

FIG.23

Result: Colour change of the slant from green to blue was observed indicating that the organism is Citrate Positive

OXIDASE TEST PROCEDURE: On a Nutrient agar plate inoculated with culture, few ml of freshly prepared1% Tetramethyl paraphenylene di amine di hydro chloride was added & decanted.

OBSERVATION: Development of purple colored colonies was seen

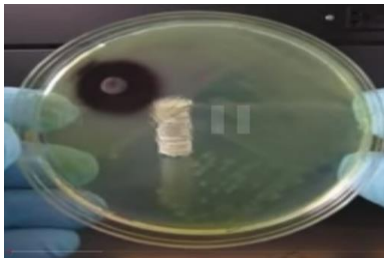

FIG.24

Result: Development of purple colour colonies was seen indicating oxidase Positive.

H_2S PRODUCTION TEST
PROCEDURE: Test organism was stab inoculated inH2s production test medium and incubated for 24 hrs.
OBSERVATION: Blackening of growth region was found

FIG.25

Result: Blackening of growth region was found indicating H_2S production test positive.

CATALASE TEST:
PROCEDURE: Prepare a suspension of agar grown culture in broth either on slide or tube. Add few drops of H_2O_2.

OBSERVATION: The bubble formation was observed.

FIG.26

Result: Bubble formation was seen. It indicates the organism catalase positive

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OBSERVATION

INDOLE TEST	NEGATIVE
METHYL RED TEST	NEGATIVE
VOGES PROSKAUER TEST	NEGATIVE
CITRATE UTILIZATION TEST	POSITIVE
OXIDASE TEST	POSITIVE
H2S PRODUCTION TEST	POSITIVE

TABLE.1

From the results of above bio chemical tests, the isolated organism was identified as "*pseudomonas* Aeruginosa".

The Pseudomonas Aeruginosa species was isolated from the soil from the municipal dumping yard. The samples were cultured on Cetrimide Agar media. Conformatory test for bacteria is done by Black sudan dye. This method is used for the production of PHB's by Pseudomonas Aeruginosa species. Bioplastics made by this process can be easily recycled when compared to simple plastics. The biopolymer Polyhydroxy butyrate (PHB) is polyester produced by certain bacteria processing glucose or starch posses same characteristics similar to Petroplastic polypropelene.

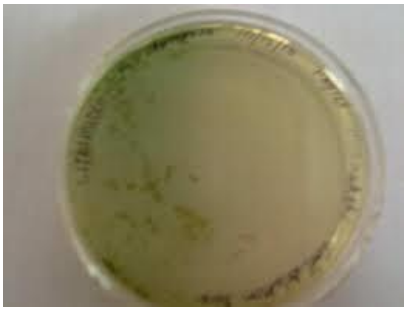

FIG.27

Pseudomonas aeruginosa growing on Cetrimide agar plates

Confirmatory test for poly –beta-hydroxybutyrate (PHB) accumulation by
sudan black dye

The samples were cultured on Cetrimide agar media. The confirmation of bacteria was done by Sudan dye. (Murray et .al1994)

Screening for PHB productionSudan black staining

1. Flame the loop and allow it to cool. Remove the cap from the culture bottle, flame the neck, remove a loopful of broth, flame the neck again and replace the cap.
2. Spread the culture on clean slide ,using the loop .The smear should cover an area about 10 mm x 30 mm. Flame the loop. Allow the smear to dry in the air.
3. Fix the smear by holding the slide with force sand passing it horizontally through a small Bunsen flame 2–3 times. Do not overheat the slide. Heat Fixing kills thebacteria by coagulating the cytoplasm. It also sticksthemto the slide.
4. Place a few drops of Sudan Black solution the fixed preparation.
5. After 5–10 minutes the ethanol in the stain should have evaporated. Any excess liquid can be carefully drawn off using the edge of a piece of filter paper.
6. Immerse the slide in xylene until it is completely decolorized (this takes about 10 seconds).Allow the slide to dry.
7. Flood the slide with the counter stain, Safranine solution.
8. After 10 seconds, gently rinse the slide with running water and allow it to dry again.
9. When the slide is completely dry add a drop of immersion oil directly to the slide (no coverslip is needed). And cells were examined by microscope.
10.In this process lipid inclusion granules are stained blue black while bacterial cytoplasm are stained light pink.

