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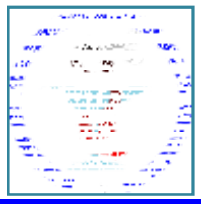

ORIGINAL ARTICLE **OPEN ACCESS**

Optimization and Characterization of Polyhydroxybutyrate (PHB) production by *Bacillus subtilis* LC535007 using cheap agro waste substrates

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ABSTRACT
Due to their relatively low cost, ease of manufacture and flexibility, the demand of plastics is ever-growing. However, plastics being man-made are not recognized by microorganisms. Polyhydroxybutyrate is a powerful replacement of non-degradable plastics. In the present study polyhydroxybutyrate production by Bacillus subtilis LC535007 strains was found to be a polyhydroxybutyrate producing bacteria by accumulation. Sudan black staining was used for the primary screening of PHB-producing B. subtilis LC535007 strains. The optimization study on different pH, temperature, incubation periods, and nutrient, carbon, and nitrogen sources were studied. The extracted PHB samples were evaluated by UV-Vis, FTIR, NMR, XRD, and SEM
Keywords: *Bacillus subtilis, PHB, Nutrient sources, Sudan black staining*

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INTRODUCTION

Plastics are high demand due to their relatively low cost, ease of manufacture, and versatility. Plastics, on the other hand, are not recognized by microorganisms because they are man-made, As a result; they take a long time to degrade, taking an average of 450 years for a plastic bottle to degrade [1]. Plastic trash is difficult to handle for nature. Since most plastics are not decomposed by microorganisms. The biodegradability of bioplastics in different environments, such as marine/fresh water and soil, is a type that makes their life cycle additional eco-friendly compared to the conventional plastics and could be a solution to this serious environmental issue.

As the natural environment is continuously polluted by these dangerous non degradable plastics, the improvement and production of environmentally-conserved biodegradable plastics is fast increasing in order to reduce our dependency on synthetic plastics [2]. In terms of the environment, the accumulation of plastic wastes has become a main concern [3-5]. Conventional plastics not only take a long time to be decomposed in nature, but they also produce toxins during the process of degradation. For this reason, there is special interest in producing plastics that can be easily removed from our biosphere in an "environmentally friendly" fashion [6].

Because our economy is still extremely oil dependent, this is a worldwide issue. Plastics are being consumed in the world at a rate of around 140 million tons per year. About 150 million tons of fossil fuels are used in the processing of these plastics, which are difficult to replace. The world's problem is to see if we can replace the non-sustainable source of these lengthy carbon arrays with a sustainable renewable supply [7]. There is a lot of interest in the development and production of biodegradable polymers or bioplastics as an alternative. Many uses, including as applications in agriculture, medical field and packaging including tissue engineering and drug delivery [8]. Despite these intriguing characteristics, industrial PHB production is still a work in progress due to its high cost. As a result, it is unable to compete with traditional plastics in the commercial market.

Plastic litter will be solved by biodegradable plastics. The successful development of biodegradable plastics will reduce the environmental impact that customers have on the environment. Bioplastics will save the lives of domestic animals while also protecting the environment. The main focus of the present study is polyhydroxybutyrate production from *Bacillus subtilis* LC535007. Sudan black staining for Screening this *Bacillus subtilis* LC535007 bacterial strains. Optimization study of carbon, nitrogen and

nutrient sources in the yield of PHB production. Characterization of extracted polyhydoxybutyrate was carried out by FT-IR, UV- Vis, NMR, XRD and SEM.

MATERIAL AND METHODS

Microorganism

Bacillus subtilis LC535007 strain was maintained on nutrient agar slant at 4°C by weekly subculture. These cultures are obtained from a waste dump yard site soil in Chidambaram local areas.

Inoculums and Substrate Preparation

Tapioca powder and Corn flour powder collected from local grocery store, Orange peel wastes are collected from Chidambaram local fruit shops in Chidambaram, Tamil Nadu, India [9]. Orange peels are washed several times with tap water for the removal of dirt and eight gram of orange peel wastes are sterilized for 20 min at 121°C followed by the addition of 200 ml sterile distilled water to pretreated material. This mixture was boiled for 30 minutes at 80°C followed by filtered through cheese cloth. The filtrate was treated with HCL 1 % (v/v) and heated at 121°C for 30 minutes [10]. Sugarcane bagasse is locally collected in Chidambaram were shred into pieces of sugarcane bagasse, and dried in the oven at 60°C after pulverizing into fine particles. They were hydrolyzed by the zinc chloride method [11].

Media

Nutrient agar medium compositions are beef extract and yeast extract (0.3%), agar (1.5 %), Peptone (0.5%), adjusted pH (7.5). a modified PHB producing Mineral salt medium compositions are $MgSO_4$ (0.20 g), glucose (50 g), NaCl (0.10g), Yeast extract (2.50 g), KH_2PO_4 (0.50 g) peptone (2.50 g),in 1000 mL [12] and using different nitrogen sources are (Tapioca powder (1%), Orange peel waste (1%), corn flour powder (1%), Sugarcane bagasse (1%) use this substrates for yield of PHB production and inoculated with 24 hours old culture of ***Bacillus subtilis*** (1%) and incubated for 96 hours at 37°C in a shaking incubator (120 rpm). The comparative standard was a modified mineral salt medium in distilled water [9].

