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Biopolymer production by Bacterial Species using Glycerol, a byproduct of biodiesel

S.Sathianachiyar*, Aruna Devaraj**

*Research Scholar, Natural Resource Management Centre, Periyakulam,Theni. Tamil Nadu
**Director Natural Resource Management Centre, Periyakulam, Theni Tamil Nadu

Abstract- Biopolymer producing bacterial species (*Bacillus* and *Pseudomonas*)were isolated from rhizosphere soil of *Jatropha curcas* by using standard morphological, cultural and biochemical characteristics.PHB production by bacterial sps were screened by Sudan black B staining method. The main aim of the present study to production of biopolymers using crude glycerol is a byproduct of biodiesel, act as a sole carbon source for PHB producing microorganisms, under the limitation of nitrogen and phosphate . Ammonium molybdate and ammonium sulphate were used in nitrogen deficient medium instead of ammonium chloride. Maximum biopolymer production were noted in *Bacillus spp13*.3 gm/100ml.*Pseudomonas spp* yielded 11.2 gm/100 ml.

Intex Terms: *Bacillus cereus,* Crude glycerol, NDMM, PHB, *Pseudomonas aeruginosa,* Sudan black B.

I. INTRODUCTION

Our whole world seems to be wrapped up in plastic. Almost every product we buy ,most of the food we eat and many of the liquids we drink come encased in plastics. Over the past 25 years, petrochemical derived plastics have become indispensable materials in modern life. These plastics however are non biodegradable and cause significant waste disposal and contamination problems. Increasing awareness of the harmful effects of non biodegradable plastics on the environment together with the growing body of legislation prevents their use in various consumer products, and created much interest in the development of biodegradable plastic material.(Sang Yup Lee,1997).Biodegradable plastic is an alternative for the petrochemical plastics must be truly degradable, non-polluting and economically priced polymer (Rawte *et al*.,2001). Biodegradable plastics, can be degraded completely into natural compounds, such as CO_2, methane, H_2O and biomass with in 1 year. This process is carried out by microorganisms.

Polyhydroxyalkanoates or PHAs are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by the bacteria to store carbon and energy resources. These are polyesters composed of repeating units of 3 hydroxyalkanoic acid which can vary in their carbon chain length(Fulal wang *et al*.,1997). In microorganisms, PHA plays an important role as a carbon and energy reservoir, it helps in the sporulation process .(Rawte *et al*., 2001).PHA granules accumulated under the conditions of limiting nutritional elements such as N,P,O,S, Mg in the presence of excess carbon source. PHA are present in the cytoplasmic members of bacteria like *Azotobacter vinelandii*, *Bacillus subtilis*, *Haemophilus influenzae* and *Escherichia coli* and also in eukaryotic membranes like in mitochondria and microsomes as complexes of calcium ions (Ca^{2+}) and polyphosphates in a ratio of 2 : 1 : 2 (Reusch,1992).

The production of biodiesel generates significant quantities of co-product stream rich in glycerol. New uses for glycerol have been the subject of much research to alleviate a market glut of this commodity and to leverage the economics of biodiesel production. One potential use of glycerol is in industrial fermentation where it can be employed as a substrate for microbial growth and the biosynthesis of microbial products. The utilization of crude glycerol will be of immense importance alleviating its disposal problem and producing a value added product. Its utilization in biodegradable polymer production will be attractive.(Raveendran Sindhu et *al*.,2011). Biodiesel production will generate about 10% (w/w) glycerol as the main byproduct. In other words, every gallon of biodiesel produced generates approximately 1.05 pounds of glycerol. This indicates a 30-million-gallon-per-year plant will generate about 11,500 tones of 99.9 percent pure glycerin. It was projected that the world biodiesel market would reach 37 billion gallons by 2016, which implied that approximately 4 billion gallons of crude glycerol would be produced.(Anand *et al*.,2011).

