Pak-Euro Journal of Medical and Life Sciences
ISSN: 2707-4471 (Print), 2707-448X (Online)
www.readersinsight.net/pjmls

Research Article

Prodcution of Polyhydroxy Butyrate (PHB) from Soil Bacterium (Bacillus megaterium TISTR 1814) with Cantaloupe Waste Extract as Potential Carbon Source

Hussana Hamid Ali[1], Zia Ur Rehman[*1], Sana Basheer[1], Ali Akbar[2] and Muhammad Kamran Taj[3]

[1]*Institute of Biochemistry, University of Balochistan, Quetta, Pakistan.*
[2]*Department of Microbiology, University of Balochistan, Quetta, Pakistan.*
[3]*Centre for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, Pakistan.*

Corresponding author: ziaasian08@gmail.com

Abstract

Background of the study: Polyhydroxybutyrate (PHB) is a natural biodegradable and biocompatible polymer having similar physical properties to those of standard conventional plastics. It is eco-friendly and can be produced economically at industrial scale via fermentation.
Purpose of the study: In this present study, a gram positive soil bacterium, *Bacillus megaterium* TISTR 1814 was selected which accumulates polyhydroxybutyrate (PHB) as stored chemical energy. This is the first detailed study, on cantaloupe waste extract as a cost effective nutrient source enriched with nitrogen sources for PHB yield were studied.
Methods: *B. megaterium* TISTR 1814 was cultivated on designed media containing cantaloupe waste extract enriched with different nitrogen sources. The monosaccharides present in cantaloupe waste extract were analyzed by HPLC method. The biomass was weighed and PHB was extracted with sodium hypochlorite for FT-IR analysis. The disrupted cells were examined under scanning electron microscope to visualize PHB granules.
Results: The highest cell density was observed at 30 °C after a period of 72 h of incubation of *B. megaterium* TISTR 1814 with highest biomass yield of 1.1 g/L using cantaloupe waste extract enriched with ammonium chloride as nitrogen source. On the other hand, maximum production of PHB was observed to be 0.46 g/g. dried biomass from 0.9 g/L dried biomass using Ammonium sulphate as inorganic nitrogen source.
Conclusions: The cantaloupe waste extract was found cost effective substrate for PHB production from *B. megaterium* strain. Incubation period after 72 h of incubation at 30 °C were the best conditions for PHB yield.

Keywords: *Polyhydroxybutyrate (PHB); Bacillus megaterium; cantaloupe waste extract; ammonium sulphate; ammonium nitrate; ammonium chloride.*

ARTICLE INFORMATION

Received: 17.04.2020
Revised: 06.05.2020
Accepted: 07.05.2020

DOI: 10.31580/pjmls.v3i1.1293
Author's Contributions: HHA conducted experiemnts, data analysis & manuscript writing; ZUR research design, funding, data analysis, manuscript writing & supervision; SB experimental work; AA technical support and MKT technical support.

INTRODUCTION

Petrochemical plastics are synthetic polymers derived from fossil fuels used in every prospects of human life (1). However, non-degradability nature of conventional petroleum-based plastics causes severe environmental problems such as landfill hazard and air pollution (2). Additionally, increased petroleum prices and alarming fossil fuels depletion limits its worth. Due to this reason, researchers and industrialists are in pursuit of alternative products alike biodegradable polymers to fulfill the global needs of plastic for packing purposes (2). Therefore, bio-based polymers are found to be green approach due to their applicability and eco-friendly nature in contrast to likely toxic non-biodegradable plastic products. Bioplastics are natural biodegradable polymers synthesized from biodegradable sources such as different microbial species and plant starch (3). Bioplastics comprises compounds of (PHAs) polyhydroxyalkanoates, polylactides, copolymers and polyesters (4). However, (PHAs) are found to be the most propitious class of green polymers with various applications (2). Moreover, polymeric esters of PHAs include polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV) and polyhydroxybutyrate-co-hydroxyvalerates (PHBHx) (5). Polyhydroxybutyric acid (PHB) is small chain length (Scl) natural biodegradable and biocompatible polymer among all the polymeric esters of PHA (3). The intracellular granules of PHB were first isolated from a rod shaped gram positive, large sized soil bacteria, *Bacillus megaterium*, primarily aerobic and spore forming bacterium widely isolated from distinct habitats (6-8). Moreover, accumulation of PHB also been reported in Gram-negative species (1). Previous studies suggest that *Bacillus* species are promising candidates for

PHB production due to its capability of producing considerable amount of bioplastic (1).