Cell Dry Weight

After incubation period was this culture centrifuged at 15 min at 10,000 rpm and discarded supernatant the cell pellet was washed twice in distilled water after wash the pellet was dried at 55°C to constant weight for cell dry biomass measurement [13].

Extraction and Quantification of PHA

Extraction of PHB from fermented broth was done. The cells are harvested the 10 ml of culture was centrifuged at 10,000 rpm for 15 minutes. Discarded supernatant and the cell pellets was dried and weighted. After harvested the cell pellets was suspended in 4% sodium hypochlorite solution and incubated for 2 hrs at 37˚C. after 2 hrs the cell pellet was centrifuged at 5000 rpm for 15 min then washed with distilled water, methanol and acetone are respectively the cell pellet was dissolved in 5 ml of hot chloroform by pouring the solution on sterile glass are kept at 4°C and weighted after evaporation [14].

Culture conditions and Optimization of PHB

Effect of different Incubation period for PHB production using different incubation period times for (24hrs, 48hrs, 72hrs, 96hrs), culture was tested for bacterial growth and polyhydroxybutyrate production by dry cell weight measurement method [15].

Effect of temperatures on PHB production

Effect of different temperature for PHB production using temperatures are (25˚C, 30˚C, 37˚C, 40˚C) for 96 hrs. PHB yield was quantified that based on optimum temperature for maximum PHB production was determined [16].

Effect of media pH on PHB production

Effect of different pH level for PHB production using different pH are (6.0, 7.0, 7.5, and 8.5) for 96 hrs. Yield of PHB was for bacterial growth and polyhydroxybutyrate production by dry cell weight measurement method [16].

Effect of different carbon sources on PHB production

The effect of galactose, lactose, sucrose and maltose on PHB production by the isolates are selected was assessed by separately adding 1% (W/V) of the sugars in a standard for the mineral salt medium at the pH level 7.0, incubation period at 150 rpm at 37°C for 48 hours followed by PHB extract weight measurement and dry biomass.

Effect of nitrogen sources on PHB production

The mineral salt medium individually augmented with 1% (v/v) nitrogen sources (Yeast extract, ammonium sulfate, malt extract, and ammonium nitrate) at the pH level 7.0 was inoculated with isolates are incubated at 150 rpm at 37°C for 48 hours. The biomass and PHB produced have been measured as above.

Characterization of PHB

UV-Vis spectrophotometer analysis of PHB

Preliminary characterization of extracted polyhydroxybutyrate was done using UV–Vis spectrophotometer [17]. The absorbance spectrum of PHB was dissolved in $CDCl_3$ and measured in the range of 800–200 nm against chloroform blank, and the spectrum was analyzed.

FTIR spectrophotometer analysis of PHB

In this present study the extracted polyhydroxybutyrate was analyzed by Fourier Transform Infra Red (FTIR) spectrum [18]. The functional group present in PHB was examined between the frequency ranges of 3,800-800 cm^{-1} by Spectrum 65 Fourier Transform – Infrared Spectroscopy [19].

NMR Analysis of PHB

Extracted PHB dissolving in chloroform analyzed on Bruker Avance III spectrometer at a concentration of 10 mg/mL using tetramethylsilane (TMS) as internal change norm, the monomer composition of PHA was calculated using ^{1}HNMR study [20].

XRD Analysis of PHB

X-ray diffraction (XRD) the crystal structure of PHB formed by powder XRD patterns was analyzed using an X-ray diffractometer recorded using the Cu K-beta radiation source. Data were recorded as described by [21].

SEM Analysis of PHB

SEM instrument is available at Annamalai University in Chidambaram; Scanning electron microscopic analysis was performed using an SEM instrument. A thin film of the sample was formed by simply dropping a very little amount of the sample on a copper grid coated with; a thin film of the sample was formed. The film was then magnified at 1,000, 5,000, and 10,000 times for cell observation [22].

Screening for PHB producing bacteria

Polyhydroxybutyrate production by *B. subtilis* LC535007 strains was found to be of polyhydroxybutyrate producing bacteria by accumulation. PHB Producing bacterial ***B. subtilis*** **LC535007** strains are primarily screened by using the Sudan black staining method. These stains are observed by light microscope examination showed (**Fig -1).**

