Impact Factor (SJIF): 5.301

International Journal of Advance Research in Engineering, Science & Technology

e-ISSN: 2393-9877, p-ISSN: 2394-2444

Volume 6, Issue 11, November-2019

Organic production of cost-effective Biopolymer in a bacterial consortium with varying substrate as sole carbon source

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I. ABSTRACT

PHAs are a good replacement for the petroleum-based plastics. Commercialization of PHAs has been limited due to their overall high production cost. The use of organic wastes, agricultural and dairy by-products and vegetable oils has been investigated as alternative substrate, for PHA production. Waste frying Soybean oil is considered as the most suitable and desirable feedstock for PHA production due to their high productivity. Ralstonia eutropha can accumulate 80% to 85% Polyhydroxyalkanoate (PHA) by dry cell weight with about 8-12 granules of polyhydroxybutyrate (PHB) per cell. The ultimate goal of this research was to investigate PHA production using waste frying Soybean oil as the sole carbon sources. Moreover, there is no report published for PHA production by bacterial consortia of Ralstonia eutropha, Cupriavidus necator and Wautersia eutropha.

II. KEYWORDS

Polyhydroxyalkanoate (PHA), Polyhydroxybutyrate (PHB), Carbon Source, Bacteria, Biodegradable.

III. INTRODUCTION

Polyhyroxyalkanotes (PHAs) are a family of polyhydroxyesters synthesized by numerous bacteria as an intracellular carbon and energy storage compound under nutrient-limiting conditions with excess carbon(Tian *et al.*, 2009). *Ralstonia Eutropha H16* is referred to as chemolithoautotrophs belonging to the β-subclass of the Proteobacteria. *Ralstonia Eutropha* or say *Cupriavidus Necator* is a Gram-negative, lithoautotrophic organism (Pohlmann *et al.*, 2006). An ideal organism to study the biosynthesis of polyhydroxybutyrate (PHB). This bacteria stockpiles organic carbon in the form of poly[R-(–)-3- hydroxybutyrate] (PHB) in special storage granules or say vacuole (Steinbüchel & Fuchtenbush, 1998). During nutrient stress condition that is, in the presence of excess carbon source wild-type *R. Eutropha* can accumulate approximately 80 % of its cell dry weight (CDW) as PHB is an intracellular carbon storage material (Budde *et al.*, 2011, Yang *et al.*, 2010).

The synthesis of polymer is governed by the bacterial strain and the carbon source used for the proper bacteria growth (Santhanam & Sasidharan, 2010). Agriculture and food industries have a large amount of carbon and other nutrient-rich waste that can be used for the economical production of PHA. As this waste can be used as a renewable source by many microorganisms thus giving it more ecological and attractive alternative use that elimination of this waste in the environment (Chee et al. 2010, S Abid et al. 2016). Vegetable oils (such as palm oil, soybean oil, sunflower oil, etc.) are preferred to sugars as the only carbon source for PHA production because they are cheaper and produce more PHA Per gram of carbon source. For the production of PHA in bacterial cells, carbon sources are included and then transmuted to hydroxyalkanoates followed by PHA polymerization and stored in the cell cytoplasm in the form of water-insoluble granules. These granules appear as spherical particles with clear boundaries and are transparent electrons. These granular PHAs are kept in an amorphous state in vivo, otherwise if they are crystallized, these granules cannot serve as a storage compound for the host producing bacterial cell (Loo and Sudesh 2007, Abid et al. 2016).

IV. MATERIALS AND METHODS

4.1 Materials:

4.1 1. Microbial Strain

- *Cupriavidus necator* MTCC 1285 [A]
- *Ralstonia eutropha* MTCC 2487 [B]
- *E.coli CGS 4401* MTCC 1302 [C]
- *Ralstonia eutropha* MTCC 1954 [D]
- *Wautersia eutropha* MTCC 6632 [E]

4.1 2. Substrate

Waste frying oil of soya bean

4.1 3. Blending Agent

- Natural rubber i.e. Latex
- Artificial Plasticizer i.e. Ethyl Cellulose

4.2 Methods:

4.2 1. Sample Collection

- Samples of microbial strain was procured from MTCC IMTECH, Chandigarh.
- Substrate was obtained locally.
  - Waste frying oil of soya bean as sole carbon source (substrate) from local cafeteria.
- Additives was obtained organically.
  - Rubber (latex) from rubber plant.

