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Research Paper

Isolation and Characterization of PHB Producing *Buttiauxella agrestis* From Slaughter House Waste Rumen Fluid

Veterinary Science

KEYWORDS : PHB, Rumen fluid, Slaughter house waste, Nitrogen Deficient Minimal Media, Crotonic acid

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ABSTRACT

Buttiauxella agrestis, a PHB producing bacterium was isolated from rumen fluid collected from slaughter house waste by sudan black staining. Buttiauxella agrestis was found to be a gram negative motile rod. It was found to be positive for Methyl red, Citrate utilization test, Glucose, Arabinose, Lactose, mannitol, Rhamnose, Sucrose and negative for Indole test, Voges proskauer, Adonitol, Sorbitol test. PHB production by Buttiauxella agrestis was confirmed by acridine orange and nile blue A staining. The bacteria produced dark yellow orange colour fluorescence at 640 nm and red fluorescence at 480 nm for acridine orange and nile blue staining respectively. Buttiauxella agrestis was grown in a nitrogen deficient minimal medium(NDMM) for PHB production. PHB was extracted by chloroform-sodium hypochlorite method. PHB yield was found to be 0.56 g/L. PHB was confirmed by FTIR analysis.

1. INTRODUCTION

Due to the negative impact of plastic material on environment and animal life, world is looking for the biodegradable material. Poly hydroxy butyrate (PHB) is a biodegradable polymer produced by living organisms, which is an alternative for the plastic material[1]. PHB is insoluble in water, resistant to ultraviolet radiation, impermeable to oxygen, and is very much suitable for use as food packaging material. PHB have attracted increasing attention due to their biodegradable, biocompatible, thermoplastic features, composted or burned without producing toxic by-products and it can be made in to films, fibres, sheets, even moulded to the shape of bags and bottle[16]. PHB has various applications like medicine, drug manufacture, drug delivery, in developing medical sutures, bone marrow scaffold, tissue engineering devices, agriculture and various industrial purposes[3].

PHB production is increased by excess of carbon source and limiting the nutrients such as nitrogen, phosphorus, sulphur, magnesium, iron, oxygen, and potassium. It is an intracellular polymer accumulated under stress conditions but with excess carbon source. *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Staphylococcus* and *Micrococcus* are some bacterial species which naturally produce PHB[12].PHB from Gram-negative organisms are widely used in a variety of products. Due to wide industrial application, PHB are considered as green plastics. The popularization of bioplastics is limited because of their high production costs. Consequently, less expensive substrates like agricultural residues, improved cultivation strategies and easier extraction techniques has to be used to bring down the cost[4,13].

Rumen of goat harbors millions of microorganisms that are important for livestock. They utilize nutrients from plant to generate energy. The microorganisms survive in the rumen under various constraints which is associated to be natural or feed related[18].Most of the PHB producing organism were isolated from soil and marine. The present study has been under taken to isolate PHB producing bacteria from slaughter house waste rumen fluid.

MATERIALS AND METHODS

2.1 Screening and Isolation of PHB Producing Bacteria From Rumen Fluid

Rumen fluid was collected from slaughter house, Chennai in an air tight container .The rumen fluid was serially diluted , plated on nitrogen deficient minimal media(NDMM). The bacterial culture plates were incubated at 37˚ C for 24hrs and stained with 1% sudan black staining solution [11]. Colonies which were black in colour are isolated and cultured for further studies.

2.2 Characterization of PHB Producing Bacteria

2.2.1 Gram staining

24 hrs of culture was gram stained and viewed under the light microscope (Olympus).

2.2.2 Biochemical characterization of PHB producing bacteria

The isolated PHB producing bacteria was characterized by using Himedia biochemical test kit. The bacteria was identified by Bergey's Manual of Determinative Bacteriology[6].