PHB with having similar physical properties to non-biodegradable polymers, however the high production cost of biopolymers limits its popularization on commercial scale compared to conventional plastics (2). Therefore, inexpensive substrate, optimized fermentation parameters, modified cultivation methods and simple downstream handling are found to be symbolic strategies to reduce the production cost of bioplastic as they alter the microbial metabolism (1). Therefore, inexpensive substrate, optimized fermentation parameters, modified cultivation methods and simple downstream handling are found to be symbolic strategies to reduce the production cost of bioplastic as they alter the microbial metabolism (1).

In the present study, *Bacillus megaterium* TISTR1814 was investigated as potential candidate for the production of PHB, cultivated on cheap nutrient source as cantaloupe waste extract. To the best of our knowledge, this is the first detailed study on the cantaloupe waste extract as a potential cheap carbon source for accumulation of PHB by the selected strain. The Cantaloupe waste extract was supplemented with three different nitrogen sources for enhanced biomass production and PHB accumulation. The PHB was then extracted and analyzed by FTIR spectra for its functional groups.

MATERIALS AND METHODS

Chemicals and Reagents

All of the chemicals, reagents and microbial culture medium used in the present study were of analytical grade and purchased from Sigma-Aldrich chemicals (USA), DAEJUNG chemicals (Korea) and Fisher scientific (UK).

Experimental Strain

In the present study, the lyophilized strain of *Bacillus megaterium* TISTR 1814 was purchased from TISTR Thailand. The selected strain was stored in a refrigerator (3566A, Thermo Fisher Scientific, China Co., Ltd) at -20°C for further use.

Cultivation Conditions

The strain was cultivated aseptically into the four 500 ml Erlenmeyer flasks, containing 250 ml of nutrient broth medium which were cotton plugged and sterilized for 30 min at 121 °C in an autoclave (HV-85L, Thailand Co., Ltd). All of the four flasks were incubated at 30 °C for 24 h in an incubator (600 Hettcube, Germany Co., Ltd). The strain was sub-cultured aseptically into the sterilized nutrient broth after every 24 h.

Preparation of Glycerol Stocks

The glycerol stocks were prepared by mixing 40% sterilized glycerol in distilled water. The active cultures were added to 2 ml cryovial tubes containing 40% glycerol and were stored in a refrigerator (3566A, Thermo Fisher Scientific, China Co., Ltd) at -20°C. The glycerol stocks were examined under optical microscope (B490B, 40X-2000X, United Scope (Ningbo) Co., Ltd) for culture purity and were used as purified seeds for further study.