Glycerol as the structural component of many lipids , is abundant in nature. Wide glycerol occurrence in nature allows different kinds of microorganisms to metabolize it as a sole carbon and energy source. Thus, in some industrial fermentation processes, glycerol can substitute traditional carbohydrates, such as sucrose, glucose, and starch (Posada *et a*l.,2010)Since purified glycerol is a high value and commercial chemical with thousands of uses, the crude glycerol presents great opportunities for new applications. For that reason, more attention is being paid to the utilization of crude glycerol from biodiesel production in order to defray the production cost of biodiesel and to promote biodiesel industrialization on a large scale. Although intensive investigations have focused on utilizing crude glycerol directly, review papers on crude glycerol utilization are scarce. Crude glycerol contains a high salt concentration and has a high pH. There is some chemical application to utilize waste glycerol ,but it requires the purification process to use it as a source of chemical. High salt and pH tolerant bacterium effectively produce bio polymer .The present study mainly address the current and potential value-added applications of glycerol from biodiesel production. PHB from glycerol requires a limitation of

an essential nutrient such as: N, P, Mg, K, O or S, and an excess of a carbon source.

The main aim of the work is the production of Biopolymers by using crude glycerol. To minimize the production cost of Bioplastics and by which making the pollution free , Eco Friendly environment from non - biodegradable plastics.

II.MATERIAL AND METHODS

Sample Collection

Rhizosphere soil samples were collected from *Jatropha curcas* plants in Bodinayakanur, Theni(Dt)Tamil Nadu .Soil was taken from 20cm depth and stored at room temperature until analysis. *Bacillus* and *Pseudomonas* spp were isolated from rhizospere soil . The morphological and physiological and biochemical properties of the isolates were investigated according to Bergey's method of Determinative Bacteriology (Holt *et al.,* 1993). and the cultures were maintained on nutrient agar at 4ºC.

Crude Glycerol Collection

Crude glycerol was collected during biodiesel production from *Jatropha* oil using alkali catalyst transesterification reaction. Un refined glycerol was used as a carbon source for PHB producers.

Inoculum Development

A loop full of *Bacillus* and *Pseudomonas* culture from nutrient agar slants was inoculated in 100 ml of carbon rich growth medium containing (gL^{-1}) peptone, 10;yeast extract ,10;meat extract ,5; pH,7.2±0.2 . Medium was inoculated at the rate of 1 % inoculum and incubated at 30ºC for 24 hrs with constant shaking at 120 rpm.

Nitrogen Deficiency Minimal Medium (NDMM) Preparation for PHB Production

For detecting accumulation of PHB, *Bacillus* and *Pseudomonas* were grown in nitrogen deficiency minimal medium (NDMM) containing (gL^{-1}) Na_2HPO_4,3.8gm;KH_2PO_4,2.65gm;NH_4CL,2gm; $Mgso_4$, 0.2 gm; glycerol, 2gm and trace minerals 1ml L^1 which contained (gL^{-1})EDTA,5 gm;$ZnSO_4$ $7H_2O$,2.2 gm; $CaCl_2$,5.45 gm; $MnCl_2$ 6H2O,5.06 gm;H_3Bo_3,0.05gm; $Feso_4$ $7H_2O$,4.79 gm;NH_4 MO,24.4 gm;$Cocl_2$ 6 H_2O,1.6 gm and $CuSO_4$ $5H_2O$,1.57gm. The PH was adjusted to 7 with KOH. -(Rapske *et al.,* 1976).The culture was incubated at 30ºC for 48 hrs on a rotatory shaker at 120 rpm. Cells were stained with lipophilic stain Sudan black B (Murray *et al*.,1994) using slide and plate method.