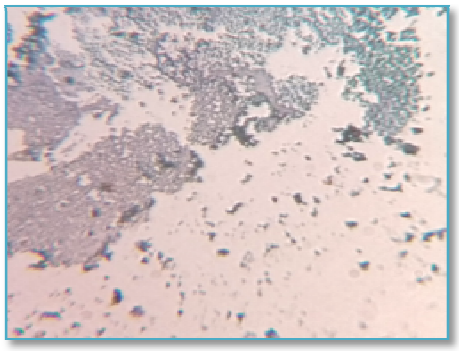
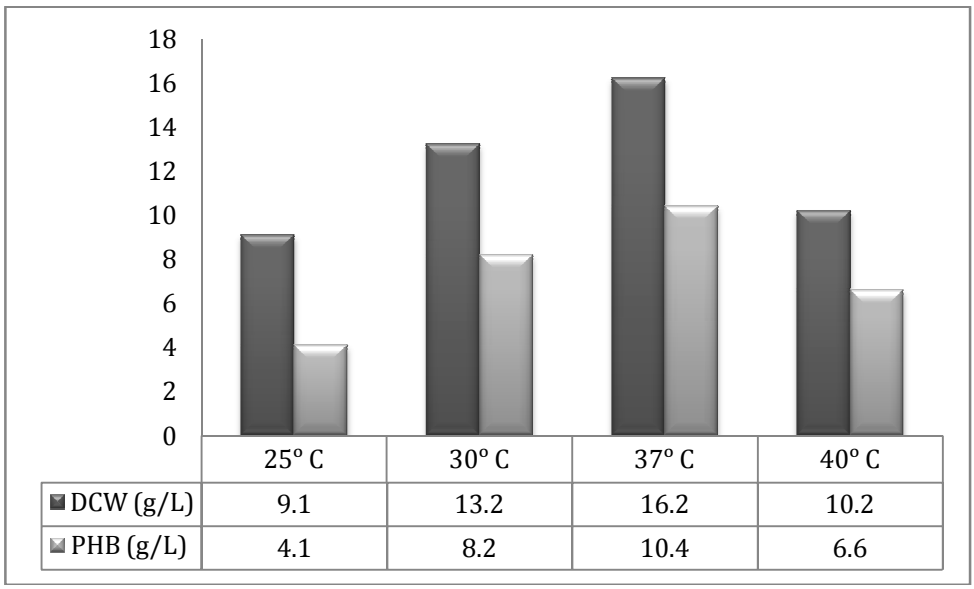


Fig – 1 PHB produced in form of dark granules

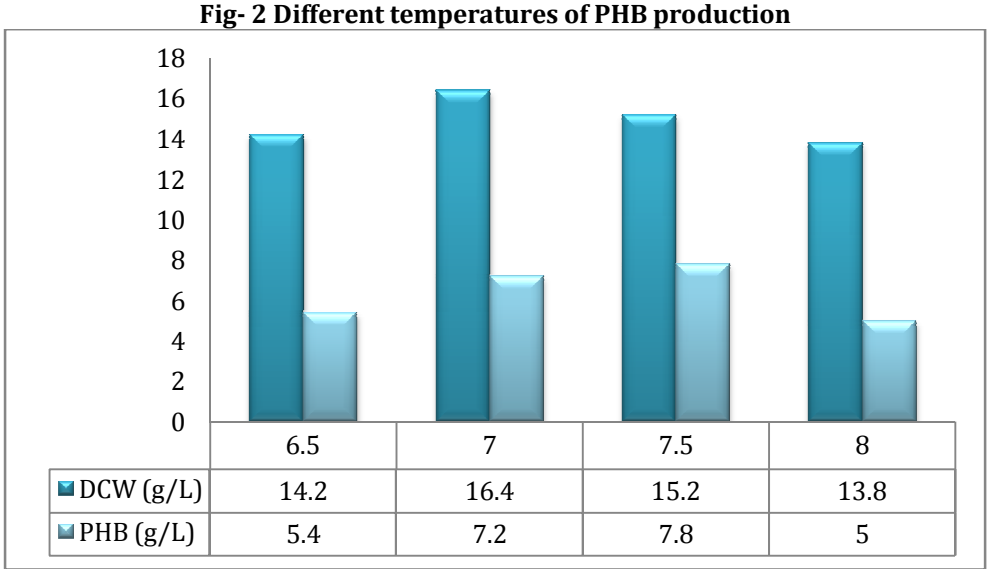
Optimization of PHB

In this current study, polyhydroxybutyrate production by *B. subtilis* under the different temperatures are **(**25°C, 30°C, 37°C, and 40°C) shown in **Figure-2**, pH ranges are (6.0, 7.0, 7.5, and 8.5), shown in **Figure-3**, Incubation periods are **(**24, 48, 72 and 96 hrs) production of polyhydroxybutyrate yield was shown in **Figure-4**. PHB production was assessed by the different cheap agro waste substrates are level of replacing glucose in the mineral salt medium were incubated at 37° C for 96 hrs (Corn flower, Tapioca powder, Sugarcane bagasse, Orange peel waste) production of PHB yield was shown in **Figure-5.** Effect of different carbon sources in the production of polyhydroxybutyrate by replacing glucose in mineral salt medium with fructose, sucrose, galactose and maltose on polyhydroxybutyrate producing *B. subtilis* LC535007 strains are adding 1% to mineral salt medium incubation period for 150 rpm at 37°C for 96 hours the results are shown in **Figure-6.** Effect of different nitrogen sources in the PHB production mineral salt medium individually augmented with 1% (w/v) nitrogen sources (Ammounium sulfate, ammonium nitrate, malt extract and yeast extract) on polyhydroxybutyrate producing *B. subtilis* LC535007 strains adding 1% to mineral salt medium were incubated 150 rpm at 37°C for 96 hours the results are shown in **(Figure-7).**