Preparation of Enriched Culture Medium for the Production of PHB

The enriched medium for the production of PHB was prepared from sterilized liquid extract of cantaloupe waste added with three different nitrogen sources, used as potential carbon source for the production of PHB. The liquid extract of cantaloupe waste was separated from the fibrous pulp by centrifugation at 10,000 rpm for 30 min in a centrifuge machine (Eppendorf- Polymer Group, 5840 R, Sichuan Shuke Co., Ltd). The chemical enrichment of the cantaloupe waste extract was carried out as (g/l) adopted from (9) with following elements : $(NH_4)_2SO_4$, 0.5(a); KH_2PO_4, 2.3; Na_2HPO_4, 2.3; $MgSO_4.7H_2O$, 0.5; $NaHCO_3$, 0.5; $CaCl_2$, 0.01; NH_4NO_3, 0.5(b); NH_4Cl, 0.5(c) and 1 ml trace element solution. The composition of trace element solution was (g/L) as follows: $ZnSO_4.7H_20$, 0.01; $MnSO_4.H_20$, 0.003; $CoCl_2.6H_20$, 0.002; H_3BO_4, 0.061; $CuSO_4.5H_20$, 0.246; $NiCl_2.6H_2O$, 0.002; $NaMO_4.2H_2O$, 0.003; $CaCl_2.2H_2O$, 0.147; $FeSO_4.7H_2O$, 0.278 and KI, 0.166. 250 ml of enrichment medium was poured into the three 500 ml Erlenmeyer flaks which were cotton plugged and sterilized for 15 min at 121°C in an autoclave (HV-85L, Thailand Co., Ltd). The pH of enrichment medium was adjusted to 7.0, In order to test the effect of different inorganic nitrogen sources on the rate of PHB, $(NH_4)_2SO_4$, ammonium chloride and ammonium nitrate were used. The pH of the medium was kept constant among all the experiments. After sterilization the flasks were cooled at room temperature and incubated at 30 °C for three different time periods (24, 48 and 72 h) in an incubator (600 Hettcube, Germany Co., Ltd). Inoculation of the sterilized enrichment culture medium was carried out aseptically by adding 4 ml of the pre-culture. The inoculated flasks were then incubated in an incubator (600 Hettcube, Germany Co., Ltd) at 30 °C for 24 h.

HPLC Analysis for Measurement of Monosaccharides

The monosaccharides in cantaloupe waste extract were analyzed by HPLC (Agilent system, HP 1100 Agilent USA), equipped with zorbax carbohydrate column (4.6 × 150 mm × 5 μm) and RID detector (Agilent). The mobile phase used was acetonitrile: deionized water at the ratio of 75:25 with constant column temperature as 40 °C.

Biomass Harvest and Drying

The biomass of *Bacillus megaterium* TISTR 1814 was centrifuged at 10,000 rpm for 20 min in a centrifuge machine (Eppendorf- polymer Group, 5840 R, Sichuan Shuke Co., Ltd). The supernatant was discarded into the flasks. The biomass was then dried over-night at 60 °C in hot air oven (Universal Mechanical Oven, 55 PLUS, Ejer Tech Co., Ltd). The dried bacterial pellet was weighed as grams per liter nutrient media. The results were taken in triplicates for all three different nitrogen sources.

Extraction and Quantification of PHB

PHB extraction was carried by adding 5 ml of sodium hypochlorite into the test tube containing dried bacterial pellet which was allowed to stand for 30 min at 37 °C in an incubator (600 Hettcube, Germany Co., Ltd) for microbial cell lysis in order to extract PHB. The suspension was centrifuged for 10 min at 10,000 rpm in a centrifuge (Eppendorf- Polymer Group, 5840 R, Sichuan Shuke Co., Ltd). After centrifugation supernatant was discarded and pellet was washed twice with 10 ml of dH_2O and was centrifuged again at 10,000 rpm for 10 min. The supernatant was discarded and the resultant PHB was washed with ethanol and acetone solution twice (1:1 v/v). Later 5 ml of boiling chloroform was added which was further evaporated by air drying (Drying oven VZLG-9070, Ejer Tech Co., Ltd). The PHB was then weighed and kept in refrigerator for further analysis.

Fourier Transform Infrared Spectroscopy (FTIR)

The algal sample was dried in an oven (Memmert: Model: 600, GmbH Co, Ltd) at 70 °C overnight. The dried biomass was ground to fine powder. The powder mixture was transferred to compression dye under high pressure to form a pellet. The pellet was kept in sample cuvette and analyzed according to standard FTIR (Perkin Elmer) test method ASTM: 1252-98 with light source in middle range infrared (4000–600cm^{-1}). The technique was employed for the determination of chemical composition of sample based on functional groups.

Scanning Electron Microscopy

The cells were examined under scanning electron microscope (SEM: Hitachi S-3400 N Japan) for overall cell lysis caused by sodium hypochlorite in order to release the PHB granules.