2.2.3 TEM analysis

The morphology of the PHB producing bacteria was identified by TEM (Tecnai-10, Phillips-Neitherland). [12]

2.3 Preparation of nitrogen deficient medium for PHB production

For the accumulation of PHB, the isolate was inoculated in nitrogen deficient minimal medium (NDMM) containing (g/L) 10g Glucose, 0.2g $MgSO_4$, 0.1g NaCl, 0.5g$KH2PO_4$, 2.5g peptone, 2.5 g yeast extract. The pH was adjusted to 7.0, incubated at 37 ºC for 48 hrs on a rotator shaker at 120 rpm[14].

2.4 Acridine orange staining

After 48hrs of incubation at 37 ºC 10 µl of culture was transferred to the eppendorf tube containing 50 µl of acridine orange and incubated at 37 ºC for 30 mins. After the incubation period culture was centrifuged at 4000 rpm for 5 min. Smear was prepared, heat fixed and viewed under fluorescent microscope at 640 nm[5].

2.5 Nile blue staining

Nile blue A is the confirmatory staining method for PHB production. Smear was prepared with 48hrs of bacterial culture grown in a NDMM broth. Slide was stained with 1% aqueous solution of nile blue A and kept at 55 ºC for 10 min and washed with water followed by 8% acetic acid. Viewed under fluorescent microscope at 480 nm.[9].

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2.6 Extraction of PHB

PHB producing isolate was inoculated in a nitrogen deficient minimal media. After 48 hrs of incubation cell mass was harvested by centrifugation at 10000 rpm for 10 minutes. Supernatant was discarded and the pellet was treated with 10 mL of 4% of sodium hypo chlorite and incubated at 37 ºC for 2 hrs. After incubation it was centrifuged at 5000 rpm for 15 min. Pellet was washed with distilled water followed by acetone and methanol respectively. After washing, the pellet was dissolved in 5 mL of boiling chloroform and allowed to evaporate at 4 ºC [7].

2.7 Quantification of PHB

Quantification of PHB was carried out by using concentrated hot sulphuric acid which converts the PHB into crotonic acid which is brown in color [19]. The crotonic acid production was measured by taking absorbance at 235nm against sulfuric acid as blank and PHB production was estimated[8,10].

2.8 FTIR analysis of extracted pHB

In order to know the functional groups present in the extracted PHB, about 1mg of extracted sample of PHB was dissolved in 5 mL of chloroform. Chloroform was allowed to evaporate to get PHB polymer and was subjected to FTIR analysis[17] by using FTIR spectrophotometer. Spectra were recorded in 4000 cm-1 to 400 cm- 1 range.

3. RESULTS AND DISCUSSION

3.1 Screening and characterization of PHB producing bacteria



Figure.1 Screening of PHB producing bacteria by sudan black staining

Out of 70 colonies obtained from rumen fluid, two colonies were found to be black in colour when stained with sudan black . These two colonies were subcultured to obtain a pure culture of PHB producing bacteria (Fig.2).One of the colony grown well after 24hrs of incubation at 37 ºC was subcultured for PHB production.

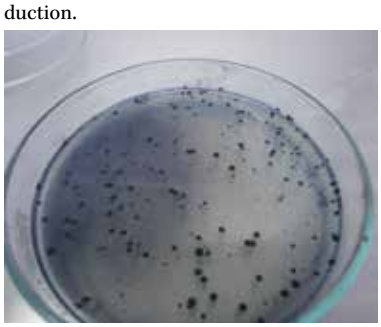

Figure.2 Colonies of sudan black stained PHB producing isolate on spread plate.