Isolation of PHB Producers

The PHB production by the microbes can be confirmed by staining with Sudan B black.

a . Slide Method

The cultures were grown in NDMM medium were heat fixed on a slide and immersed in 0.5% (w/w) Sudan black B staining with ethylene glycol for 5min.Then the slide was air dried, the excess amount of stain was destained using xylene several times and blot dried with absorbent paper. Then the counter stain (0.5%w/v aqueous saffranin) 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.

b . Plate Method

Nitrogen Deficiency Minimal Medium agar plates were prepared and the test cultures were inoculated by using spread plate method. The plates were incubated at 37ºC for 48 to 72 hrs. After incubation, the plates were flooded with Sudan black B solution for 20min. Later, the solution was destained with ethanol solution .Finally the solution was drained off and observed for screening of PHB producers.

PHB Production in shake flask

PHB production occurs only under nitrogen limiting conditions (Takahiro,1986),it was carried out in two steps: i) PHB producers were grown in 500 mL Erlenmeyer flask containing 100 mL carbon rich growth medium.(Jung *et al*.,1997).Flask were inoculated at the rate of 1% inoculums (v/v)and incubated at 30ºC for 24 hrs with constant shaking at 120 rpm and ii) after getting optimal cell concentration ,cell mass was centrifuged at 10000 rpm for 10 min, washed with sterile distilled water, inoculated in nitrogen deficient minimal medium and incubated for 48 hrs at 37ºC on a rotatory shaker(120 rpm).

Optimization of cheap carbon source (Glycerol) and various nitrogen sources for PHB production.

- The effect of crude glycerol from biodiesel production on PHB production by *Bacillus*,*Pseudomonas spp* were performed in N and P limiting conditions.
- Nitrogen sources such as Ammonium molybdate, Ammonium sulphate were used for Biopolymer production instead of Ammonium chloride.
- Glycerol (carbon source) individually added at the rate of 2g/l in NDMM medium devoid of fructose. Over night culture of PHB producers was inoculated (1%)in to NDMM medium. After 24 h incubation at 30ºC with shaking (120 rpm) the culture was used for estimation of PHB.

Dry weight measurement of PHB producers

48 hours incubated PHB producers in NDMM broth allowed centrifuged at 4000 rpm for 15 mins. The cell pellets were collected and washed with distilled water and kept allowed to dry, then the final weight measured and tabulated .

Extraction of PHB

After 48 hrs incubation cell mass was harvested by centrifugation at 10000 rpm for 10 minutes. Supernatant was discarded and the pellet was treated with equal quantity of sodium hypo chloride and the mixture was centrifuged with 5000 rpm for 15 min and then washed with distilled water,acetone respectively for washing and extraction. After washing, the pellet was dissolved in 5 mL of boiling chloroform and allowed to evaporated on

sterile glass plate and kept at 4°C. After evaporation the powder was collected for further analysis.

Assay of PHB

Extracted PHB was dissolved in minimum volume(10ml) of chloroform and subjected to grarvimetric and spectrophotometric analysis. Sample containing 5 to 50 µg polymer in chloroform was transferred to a clean test tube, allowing chloroform to evaporate and 10 ml of concentrated H_2SO_4 was added the tube is capped with a glass marble and heated for 10 min at 100 °C in a water bath the solution was cooled and after thorough mixing, sample was transferred to a silica cuvette and the absorbance at 235 nm was measured against a sulfuric acid blank. standard curve was established with PHB concentrations ranging from 2-20µ g/ml PHB (Dekwer *et al*., 1999).

Characterization of PHB
Fourier Transform Infrared Spectroscopy (FTIR)

The presence of different functional groups in PHB was checked by FTIR. Extracted PHB (2 mg) was dissolved in 500 µl of chloroform and layered on NaCl crystal. After evaporation of chloroform, PHB polymer film was subjected to FTIR.

Statistical Analysis

Significant differences between bacterial treatments were tested by analysis of variance by using ANOVA.The results of different nitrogen sources were subjected to identify significant differences between bacterial treatments. Probability (P) values of <0.05 were considered significant.