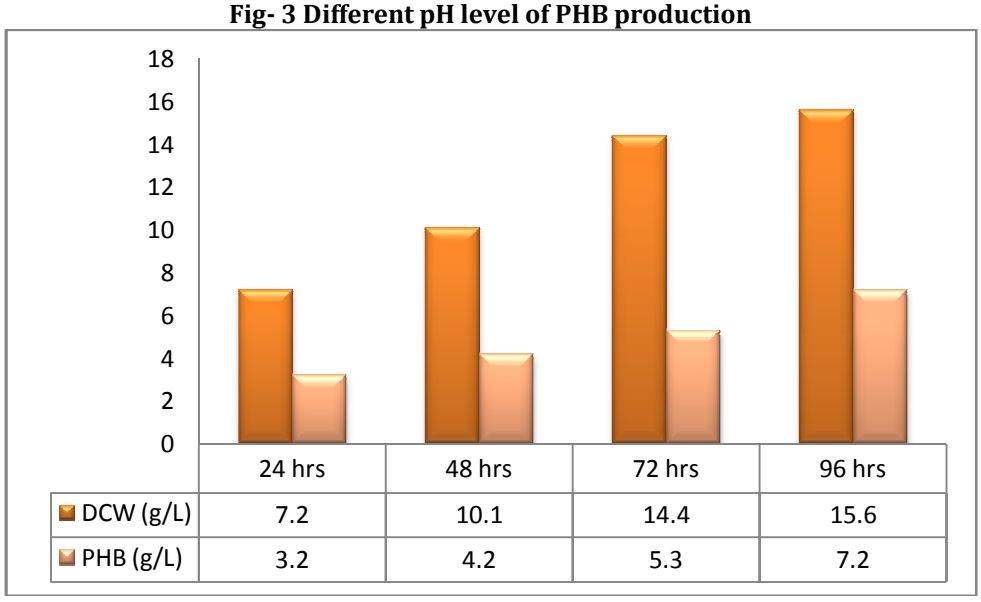
	25° C	30° C	37° C	40° C
DCW (g/L)	9.1	13.2	16.2	10.2
PHB (g/L)	4.1	8.2	10.4	6.6

Fig- 2 Different temperatures of PHB production



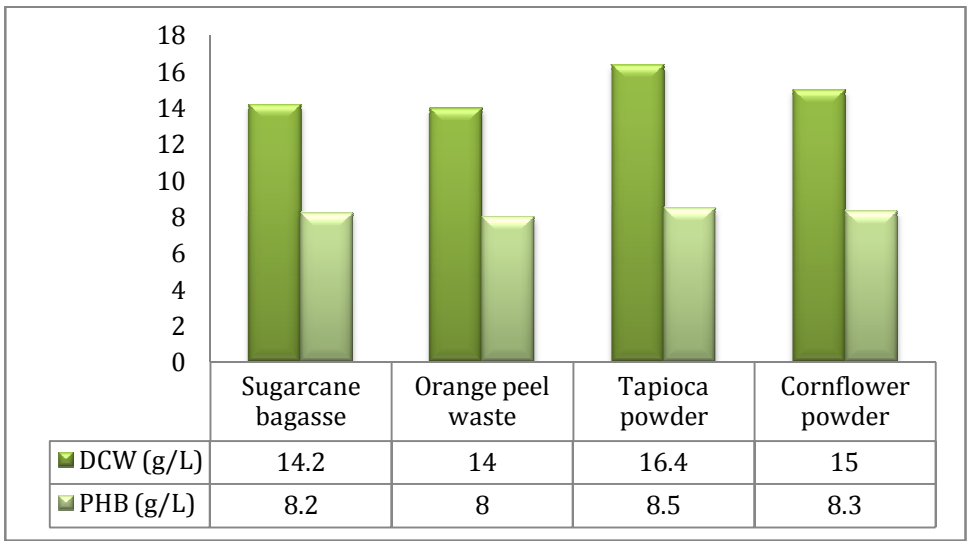
	6.5	7	7.5	8
DCW (g/L)	14.2	16.4	15.2	13.8
PHB (g/L)	5.4	7.2	7.8	5

Fig- 3 Different pH level of PHB production



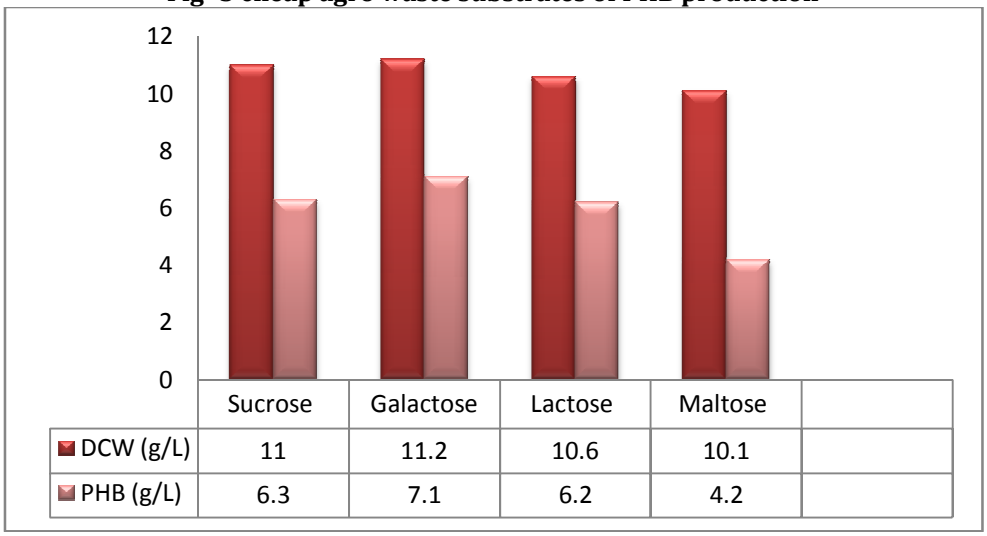
	24 hrs	48 hrs	72 hrs	96 hrs
DCW (g/L)	7.2	10.1	14.4	15.6
PHB (g/L)	3.2	4.2	5.3	7.2