4.2 2. Growth Media and Cultivation Conditions (Lu *et al.*, 2013)

- All strains were cultivated aerobically in rich and minimal media at 30 °C.
- Rich medium consisted of 2.75 % (w/v) dextrose-free Tryptic Soy Broth (TSB).
- Carbon sources used in minimal medium cultivations are 2 % (final w/v) fructose or sodium gluconate.

4.2 3. Revival of Culture (Wang *et al., 2013,* Compos et al., 2014)

- The microorganisms were stored below 5 ^{o}C in nutrient agar (NA) that comprises of 5.0 g L^{-1} meat peptone, 3.0 g L^{-1} beef extract, and 3.75 g L^{-1} agar.
- Periodic replating was performed every 15 days, and inoculation was done in nutrient broth (NB, 5.0 g L^{-1} bacteriological peptone, 3.0 g L^{-1} beef extract, and distilled water) over a 24 h period.
- A mineral media was used as the first culture (FC), without nitrogen limitation, and a second culture (SC) same as mineral media with nitrogen limitation.

4.2 4. Shaker Flask Cultivation (Campos *et al.*, 2014)

Experiments were performed in triplicate in 250 mL flasks which contains 50 mL of nutrient broth medium and 2 mL of pre-culture inoculum, which were incubated at 30 ◦C in a shaker flask without baffles for 24 h at 150 rpm. Timely at an interval of 2 hr Optical Density of the sample was determined at 600nm.

4.2 5. Cellular Separation and PHA Extraction(Campos et al., 2014)

- The cell cultures was harvested by centrifugation at 5000 rpm for 30 min at 5 ^{o}C.
- Washing was done twice with distilled water.
- Transferred to round bottom flasks (50 mL), and freeze at −80 ^{o}C for subsequent lyophilization at −42 ^{o}C for 24 h.
- PHA was extracted from the freeze-dried cells using chloroform at 60 ^{o}C for 2 h with vigorous shaking on a magnetic stirrer plate with heating.
- Biomass production and PHA (obtained after extraction) were calculated using a gravimetric method and expressed in g L^{-1}. The percentage of extraction of PHA is calculated by the ratio of the concentration of PHA to the concentration of biomass according to Eq.

$$\text{PHA extraction}(\%) = \frac{\text{PHA}(gL^{-1})}{\text{Biomass}(gL^{-1})}$$

4.2 6. PHA Measurement

The amount of PHA were determined spectropho-tometrically and chemical method could be used to extract PHA(Senior *et al.*, 1972, Sabra, 1999, Sepahei *et al.*, 2006).

- The samples (10 mL) obtained from the shake flasks were centrifuged at 5000 g for 45 min.
- The solid pellets were re-suspended, washed with 1 ml equal portions of water, acetone, and ether.
- Centrifuged for 30 min at 5000 g.
- Subsequently, chloroform was added and allowed to boil in an ultrasonic bath at 100°C for 10 min.
- Incubation at 30°C for 24 h to evaporate chloroform.
- The obtained white powder was dissolved in concentrated H_2SO_4(2.5 mL 96%) and heat at 100°C for 15 min.
- After cooling down to room temperature, the amount of PHA in the solution would be determined photo metrically at 235 nm against sulfuric acid blank.

A standard curve, correlating PHA concentration to the absorbed light intensities, was generated by using pure PHA. A sample containing 5 to 50 µg PHA in chloroform was transferred to a clean test tube. When the chloroform evaporates, 10 mL of the concentrated H_2SO_4 is added and heated in a water bath at 100°C for 10 min. After cooling down, its absorbance could be measured at 235 nm against sulfuric acid blank.

4.2 7. Preparation of Bioplastic Film

Stock preparation comprises of:

- PHA was dissolved in chloroform at concentration of 0.15g of PHA in 30ml of Chloroform.
- Rubber was dissolved in n-Hexane at concentration of 0.15ml in 30 ml of n-Hexane a clear solution was prepared.
- Artificial Plasticizer Ethyl Cellulose was dissolved in chloroform at concentration of 0.15g in 30ml of chloroform.