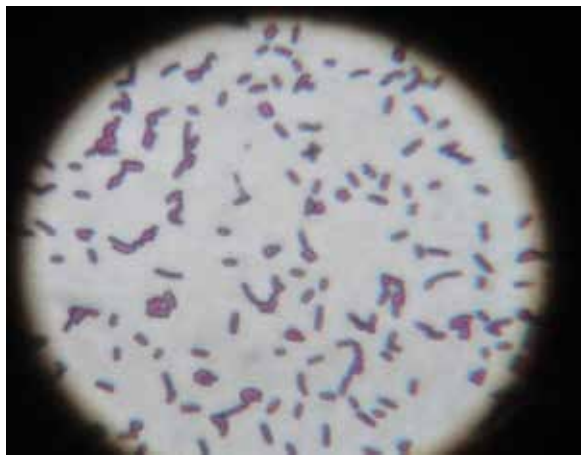

Figure.3 Photomicrograph of Buttiauxella agrestis by gram staining (1000x)

PHB producing isolate was found to be Gram negative motile rods (Fig.3).



Figure 4 Biochemical test results of PHB producing isolate

The PHB producing bacteria isolated from rumen fluid was found to be positive for Methyl red, Citrate utilization test, Glucose, Arabinose, Lactose, mannitol, Rhamnose, Sucrose (Fig.4) and negative for Indole test, Voges proskauer, Adonitol, Sorbitol test . The PHB producing organism was identified as *Buttiauxella agrestis* as per the Bergey's manual of determinative bacteriology.

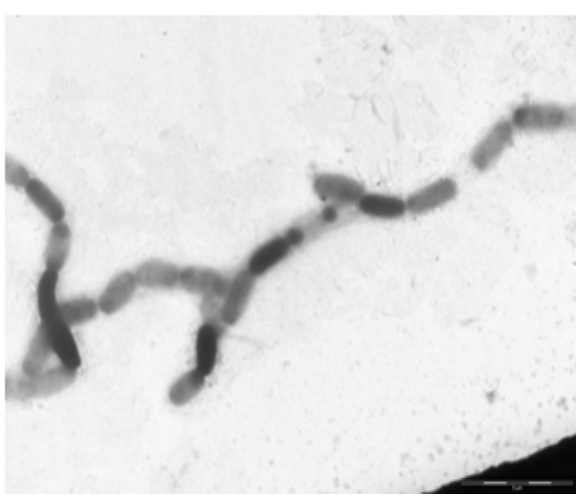

Figure.5 Transmission electron micrograph of *Buttiauxella agrestis*

Transmission electron microscope analysis showed PHB producing rods in chains. Bacteria (Fig.5)

3.2 Confirmation of PHB Production by *Buttiauxella agrestis*

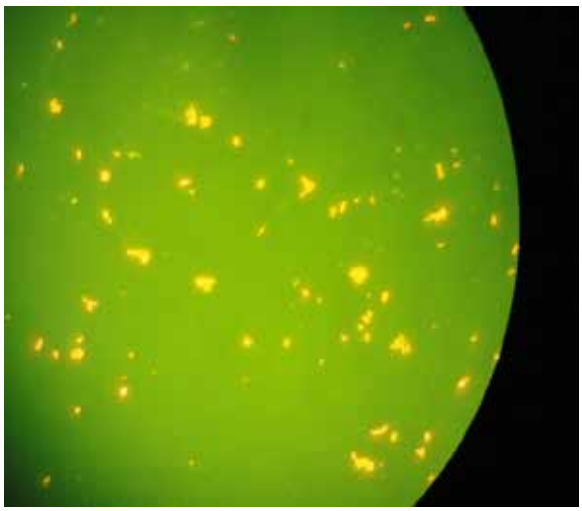

Figure.6 Photomicrograph of *Buttiauxella agrestis* on acridine orange staining.

Buttiauxella agrestis showed the fluorescence of PHB granules at 640 nm as yellow orange (Fig.6). when stained with acridine orange, which confirms the production of PHB.