III RESULTS & DISCUSSION

Screening of PHB producers

Microbial isolates from rhizospere soil of *Jatropha curcas* were subjected for partial identification based on various biochemical tests according to Bergey's method of systematic bacteriology and confirmed as *Bacillus* and *Pseudomonas*. PHB productions were screened in bacterial sps by plate and slide methods using Sudan black B staining. In the plate method blue blackish colored granules, the intensity of the color increases as the increased amount of PHB content more in the colonies. In the slide method, blue colored granules were observed. Sudan black b is slightly basic dye and accumulate in the acidic groups of fat globules in the PHB producers. This stain is specific for lipids.

Effect of glycerol on PHB production

Present study focused the effect of glycerol on PHB production under nitrogen and phosphate limitation.PHB producers used crude glycerol as carbon source. The total PHB accumulation was found to be slightly greater in the P- limit media than the N-limit media..The PHB production were compared in constant pH(7) ,temperature(37ºC),and duration (48 hrs) using two bacterial species. Maximum PHB biomass production and yield were noted in *Bacillus* spp(13.3gm/ 100 ml). In *Pseudomonas* the biopolymer yield was 11.2gm/100 ml respectively ,when using 2% (v/v) Crude glycerol + methanol as a feedstock over 48 h of growth in 37º C for 48 hrs.The relationship between biomass and PHB production yield were statistically significant ($p<0.005$). Regarding PHB yield ,the results of this study compares similarly to those of Dobroth *et al*., (2011) (0.10 g PHB per g glycerol + methanol) and Zhao *et al*., (1993) (0.12 g PHB per g methanol). However, PHB yields observed by Braunegg *et al*., (1999) (0.23 g PHB per g methanol + glycerol), Ibrahim and Steinbuchel (2010) (0.29 g PHB per g glycerol), and Cavalheiro *et al*., (2009) (0.36 g PHB per g glycerol) suggest that higher PHB yields on CG could be achieved. PHB synthesis was driven by a macronutrient deficiency. In crude glycerol both glycerol and methanol used by PHB producers to produce PHB.The bio-energetic perspective microbes (*Bacillus,Pseudomonas*) preferentially use methanol first (higher oxidation state).The results indicate methanol as a primary precursor for PHB synthesis . Dobroth *et al*.,(2011) reported that longer HRTs (Hydraulic residence time) were considered to be favorable for maximizing microbial growth and PHB production on the bio-available nutrient-poor CG. Notably, the reactor with no ammonium chloride added produced no PHB, which suggested that a minimum amount of readily bio-available nitrogen must be added to stimulate PHB synthesis and glycerol concentrations remained relatively constant, while methanol concentrations were significantly reduced (to near complete depletion). Considering the favorable microbial energetics for methanol use over glycerol (i.e., methanol exhibits a higher oxidation state), these results and conclusion align with the observations from reactors fed glycerol only. Enriched methylotrophic population was metabolizing methanol to form PHB.

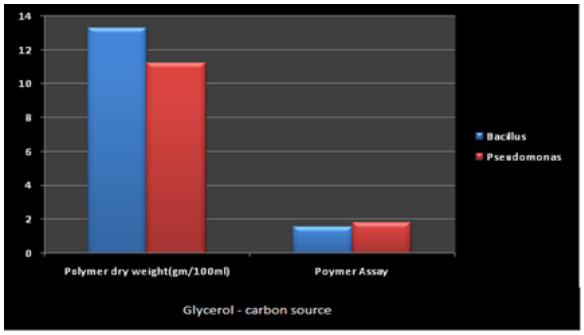


Fig .1 .Biopolymer yield and biopolymer assay in Crude glycerol Medium

Optimization of Nitrogen and Phosphate limitation for PHB production

The maximum PHB production were observed in Ammonium sulphate (0.19gm /30 ml)than other nitrogen sources in *Pseudomonas* spp. Ammonium molybdate yielded (0.11gm/30ml) maximum PHB concentration in *Pseudomonas* spp. Maximum PHB production(0.23mg/30ml) in phosphate

deficiency noted in *Pseudomonas* spp. The values were statically significant ($p<0.005$).