Each stock was poured at different concentration such as 50%, 60%, 70%, 80% and 90%. Poured sample was fabricated into thin films by casting into clean, dry, Silicon moulds. Chloroform and n-Hexane was evaporated slowly at temperature of 40ºC. A complete evaporation resulted in formation of films and further allowed to stand for 24 h resulted in weight stabilization in air.

4.2 8. Fourier Transform Infrared Spectroscopy (FTIR)

To identify the kind of the PHAs produced, preliminary analysis of the polymer was performed using FTIR. Approximately 5 mg of the polymer was used for the study. The analysis was performed under the following conditions: Spectral range 4000 to 400 cm-1; window material, CsI; 10 scans and resolution 4 cm^{-1}. The analysis was carried out at the School of Pharmacy RGPV Bhopal.

V. RESULTS AND DISCUSSIONS

5.1 PHAs Synthesizing Bacterial Colonies

In recent years, hundreds of microorganisms with the ability to produce different types of PHA have been studied. They have been recognized in a wide range of gram-positive and gram-negative bacteria and in the archea. Most of them cannot be considered as hosts in industrial production because their ability to synthesize PHAs is insufficient. One of the bacterial species that can accumulate PHA in satisfactory amounts is the Cupriavidus necator (Chen, 2010). Fig 1 below shows the colony of PHA synthesizing bacteria which could be easily seen through naked eyes. Fig 2 shows the microscopic view of PHA synthesizing bacterial colonies.

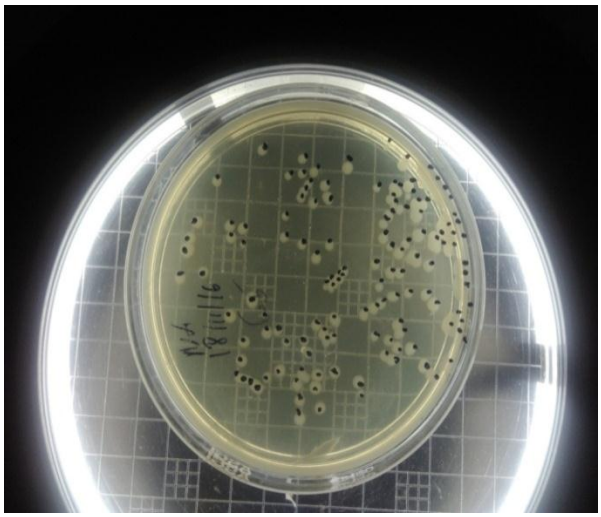

Fig 1. PHA synthesizing bacterial colonies.

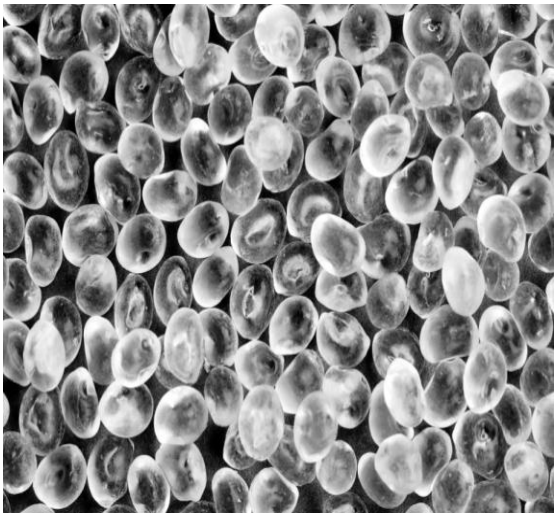

Fig 2. Microscopic view of Bacterial colonies.

5.2 Growth Profiling of the Strains

Below is the graph showing specrophotometric analysis of PHA production of Cupriavidus necator MTCC 1285 as TBA, Ralstonia eutropha MTCC 2487 as TBB, E.coli CGS 4401 MTCC 1302 as TBC, Ralstonia eutropha MTCC 1954 as TBD, Wautersia eutropha MTCC 6632 as TBE. Synthetic media used is Trypton broth with Incubation period of 60 hrs maintained at 30º C of temperature. From the below graph we can determine all the 5 bacteria have similar growth kinetics.