RESULTS AND DISCUSSIONS

Currently conventional plastics waste management is threatening to our environment and leads to the development of great interest in the production of biodegradable plastics (10). Exploration of soil bacterium *Bacillus megaterium* TISTR 1814 leads to the production of PHB from cheap bio-waste (cantaloupe waste extract) could probably replace conventional polymers which are presently used. The scope of the recent study is optimization of fermentation conditions, with distinct parameters of inorganic nitrogen sources for enhanced PHB production from *Bacillus megaterium* TISTR 1814 using cantaloupe waste extract as cheap carbon source.

Biomass Production

Microbial growth was confirmed by taking weighed of the cultivated broth with different nitrogen sources at different incubation period (h) as shown in Fig. 1. Biomass yield (g/L) was recorded at the interval of 24 h, while incubation temperature was kept constant at 30°C which was considered the optimum temperature for accumulation of PHB as reported from previous research studies with this species**.** Maximum biomass yield was observed at incubation period of 72 h, which is in agreement with earlier study by (2) using low cost agricultural residues and sewage samples for maximum PHB production from *Bacillus* sp. Moreover, (11) revealed the maximum production of PHB (41% w/w) at incubation period of 72 h from pea-shells as cheap bio-waste. Comparable another study was reported the maximum production of PHB from glycerol after 72 h (12). However, few studies are not in accordance with present result that found the maximum bacterial growth after 24 h of incubation (13-15).

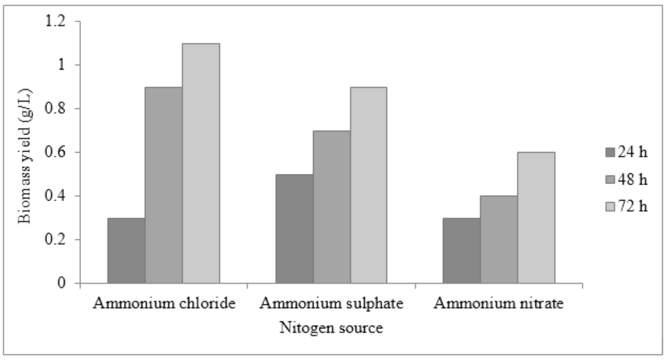

Fig. 1. Biomass yield (g/L) of *Bacillus megaterium* TISTR 1814 cultured with different nitrogen sources at different incubation time (h).

Effect of Different Inorganic Sources on PHB Yield

The effect of different inorganic sources (ammonium sulphate, ammonium nitrate and ammonium chloride) were tested for overall biomass yield (g/L) and PHB production. Enrichment of cantaloupe waste extract enriched with inorganic nitrogen effected the yield of PHB and biomass due to the fact that their insufficiency in the fermentation medium undergo the production of acetyl-coA by activating TCA cycle (16). It has been reported that reduction of nitrogen in fermentation media decreased the protein content while increased PHB accumulation (17).

As discussed above, in present study, the cantaloupe waste extract was enriched with three nitrogen sources for its effect on biomass and PHB yield. Ammonium chloride was found to enhance biomass while ammonium sulphate enhanced PHB production. Ammonium nitrate was found to be least effective both in case of biomass and PHB yield. From HPLC analysis, the cantaloupe waste extract was found to contain 5.5 g/100 g fructose and 6.27 g/ 100 g glucose which are essential for bacterial growth (Fig. 2)

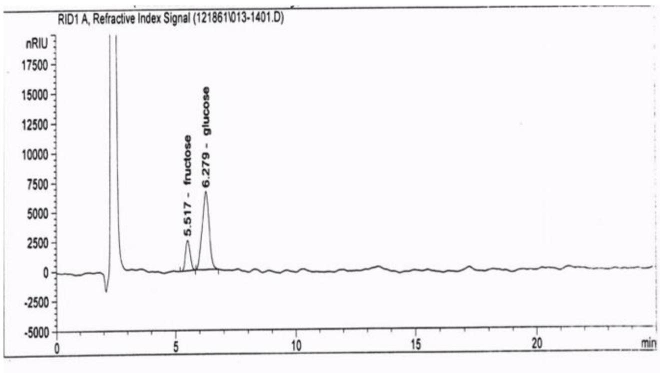

Fig. 2. HPLC chromatogram of glucose and fructose present in cantaloupe waste extract.