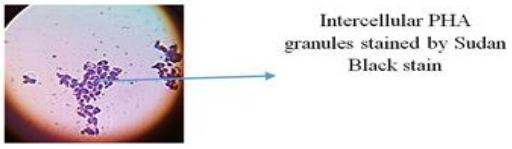

FIG.28

The appearance of black colored granules in the cell indicates PHB production

Table .2

TEST	OBSERVATION	RESULT
Gram staining	Rod shaped ,pink colour bacteria are seen	gram-negative
Oxidase test	purple to black colouration	oxidase-positive
Conformatory test for phb accumlation	PHB inclusions are stained blue green to black	positive

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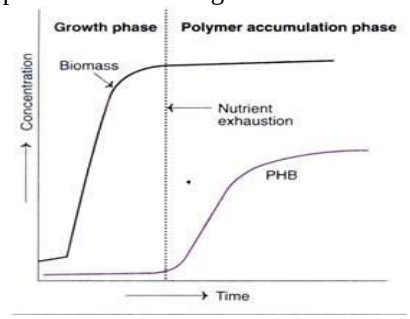
PRODUCTION OF POLY BETA HYDROXY BUTYRATE FROM PSEUDOMONAS AERUGINOSA

Polyhydroxybutyrate is mostly grown by Batch Culture. PHB production occurs when there is an excess supply of carbon source, and limitation of some other essential nutrient such as nitrogen, phosphorus of sulfur source. The production/ accumulation of PHB is depicted in graph. There are two distinct phases—a growth phase and a polymer accumulation phase. As the growth phaseceases, due to nutrient exhaustion, synthesis of polymer(PHB) commences. It is also possible to produce PHB during anaerobic conditions.



Graph.1

PHB EXTRACTION FROM BROTH

1ml of ***PseudomonasAeruginosa*** culture was inoculated in 250ml of specific broth composed of

The flasks were incubated at 300^0C in a rotary shaker at 150 rpm for 48 hours.
After incubation, PHB produced by the isolates were quantified spectrophotometerically by John and Ralph (1961).

Nutrient broth (high media)	13g
MgCl2	10g
Nacl	10g
KH2PO4	10g
Distilled water	1000ml

TABLE.3

PHB EXTRACTION:

After incubation of48hrs the pseudomonas Aeruginosa was sub cultured and inoculated in specific nutrient broth and incubated that supports the production of poly beta hydroxy butyrate.

After incubation each sample was centrifuged for 15 minutes at 6000 rpm. Pellet was washed twice with sterile deionized water and dried for24 hrs at 100°C.

The total dry weight of Bacteria was determined. Add Sodium hypochlorite to dry cell mass and then incubated at60^0C FOR 1hour to break the cell wall of bacteria

This sample was centrifuge at 6000 rpm for 15 minutes and supernatant was used for further treatment.

Using 96% v/v ethanol: acetone (1:1) cell lipid and other molecules, except PHB were extracted from supernatant. PHB extraction was done by hot chloroform method (adding chloroform to the tube containing supernatant in water bath). PHB crystals were obtained after evaporation of chloroform.

Powdered PHB was collected and finally weighed.

It was found to be that *PseudomonasAeruginosa* can produce 1.32gm of PHB from 250 ml of broth.

FIG.29

IV. DISCUSSION

PHB, the bio plastic due to its biodegradable nature can be used on commercial scale to replace the synthetic plastic.

Since the plastic degradation in the nature occur very slowly, huge amounts of plastic was found none decomposed on the earth surface. This is leading to many implications to all the living organisms on the earth.

If the bioplastic is used as an alternative to the synthetic plastic, due to its biodegradability it doesn't cause any harm to mankind.

V. CONCLUSION

From the present study, we can conclude that we can produce the PHB from an industrial effluent (sugarcane effluent) and the degradation of PHB by microorganisms on soil-garden soil. The convenient carbon, nitrogen and vitamin supplementations were used in order to improve the PHB production rate. The effluent discharged from the industries spoils the agricultural practice and aquatic organisms, thereby affecting the ecosystem around them. These industrial effluents utilization in an effective way will be a boom for agriculturalist and industrialists to get rid of the hazardous problems. This study thus revealed that this effluent can be used as substrate for PHB (bio-plastic) production. These can be degraded by microorganisms without any chemicals. In case of using chemicals it may cause environmental pollutions but microbes do not cause any serious problems. So we can conclude bio-plastic, an eco-friendly and very valuable product.

FUTURE ASPECTS OF PHB

PHBs are already widely used in Europe and Japan because they are much more environmentally conscious.

Wastes produced from agricultural and food industries can be either treated or converted into high valuable compounds making use of a sustainable technology. The uses of waste materials as substrate for the production of PHB reduce both the disposable costs and lower the price of a costly product.

This could be passed in all the countries such that these products can be biodegradable in any environment in which they might be left

VI. ACKNOWLEDGEMENT

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