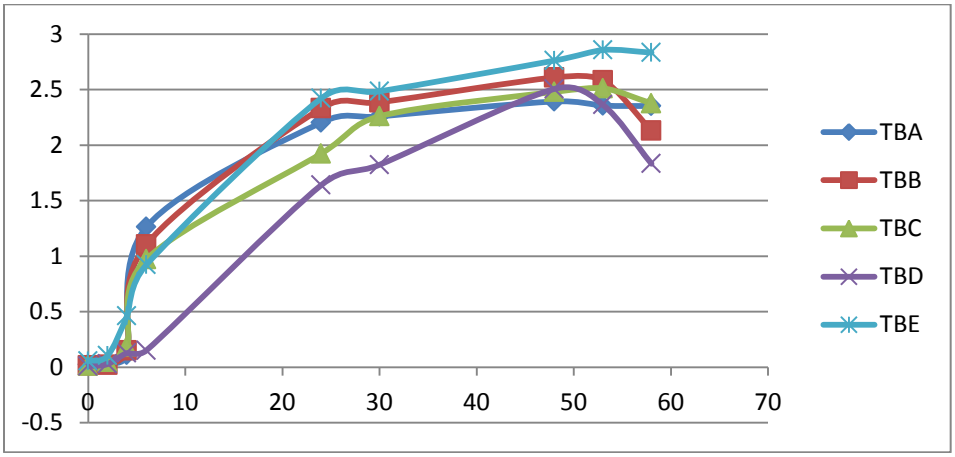

Fig 3. PHA production with Trypton Broth

When Trypton Broth is used as substrate the strain A and C yield approximately similar amount of biomass i.e. around 12 gm/L, while the other 3 strain that is B, D, E yield similar amount of biomass i.e. around 8 gm/L .

5.3 Growth Kinetics of Bacteria with Waste Frying Soybean Oil

Below is the graph showing specrophotometric analysis of PHA production of Cupriavidus necator MTCC 1285 as MMA, Ralstonia eutropha MTCC 2487 as MMB, E.coli CGS 4401 MTCC 1302 as MMC, Ralstonia eutropha MTCC 1954 as MMD, Wautersia eutropha MTCC 6632 as MME. Minimal media is used as nutrient rich media and waste frying oil is used as substrate. Incubation period of 72 hrs is maintained at 30º C of temperature. From the below graph we can determine all the 5 bacteria have similar growth kinetics with waste frying oil as carbon source.

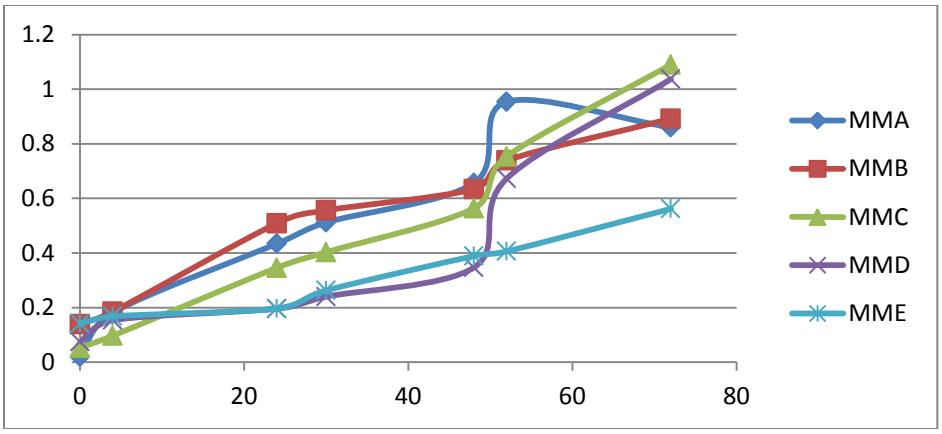

Fig 4. PHA production with Minimal media and waste frying oil.