Fig- 4 Different incubation periods of PHB production



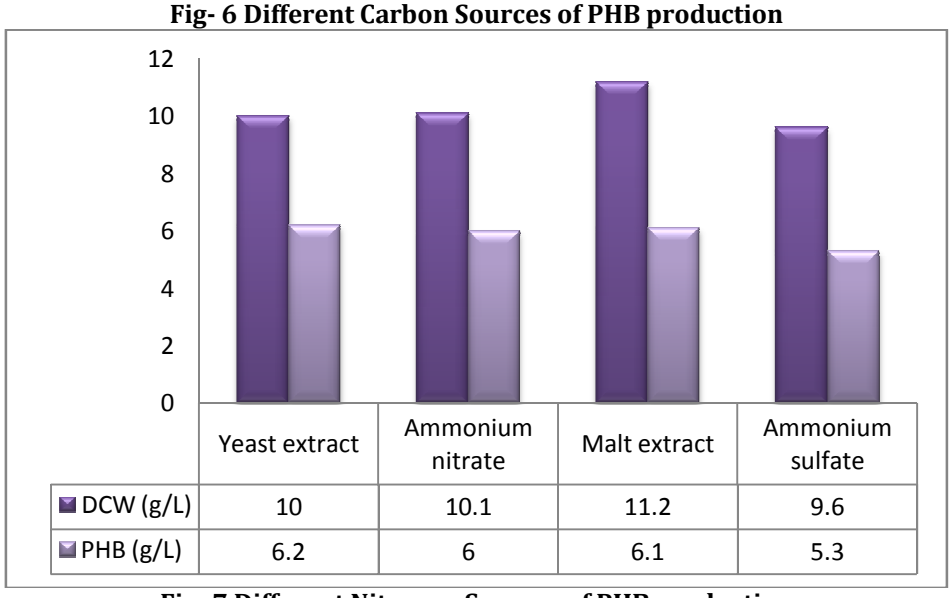
	Sugarcane bagasse	Orange peel waste	Tapioca powder	Cornflower powder
DCW (g/L)	14.2	14	16.4	15
PHB (g/L)	8.2	8	8.5	8.3

Fig- 5 cheap agro waste substrates of PHB production



	Sucrose	Galactose	Lactose	Maltose	
DCW (g/L)	11	11.2	10.6	10.1	
PHB (g/L)	6.3	7.1	6.2	4.2	

Fig- 6 Different Carbon Sources of PHB production



	Yeast extract	Ammonium nitrate	Malt extract	Ammonium sulfate
DCW (g/L)	10	10.1	11.2	9.6
PHB (g/L)	6.2	6	6.1	5.3

Fig- 7 Different Nitrogen Sources of PHB production

Characterization of PHB

The preliminary confirmation of polyhydroxybutyrate extract was done by Scanning with a UV-Vis spectrophotometer by measuring absorbance records in the 200-800 nm range **(Figure- 8).** An absorbance sharp peak was observed at **261 nm** [23].

FT-IR technique for identifying functional groups present in **a** polyhydroxybutyrate [24]. Extracted PHB from *B. subtilis* LC535007 strains was subjected to FTIR in the frequency range of 3,800– 800 cm-1 to analyze the characteristic functional groups present (**Figure - 9**). PHB extract was identified functional groups –CH2, –OH, C=O amide protein, C=O ester,–C–O–, C=O ester, CH3, alkyl halides. ^{1}H NMR technique was used to determining the composition and monomeric structure of the PHB extract.

In ^{1}H NMR, extracted polymer produce characteristics of magnetic resonance and any changes in these groups can be easily identified. ^{1}H NMR spectrum of extracted PHB from *B. subtilis* LC535007 indicated three characteristic peaks that are specific to polyhydroxybutyrate molecules [25] a double at 2.07 ppm contributed by a methyl group, a doublet of quadruple at about 3.14 ppm which was a characteristic of methylene group and a multiple at 4.01 ppm which is attributed to methylene group (**Figure–10).**

X-ray diffraction analysis (XRD) of polyhydroxybutyrate was used in this study to indentify whether the PHB extract was crystalline or amorphous [26]. The XRD pattern is recorded (**Fig-11)** revealed peak values of 2θ at 11.3, 14.3, 19.6, 27.5, 32.4, 46.5, 53.9, 66.9, 76.0, 78.0, and 84.5 which are characteristic of PHB molecules. The increased intensity of peaks at 110.0 and 116.1 showed that the polymer may have a more organized/packaged crystalline structure.