Nutrient broth yielded maximum PHB (0.37 g/L) from 0.97 g/L dried biomass. Similar results have been reported by previous studies (18,19). Using glucose as a carbon source, *Bacillus spp* accumulated 55.6% on optimum tmperature of 37 °C for 48 h (20). Furthermore, among standard and all inorganic nitrogen sources, ammonium sulphate amended medium gave maximum production of PHB as 0.46 g/L from 0.9 g/L dried biomass. The PHB yield from ammonium chloride and ammonium nitrate was 0.33 g/L and 0.2 g/L respectively as given in Fig.3.

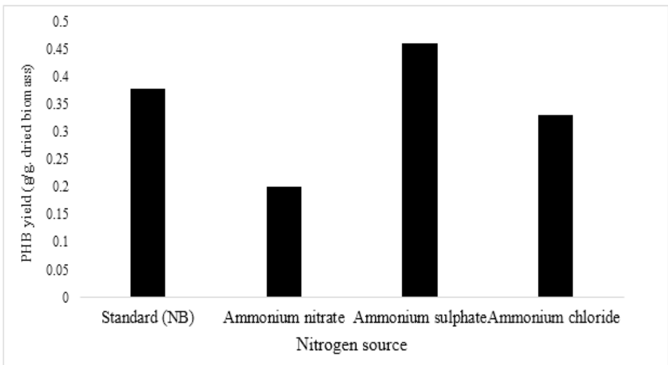

Fig. 3. Production of PHB (g/g. dried biomass) from *Bacillus megaterium* TISTR 1814 using cantaloupe waste extract enriched with 0.5 g/L of inorganic N- sources.

This result is comparable with previous studies having ammonium sulphate as an efficient inorganic nitrogen source with maximum PHB yield of 30.75% from *C. necator*, due to the fact that existence of sulphate anion elevates the nitrogen uptake hence making $(NH_4)_2SO_4$ an efficient nitrogen source (21). Most recently, *C. necator* was cultivated on cashew apple juice as a low cost carbon source supplemented with urea as nitrogen source for high yield of bioplastic (15.78 g/L) (22). Additionally, other previous studies also support this present study, in which ammonium sulphate was found as an efficient nitrogen source followed by ammonium nitrate to produce PHB from *Bacillus sp.* (23,24). Moreover, diverse microbial strains having different preferences of nitrogen sources for accumulation of biological polymers (23). The scanning electron micrograph clearly shows the PHB granules inside lysed bacterial cells (Fig.4).

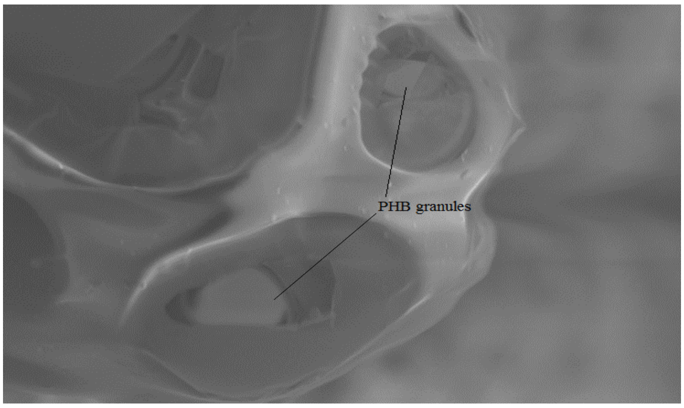

Fig. 4. Lysed *Bacillus megaterium* TISTR 1814 cell showing PHB granules.