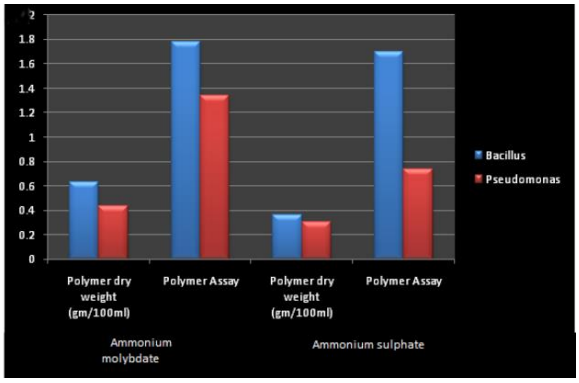


Fig. 2. Biopolymer yield and biopolymer assay in various nitrogen sources.

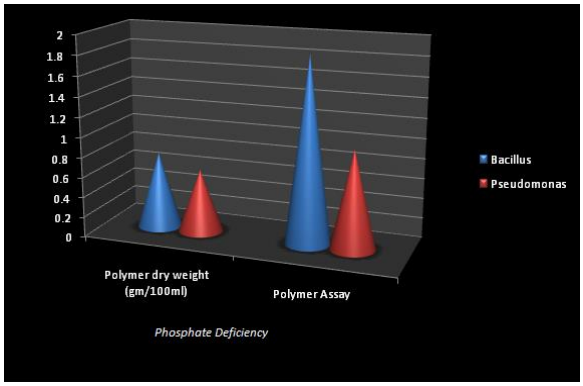
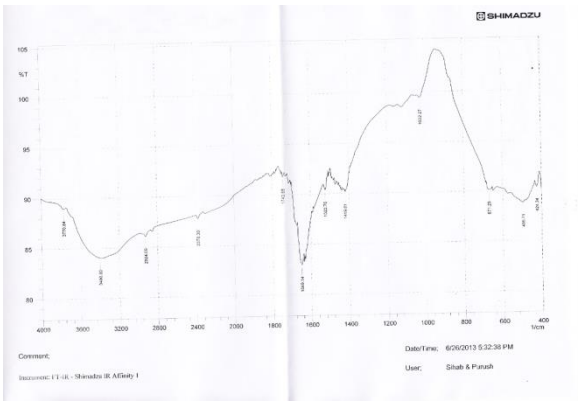


Fig.3. Biopolymer yield and biopolymer assay in Phosphate deficiency medium.

FT-IR Analysis

The FT-IR spectra are dominated by the absorbances for the major cellular constituents, namely, PHB (in the case of PHB-producing microorganisms) and cellular proteins from crude glycerol. FTIR spectra of extracted polymer shown peaks at 1743.65 and 1649.14 cm^{-1} corresponding to C=O stretch of methyl group present in the PHB molecular chain. The peak at 1523.76 and 1419.61 corresponding to N-H Bend. These are the amide I band at 1,649.14 cm^{-1}, which is due primarily to the amide(methanamide) carbonyl stretching vibration, and the amide II band at 1,523.76 cm^{-1}, which is due mostly to N-H bending vibrations. FTIR spectra revealed the functional groups of PHB.



IV CONCLUSION

The research study presented that *Bacillus* and *Pseudomonas* have potential to produce PHB from crude glycerol with methanol .The PHB producers synthesise PHB from methanol from crude glycerol with PHB synthesis seemingly driven by a macronutrient deficiency under the optimized conditions using nitrogen and phosphate limitation .Crude glycerol reduce PHB production cost by using the inexpensive carbon source for industrial use.