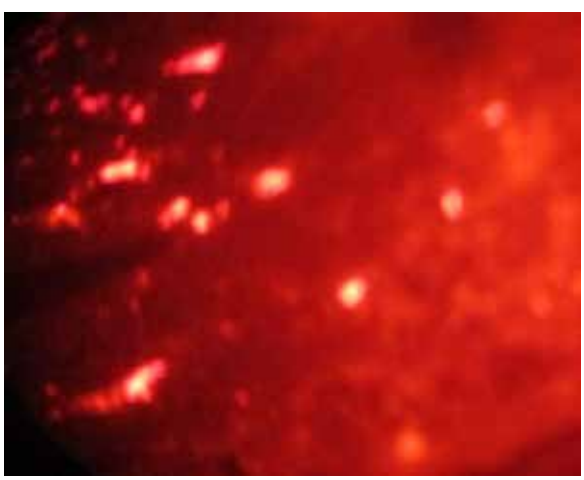

Figure.4 Photomicrograph of *Buttiauxella agrestis* on nile blue A staining

PHB production of isolate was further confirmed by nile blue A staining. Intra cellular lipid granules (PHB) exhibited a red colour fluorescence at 480 nm.

3.3 EXTRACTION OF PHB

In the present study *Buttiauxella agrestis* found to produce 0.56 g /L of PHB. PHB production has been reported among diverse bacteria including *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium*, *Ralstonia eutropha*, *Rhizobium spp*. Bhairavi *et.al.*,[2] obtained 0.282 g/L PHB content from *Bacillus subtilis* with Neera as source of carbon and *Bacillus cereus* gave 0.152 g/L PHB using Neera. *Bacillus subtilis* gave lowest yield of 0.027g/L with Cashew and Apple as a carbon source. *Bacillus cereus* with Jawar stem gave 0.034g/L.

Pinal Chauhan et.al.,[15] recovered the PHB from *Bacillus cereus*, *Bacillus megaterium*, *Ralstonia eutropha*, *Rhizobium spp*. The amount of PHB produced was 0.594, 0.537, 0.694 and 0.389. Results obtained in the present study was comparable with *Bacillus megaterium, Ralstonia eutropha*. The amount of PHB produced by *Buttiauxella agrestis* (0.56g/L) was higher than the PHB produced by *Rhizobium spp*.

3.4 CHARACTERIZATION OF PHB

3.4.1 FTIR analysis

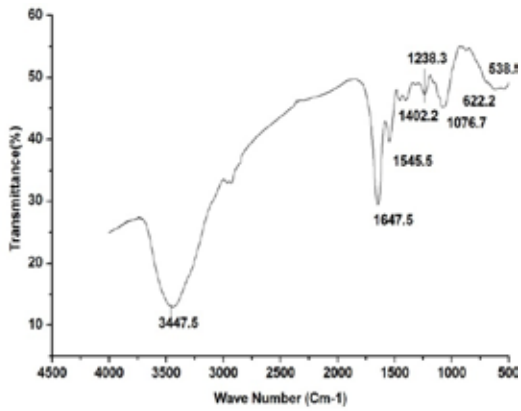

Figure.7 FTIR spectrum of PHB produced by *Buttiauxella agrestis*

PHB was characterized by FTIR which gave ester group which indicates Polyhydroxybutyric acid. FTIR spectra of the extracted polymer show peaks at 1647.5 cm^{-1} and 1238.3cm^{-1} corresponding to C=O and C-O stretching groups. The peaks for – CH, CH_2, CH_3, and terminal OH group occurred at 1402.2 cm^{-1}, 1238.3 cm^{-1}, 1076.7 cm-1and 3447.50 cm^{-1}, respectively (Fig.7).

4.0 CONCLUSION

The PHB producing organism isolated from slaughter house waste rumen fluid was found to be *Buttiauxella agrestis*. *Buttiauxella agrestis* was able to produce 0.56 g/L PHB, which was obtained by sodium hypochlorite extraction method. FTIR spectra of the synthesised PHB revealed similar spectra with commercial PHB. The isolated *Buttiauxella agrestis* is found to be a potential PHB producer.

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