When Minimal media is used as substrate and waste frying Soybean oil is used and carbon source the strain B and E yield approximately similar amount of biomass i.e. around 12.5 gm/L, while the other 3 strain that is A, C, D yield similar amount of biomass i.e. around 9gm/L .

5.4. Consortium Preparation

Consortium was prepared by focusing on two things.

Firstly by using the formula of cfu/ml (colony forming unit per milliliter). Since growth pattern or rate of division was not same for all the microorganism so to standardize it, it was necessary to calculate the cfu/ml; from which one get to know how much amount of sample should be added .

- **Cfu/ml** = (no. of colonies × dilution factor) / volume of culture plate
- No. of colonies seen by naked eyes after 12 hours of incubation were A – 81 colonies, B – 62 colonies, C – 132 colonies, D – 08 colonies, E – 138 colonies.
- Dilution factor – 10^{5}
- Volume of culture plate - 0.1 ml

Thus by putting all the respective values in the formula of cfu/ml for all the strains following values were obtained. A = 81×10^{6} , B = 62×10^{6}, C = 132×10^{6}, D = 8×10^{6}, E = 138×10^{6}

Out of all the values one reference value was selected so that others have to be set according to that value. So according to all calculated cfu/ml values the minimum would be set as reference because other would possess that. Therefore; 8×10^{6} was the reference, so other values were standardize accordingly; by the method of normalization consortium was prepared for 1ml of solution with culture concentration as A = 98.76 µl, B = 129.032 µl, C = 60.6 µl, D = 1000 µl, E = 57.9 µl.

5.5 Growth Kinetics of Consortia with Varying the concentration of Substrate

Graph below shows the growth of mixed consortia with varying the concentration of substrate and optimizing their growth parameter.

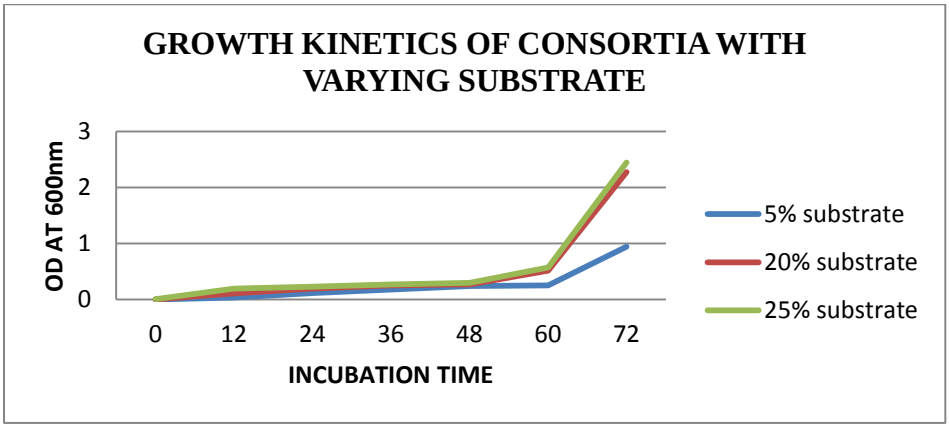

Fig 5. PHA production with varying concentration of waste frying oil.

By varying the concentration of Waste frying oil biomass obtained from mixed consortia was highest for 20% substrate concentration.

5.6 Bioplastic Thin Film Formation

PHA obtained was dissolves in chloroform for formation of thin film. In the experiment natural rubber was used as blending agent. Latex was dissolved with n-Hexane. To provide strength to the biopolymer sheet artificial plasticizer Ethyl Cellulose was added. Figure below shows Biopolymer film cast with varying the concentration of additives.