Extracted PHB was magnified at 1K, 5K, and 10K. Scanning electron microscope (SEM) analysis was done for the polyhydroxybutyrate these granules are either single or in budding groups **(Figure- 12).**

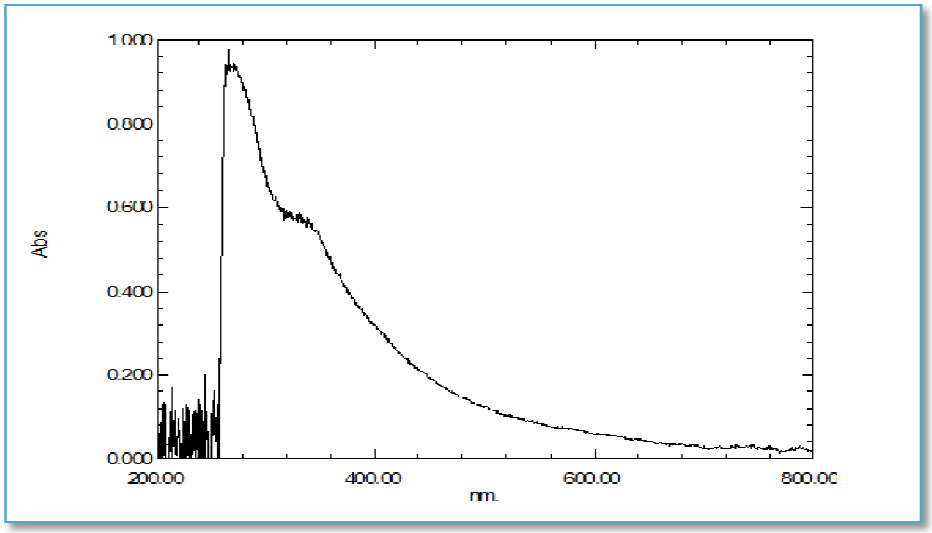


Fig- 8 UV- Vis Spectrophotometer Scanning Spectrum of PHB Compound

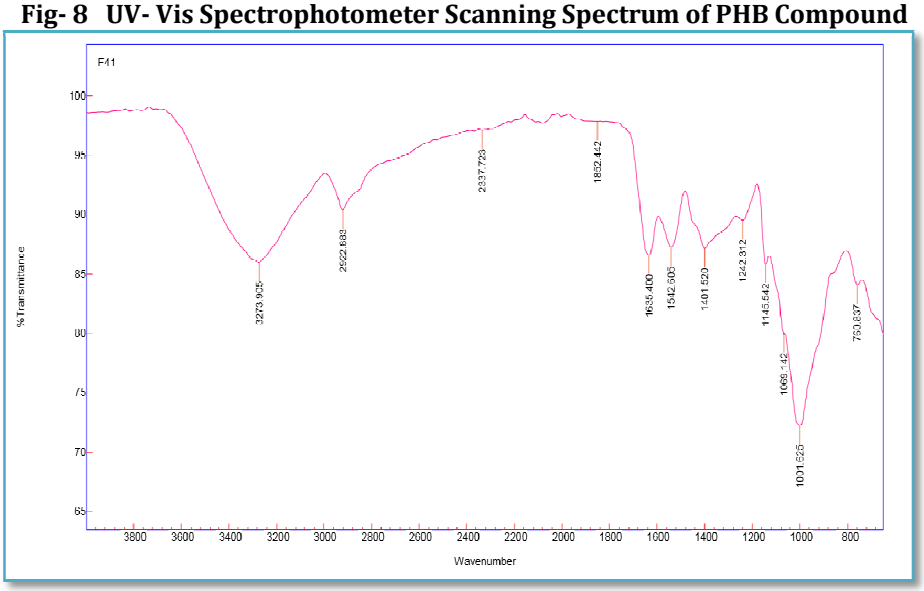


Fig- 9 FT-IR analysis of Polyhydroxybutyrate

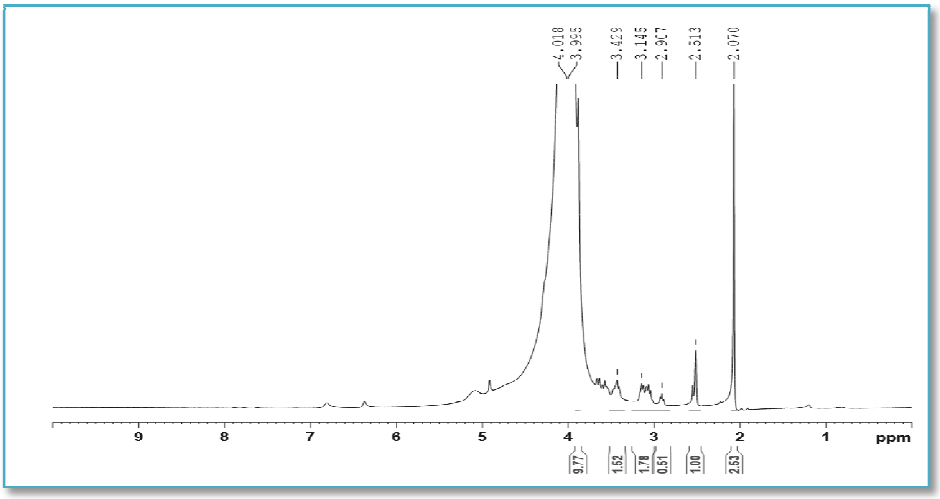


Fig- 10 ^{1}H NMR Spectrum of extracted Polyhydroxybutyrate

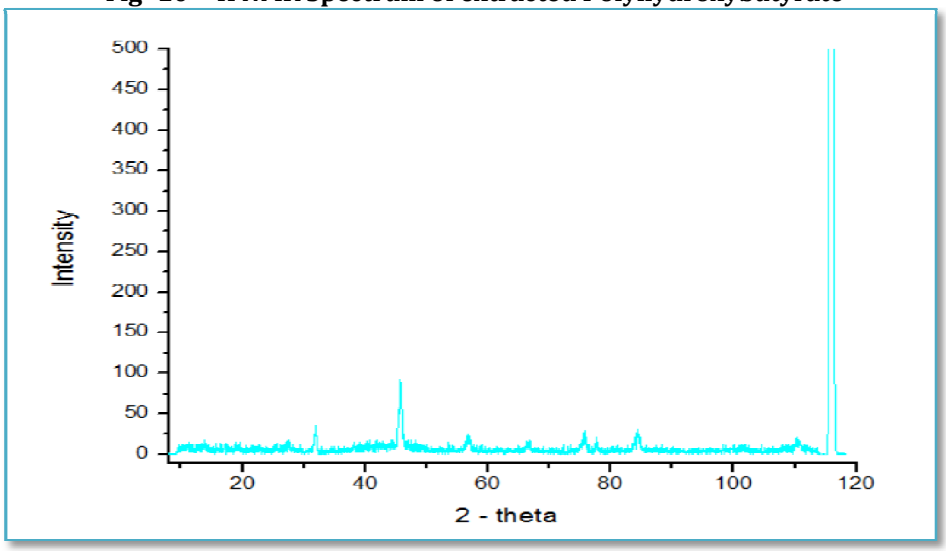


Fig- 11 XRD Spectrum of extracted Polyhydroxybutyrate

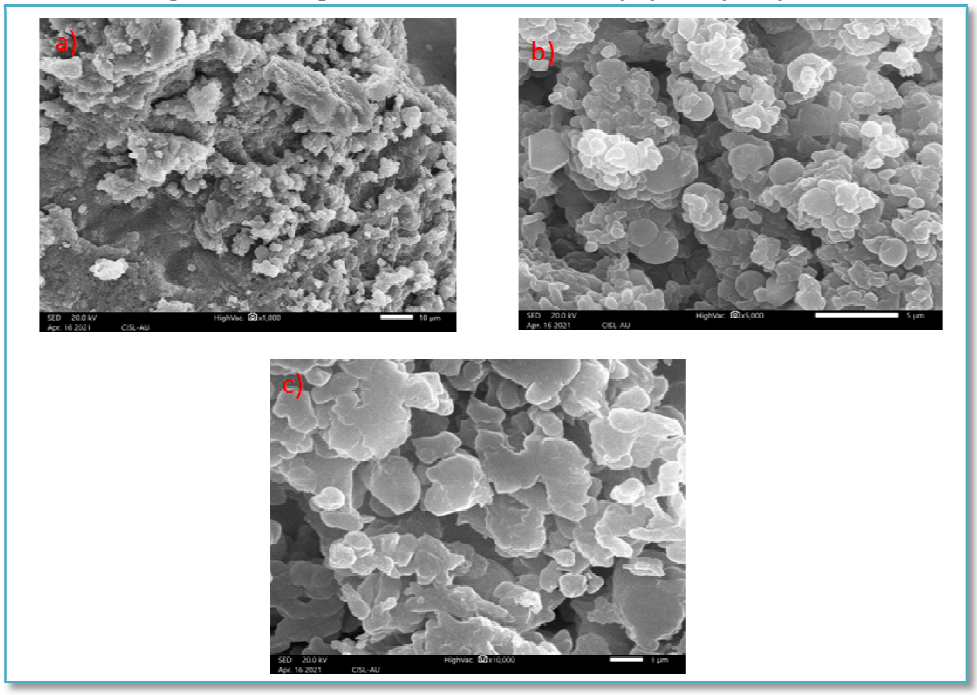


Fig- 12 SEM images of extracted Polyhydroxybutyrate (a) 1k (b) 5k (c) 10k Magnifications

CONCLUSION

Researchers are focusing on bioplastic production from microorganisms for developing biodegradable plastics. Reduce the biggest environmental pollution and also solving the agricultural waste disposable problems. Polyhydroxybutyrate is a powerful replacement of non- degradable plastics. The present study was concluded that PHB producing *Bacillus subtilis* LC535007 bacterial strains by Sudan Black staining method showed positive results by dark black granules are PHB producing bacteria. PHB was extracted from using different carbon sources (e.g. lactose, sucrose, maltose, galactose), nitrogen sources (e.g. ammonium sulfate, ammonium nitrate, malt extract, yeast extract) and cheap agro waste substrates(e.g. sugarcane bagasse, orange peel waste, tapioca powder, cornflower powder) for a yield of polyhydroxybutyrate production. Characterization of polyhydoxybutyrate production was carried out by FT-IR, UV- Vis, NMR, XRD and SEM.