REFERENCES

1. P.Anand, R.K.Saxena, A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundi***.** New Biotechnol .2011**,** pp.1-7.
2. G.Braunegg, K.Genser, R.Bona, Haage, G., F.Schellauf, E.Winkler, Production of PHAs from argicultural waste material.Macromolecular Symposia. 1999;144, 375–383.
3. J.Cavalheiro, M.de Almeida, C.Grandfils, M.M.R.da Fonseca, Poly(3-hydroxybutyrate) production by Cupriavidusnecator using waste glycerol.Process Biochemistry.2009; 44 (5), 509–515.
4. Doan, Thuoc Van, Nguyen, Binh Thi, Polyhydroxyalkanoates production by a bacte- rium isolated from mangrove soil samples collected from Quang Ninh province. J. Viet. Env. 2012; 3(2); 76-79.
5. Z.T.Dobroth, Shengjun Hu,E.R. Coats,A.G. McDonald, Polyhydroxybutyrate synthesis on biodiesel wastewater using mixed microbial consortia. Bioresource Technology 102 (2011) 3352–3359.
6. D.Dekwer ,D.C. Hempel , Microaerophilic production of alginate by *Azotobacter vinelandii*,Von der Gemeinsamen Naturwissenscha ftlichen, Fakultat der Technischen UN. Carolo-Wilhelmina zu Braunschweig, Edited by Wael Sabra, aus Alexandria, Agypten.1999: 37-54.
7. Fulal wang , Sang yup lee, Poly C3 – hydroxybutyrate production with high productivity and high polymer content by a fed – batch culture of *A. latus* under Nitrogen Limitation Appl. Environ. Microbiol.1997; 63 : 3706 – 3706.
8. Holt Bergey's Manual of Bateriology.1997, Volume1, Second Edition.

9. M.H.A.Ibrahim, A.Steinbuchel, Zobellella denitrificans strain MW1, a newly isolated bacterium suitable for poly(3-hydroxybutyrate) production from glycerol. Journal of Applied Microbiology .2010;108 (1), 214–225.
10. J.A.Posada, C.A.Cardona, Design and analysis of fuel bioethanol production from raw glycerol, Energy, Article in Press. doi:10.1016/j. energy.2010.07.036.
11. Y.M.Jung , Y.H.Ley,Investigation of regulatory mechanism of flux of acetyl CoA in *Alcaligenes eutrophus.* J Microbiol Biotech .1997;24:222-225.
12. R.G.E.Murray, R.N.Doetsh, C.F.Robinow. Determinative and cytological light microscope. In method for General and Molecular Bacteriology, Eds., P.Gerhardt , ,R.G.E.Murray, W.A.Wood , N.R.Krieg.Washington D C:American Society for Microbiol.1994:21-41.
13. R.Rapske,A.C.Rapske,Quantitative requirements for exponential growth of *A.eutrophus* . Appl. Environ. Microbiol.1976; 32 : 585 – 5591.
14. Raveendran Sindhu, Balakrishnan Ammu, Parameswaran Binod,S.K.Deepthi, Production and characterization of poly-3-hydroxybutyrate from crude glycerol by *Bacillus sphaericus* NII 0838 and improving its thermal properties by blending with other polymers. Braz. arch. biol. technol. vol.54 no.4 Curitiba July /Aug. 2011
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15. T.Rawte ,S. Mavinkurve ,Biodegradable plastics – Bacterial polyhydroxy alkanoates. Indian J Microbiol .2001;47: 233 – 245.
16. R.N.Reusch ,Biological complexes of poly β- hydroxyl butyrate FEMS Microbio Revs .1992;103 : 119 – 130.
17. Sang yup lee,Fulai wang . PHB production with high productivity and high polymer content by a Fed – batch culture of *A.latus* under nitrogen limitation.1997; 63 : 3703 – 3706.
18. S.Takahiro ,Y.Tsuneo,S.Soichi,Kinetics and effect of nitrogen source feeding on production of PHB by fed batch culture.Appl Microbiol Biotechnol.1986;24:366-369.

AUTHORS

First Author- S. Sathianachiyar, Research Scholar, Natural Resource Management Centre,Periyakulam,Theni(Dt),Tamil Nadu.
E.mail:sathyaprabhu84@gmail.com.

Second Author - Dr. Aruna Devaraj, Director, Natural Resource Management Centre,Periyakulam,Theni(Dt),Tamil.
E.mail:snakearuna@yahoo.com.