Fig. 6 (A) 50% Composition of Bioplastic

Fig. 6 (B) 60% Composition of Bioplastic

Fig. 6 (C) 70% Composition of Bioplastic

Fig. 6 (D) 80% Composition of Bioplastic

Fig. 6 (E) 90% Composition of Bioplastic

5.7 Fourier Transform Infrared (FT-IR) Spectroscopy of Casted Biopolymer

The structural change in the Biopolymer surface was investigated using the FTIR spectrometer. Preliminary confirmation of the chemical structure of the polymers produced with different concentration of PHAs, Latex and Ethyl Cellulose was carried out using FTIR. FTIR analyses of the polymers produced from different concentration of additives confirmed the presence of the characteristic marker ester carbonyl bond and C-O stretching bond in the range between 1728-1736 cm-1 and 1159-1162 cm-1 respectively. The bands at 2954-2956cm-1, 2922-2928 cm-1 and 2853-2858 cm-1 correspond to the aliphatic C-H group of the polymer backbone (Sánchez *et al.*, 2000). Hence, the results indicated that the polymers produced contain similar elemental characteristic. In general, the IR spectrum can be split into four regions for interpretation:

- 4000 – 2500 cm^{-1} : Absorption of single bonds formed by hydrogen and other elements e.g. O–H, N–H, C–H
- 2500 – 2000 cm^{-1} : Absorption of triple bonds e.g. C≡C, C≡N
- 2000 – 1500 cm^{-1} : Absorption of double bonds e.g. C=C, C=O
- 1500 – 400 cm^{-1} : This region often consists of many different, complicated bands.

This part of the spectrum is unique to each compound and is often called the ***fingerprint*** region. It is rarely used for identification of particular functional groups.