REFERENCES

1. LeBlanc, R. (2017). How Long Does It Take Garbage to Decompose? the balance.
2. Steinbuchel, A. (2001). Perspectives for biotechnological production and utilization of biopolymers: Metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a ssuccessful example. Macromolecular Bioscience 1: 1-24.
3. Guillet, J. (2002). Plastics and environment. In: Scott G, editor. Degradable polymers: principles and applications. Dordrecht, the Netherlands: Kluwer Academic Publisher; 413–48.
4. Derraik J.G. (2002). The pollution of the marine environment by plastic debris: a review. Mar Pollut Bull 44: 842–52.
5. Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., et al. (2004). Lost at sea: where is all the plastic? Science, 304:838.
6. Gross, R.A., and Kalra, B. (2002). Biodegradable Polymers for the Environment. Sci. 297: 803-807.
7. Pornpa Suriyamongkol, Randall Weselake, Suresh Narine, Maurice Moloney, Saleh Shah. (2007). Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants - A review, Biotechnology Advances, 25, 148–175.
8. Rydz, J., Wanda, S., Mariya, K., and Darinka, C. (2015). Polyester-Based (Bio) Degradable Polymers as Environmentally Friendly Materials for Sustainable Development. International Journal of Molecular Sciences, 16(1): 564–596.
9. Mirdul umesh, Kumaresan Priyanka Basheer and Kathirvel Preethi. (2018). Biogenic PHA nanoparticle synthesis and characterization from *bacillus subtilis* NCDC0671 using orange peel medium. International journal of polymeric materials and polymeric biomaterials, vol- 67, No. 17 996-1004.
10. Pumiput, P.; Chuntranuluck, S.; Kitpreechavanich, V.; PilaneeVaithanomsat, V. P. Production Process of Hydrolysate from Steam Explosion of Oil Palm Trunk for Xylitol Fermentation. Kasetsart J. 2008, 42, 73.
11. Chen, G., and Wang, Y. (2013) Medical applications of biopolyesters polyhydroxyalkanoates. Chin J PolymSci 31: 719–736.
12. Ramsay, J.A., Berger, E., Ramsay, B.A., Chavarie, C. (1990). Recovery of Poly-3-Hydroxyalkanoic Acid Granules by a Surfactant- Hypochlorite Treatment. Biotechnol. Tech. 4, 221–226. DOI: 10.1007/bf00158833.
13. Du, G., Chen, J., Yu, J,. and Lun, S. (2001). Continuous production of poly-3hydroxybutyrate by *Ralstonia eutropha* in a two stage culture system. J. Biotech., 88: 59-65.
14. Anteneh Getachew and FantahunWoldesenbet. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low-cost agricultural waste material. Getachew and WoldesenbetBMC Res Notes 2016, 9:509 DOI 10.1186/s13104-016-2321-y.
15. Shah, K.R. (2014). Optimization and production of Polyhydroxybutarate (PHB) by Bacillus subtilis G1S1from soil. Int J Curr Microbiol App Sci, 3(5):377-387.
16. Singh, G., Mittal, A., Kumari, A., Goel, V., Aggarwal, N.K., Yadav, A. (2011). Optimization of Poly-B-Hydroxybutyrate Production from Bacillus species. Eur J Bio Sci, 3(4):112-116.
17. Poindexter, J. S.; Eley, L. F. Combined Procedure for Assays of Poly-β-Hyroxybutyric Acid and Inorganic Polyphosphate. J. Microbiol. Methods 1983, 1, 1–17. DOI: 10.1016/0167-7012(83) 90002-7.
18. Padermshoke, A., Katsumoto, Y., Sato, H., Ekgasit, S., Noda, I. (2004). Surface melting and crystallization behavior of polyhydroxyalkanoates studied by attenuated total reflection infrared spectroscopy. Polymer, 45: 65476554.
19. Hong, K., Sun, S., Tian, W., Chen, G.Q., Huang, W.A. (1999). rapid method for detecting bacterial PHA in intact cells by FT-IR. Appl Microbiol Biotechnol. 51:523–6.
20. Yoshie, N., Goto, Y., Sakurai, M., Inoue, Y., Chûjô, R., Doi, Y. (1992). Biosynthesis and NMR Studies of Deuterated Poly(3-Hydroxybutyrate) Produced by Alcaligeneseutrophus H16. Int. J. Biol. Macromol. 14, 81–86. DOI: 10.1016/0141-8130(92)90003-q.
21. Jayachandra Yaradoddi, Vinay Patil, Sharanabasava Ganachari, Nagaraj Banapurmath1, Anand Hunashyal, Ashok Shettar. (2016). "Biodegradable plastic production from fruit waste material and its sustainable use for green applications." *Int. J. Pharm. Res. Allied Sci* 5(4), 72-81.
22. Sasikala Sadasivam, Santhosh Sigamani, Hemalatha Venkatachalam & Dhandapani Ramamurthy. (2018). A New Method for the Production of Polyhydroxyalkanoates by Bacillus sp. and Detect the Presence of PHA Synthase. Smart Science. 6(2):105-16.

23. Santhanam, A., Sasidharan, S. (2010). Microbial Production of Polyhydroxyalkanoates from Alcaligens spp and Pseudomonas Olevoransusing Different Carbon Sources. Afr. J. Biotechnol. 9, 3144–3150.
24. Naumann, D., Helm, D., Labischinski, H. (1991). Microbiological Characterizations by FT-IR Spectroscopy. Nature, 351, 81–83. DOI: 10.1038/351081a0.
25. Lakshman, K., Shamala, T.R. (2006). Extraction of Polyhydroxyalkanoate from *Sinorhizobiummeliloti* Cells Using Microbispora sp. Culture and its Enzymes. Enzyme Microb. Technol. 39, 1471. DOI: 10.1016/j. enzmictec.2006.03.037.
26. De Rooy, S. L., Wahyuni, E. T, Wiratni, W., Syamsiah, S., Ismail, J. (2010). Purification and Characterization of Poly-Hydroxybutyrate (PHB) in Cupriavidus necator. Indones. J. Chem. 7, 243–248.

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