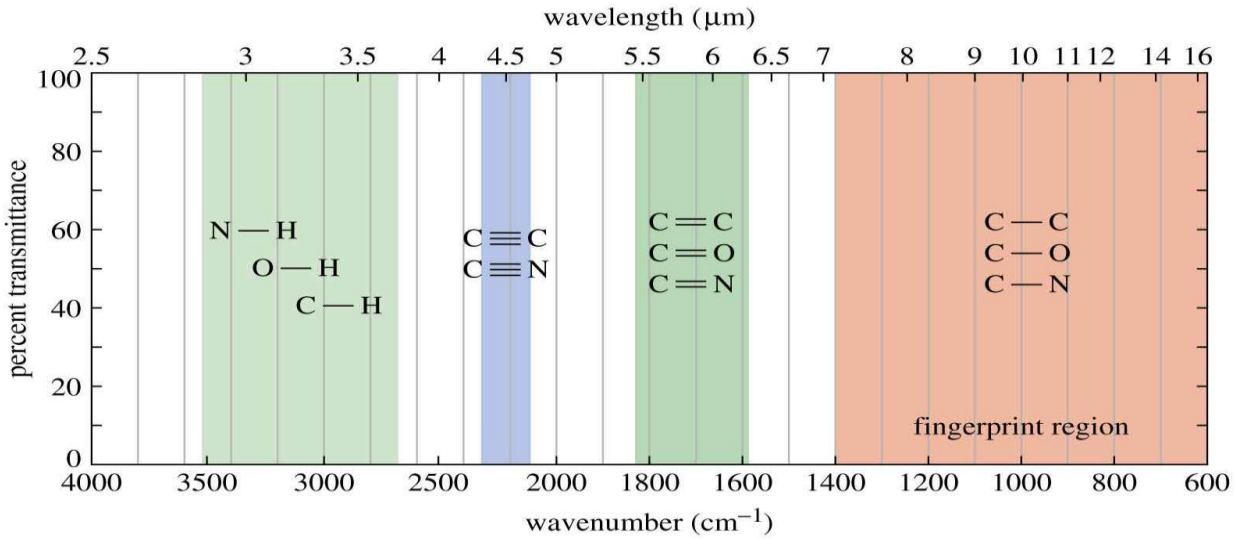

Table below shows the comparative study of the FTIR report of biopolymer film

S.No.	Absorbance Band of different bond	Bioplymer Film 50%	Bioplymer Film 60%	Bioplymer Film 70%	Bioplymer Film 80%,	Bioplymer Film 90%
1	Absorbance of **N-H, O-H, C-H** in cm-1 (3500-3000)	2864.29 2611.62	2960.73 2866.22 2779.42 2696.48 2611.62	2962.66 2900.94 2864.29 2787.14 2700.34 2613.55	2962.66 2866.22 2787.17 2702.27 2613.55	2962.66 2862.36 2775.57 2671.41 2611.62
2	Absorbance of **C≡C, C≡N** in cm-1 (2500-2000)	No band obtained	2266.36 1953.69	2183.42	2264.43 1969.32	2360.87 2333.87 2266.36 1644.25
3	Absorbance of **C=C, C=O, C=N** in cm-1 (1800-1600)	1734.01	1739.79	1732.08	1735.93	1739.79
4	Absorbance of **C-C, C-O, C-N** in cm-1 (1400-600)	1261.45 1095.57 1024.20	1446.61 1261.45 1093.64 1024.20 873.75 802.39	1446.61 1261.45 1093.64 1024.20 871.82 802.39	1448.54 1261.45 1093.64 1026.13 877.61 804.32	1448.54 1261.45 1093.64 1026.13 802.39

VI. CONCLUSION

PHAs are a good replacement for the petroleum-based plastics. Commercialization of PHAs has been limited due to their overall high production cost. A major factor that adds to their relatively high production cost is the cost of expensive carbon feedstock for the organism. Hence, to make PHA production more economical, much research is involved in identifying and using renewable and cheap carbon sources, which not only reduces the production costs but also increases polymer yields.

The use of organic wastes, agricultural and dairy by-products and vegetable oils have been investigated as alternative substrate, for PHA production. Waste frying Soybean oil is considered as the most suitable and desirable feedstock for PHA production due to their high productivity. According to the literature maximum yield of Polyhydroxyalkanoate (PHA) was estimated when the substrate concentration used is 20% v/v. *Ralstonia eutropha* can accumulate 80% to 85% Polyhydroxyalkanoate (PHA) by dry cell weight with about 8-12 granules of polyhydroxybutyrate (PHB) per cell. The inexpensive substrates have been explored for polyhydroxybutyrate (PHB) production to reduce the feedstock cost.

The ultimate goal of this dissertation was to investigate PHA production using waste frying Soybean oil as the sole carbon sources. Moreover, there is no report published for PHA production by bacterial consortia of *Ralstonia eutropha, Cupriavidus necator* and *Wautersia eutropha.* Growth profiling of bacteria was done in order to determine the growth kinetic of each bacteria and the mixed consortia. The amount of PHA obtained was determined by Biomass estimation. Further natural additive like Rubber (Latex) was used to provide strength to the polymer.

On the other hand, the industry requires optimization of fermentation processes for well-known microbes in order to improve the concentration of the biomass and the PHA content with the desired properties. In order to reduce the costs of this bioproduct, additional work should be carried out, in particular, on a high cell density culture method in conjunction with bioproduction of PHAs using cheap carbon sources and easier and non-harmful isolation and purification of Polymers of PHA. It is expected that there is an increasing demand for bacterial polymers with special properties that are suitable for applications in many fields. Using current knowledge and advances in genetic

engineering and synthetic biology, it seems possible to build an ideal PHA producer who can biosynthesize new biopolymers and could be used profitably for industrial production.

VII. REFERENCES

1. Bhubalan, K., W.-H. Lee, C.-Y. Loo, T. Yamamoto, T. Tsuge, Y. Doi and K. Sudesh, 2008: Controlled biosynthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) from mixtures of palm kernel oil and 3HV-precursors. *Polymer Degradation and Stability*,93, 17-23.
2. Budde, C. F., S. L. Riedel, F. Hübner, S. Risch, M. K. Popović, C. Rha and A. J. Sinskey, 2011: Growth and polyhydroxybutyrate production by *Ralstonia Eutrophain* emulsified plant oil medium. *Applied microbiology and biotechnology*,89, 1611-1619.
3. Campos, M. I., T. V. B. Figueiredo, L. S. Sousa and J. I. Druzian, 2014: The influence of crude glycerin and nitrogen concentrations on the production of PHA by *Cupriavidus necator* using a response surface methodology and its characterizations. *Industrial Crops and Products*,52, 338-346.
4. Chee, J.Y., Yoga, S.S., Lau, N.S., Ling, S.C., Abed, R.M., Sudesh, K., 2010. Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics, in: Méndez-Vilas A (Eds), Current Research, Technology and Education Topics in *Applied Microbiology and Microbial Biotechnology* Vol. 2. Badajoz: Formatex, pp. 1395-1404.
5. Chen, G.Q., 2010. Plastics completely synthesized by bacteria: polyhydroxyalkanoates, in: Plastics from bacteria. *Springer Berlin Heidelberg*, pp. 17- 37.
6. Chen, Y.J., Huang, Y.C., Lee, C.Y., 2014. Production and characterization of medium chain- length polyhydroxyalkanoates by *Pseudomonas mosselii* TO7. J. *Biosci. Bioeng*. 118, 145-152.
7. Galego, N., Rozsa, C., Sánchez, R., Fung, J., Vázquez, A., Tomás, J.S., 2000. Characterization and application of poly(β-hydroxyalkanoates) family as composite biomaterials. *Polym. Test*. 19, 485-492.
8. Kamilah, H., Tsuge, T., Yang, T.A., Sudesh, K., 2012. Waste cooking oil as substrate for biosynthesis of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3- hydroxyhexanoate): turning waste into a value-added product. Malaysian J. Microbiol. 9, 51-59.
9. Lee, W.-H., C.-Y. Loo, C. T. Nomura and K. Sudesh, 2008: Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. *Bioresource technology*,99, 6844-6851.
10. Lu, J., C. J. Brigham, C. Rha and A. J. Sinskey, 2013: Characterization of an extracellular lipase and its chaperone from *Ralstonia Eutropha H16*. *Applied microbiology and biotechnology*,97, 2443-2454.
11. Pohlmann, A., W. F. Fricke, F. Reinecke, B. Kusian, H. Liesegang, R. Cramm, T. Eitinger, C. Ewering, M. Pötter and E. Schwartz, 2006: Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia Eutropha H16. Nature biotechnology*,24, 1257-1262.
12. S. Abid, Z. A. Raza, T. Hussain, 2016: Production kinetics of polyhydroxyalkanoates by using *Pseudomonas aeruginosa* gamma ray mutant strain EBN-8 cultured on soybean oil. *Springer Biotech* (2016) 6:142.

13. Sabra, W., 1999: Microaerophilic production of alginate by *Azotobacter vinelandii*. Ph. D. dissertation. Technische Universität Braunschweig, Braunschweig, Germany.
14. Santhanam, S.Sasidharan, 2010: Microbial production of polyhydroxy alkanotes (PHA) from *Alcaligens spp*. and *Pseudomonas oleovorans* using different carbon sources. *African Journal of Biotechnology*, Vol. 9(21), pp. 3144-3150.
15. Senior, P., G. Beech, G. Ritchie and E. Dawes, 1972: The role of oxygen limitation in the formation of poly-beta-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii. Biochem*. J,128, 1193-1201.
16. Sepahei, A. A., A. Khanafari and M. Mogharab, 2006: Production and recovery of poly-3-hydroxybutyrate from whey degradation by *Azotobacter*. *Iranian Journal of Environmental Health Science & Engineering*,3, 1-2.
17. Steinbüchel, A. and B. Füchtenbusch, 1998: Bacterial and other biological systems for polyester production. *Trends in biotechnology*,16, 419-427.
18. Steinbüchel, A. and Y. Doi, 2002: Biopolymers, vol. 4. Wiley-VCH Verlag GmbH, Weinheim.
19. Tian, P., L. Shang, H. Ren, Y. Mi, D. Fan and M. Jiang, 2009: Biosynthesis of polyhydroxyalkanoates: current research and development. *African Journal of Biotechnology*,8.
20. Wang, B., R. R. Sharma-Shivappa, J. W. Olson and S. A. Khan, 2013: Production of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using sugarbeet juice. *Industrial Crops and Products*,43, 802-811.
21. Yang, Y.-H., C. J. Brigham, C. F. Budde, P. Boccazzi, L. B. Willis, M. A. Hassan, Z. A. M. Yusof, C. Rha and A. J. Sinskey, 2010: Optimization of growth media components for polyhydroxyalkanoate (PHA) production from organic acids by *Ralstonia eutropha. Applied microbiology and biotechnology*,87, 2037-2045.