FTIR Analysis

The FTIR spectroscopy is considered as an important analytical technique for qualitative analysis of chemical structures. This technique is basically used for the identification and verification of functional groups present in PHB polymer. The spectral result of PHB produced by *Bacillus megaterium* TISTR 1814 is given in Fig. 5. The noticeable peak of 1727 cm^{-1}, corresponded to the presence of carbonyl groups of ester compound present in PHB molecules (25). Additionally, the peaks in the region of 1261 and 1166 cm^{-1} were matched to C-O-C groups while 1057 and 979 cm^{-1} were attributed to C-O bonds. The FTIR spectra from each sample is in agreement to PHB spectra obtained from commercial PHB *and Bacillus shackletonii* K5 (26).

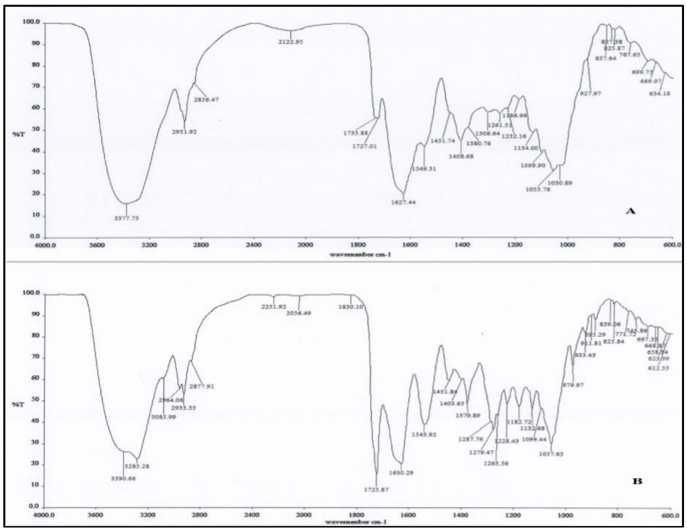

Fig. 5. FTIR spectra of PHB extracted from *B. megaterium* TISTR 1814 biomass cultivated on cantaloupe waste extract enriched with ammouniumsulphate **(A)** and ammonium chloride **(B).**

The C-H stretch bonds in the polyester were assigned to the bands located in the spectral region around 2900 cm^{-1}. The obtained FTIR absorption peaks from the culture were in agreement with the corresponding spectra to pure PHB. Based on the above results, it was concluded that the extracted compound from *B. megaterium* TISTR 1814 should be PHB.

CONCLUSIONS

In the present study, for the first time cantaloupe waste extract as cost effective substrate was used to produce the considerable amount of PHB from *B. megaterium* strain. Incubation period after 72 h of incubation at 30°C were the best conditions for PHB yield, and the maximum PHB (0.7g/L) yield has been adapted when Ammonium sulphate followed by other nitrogen sources were used. This cost effective and eco-friendly strategy can fulfill the potential development objective of PHB production.

Conflict of Interest: There is no conflict of interest.

REFERENCES

1. Asad-ur-Rehman M, Aslam A, Masood R, Aftab MN, Ajmal R, Haq IU. Production and Characterization of Thermostable Bioplastic (Poly-beta-Hydroxybutyrate) from Bacillus Cereus NRRL-B-3711. Pakistan Journal of Botany. 2016;48(1):349-56.

2. Kumaran B, Santhi R, Sridharan J. Exploitation of agro industrial residues as a substrate for the biodegradable polymer production using polyhydroxybutyrate accumulating bacteria. 2017;6:702-706.

3. Singh P, Parmar N. Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria. African Journal of Biotechnology. 2011;10(24):4907-19.

4. Luengo JM, García B, Sandoval A, Naharro G, Olivera ER. Bioplastics from microorganisms. Current opinion in microbiology. 2003;6(3):251-60.

5. Steinbüchel A. Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. Macromolecular Bioscience. 2001;1(1):1-24.

6. Gowda V, Shivakumar S. Agrowaste-based Polyhydroxyalkanoate (PHA) production using hydrolytic potential of Bacillus thuringiensis IAM 12077. Brazilian Archives of Biology and Technology. 2014;57(1):55-61.

7. Periago PM, Conesa R, Delgado B, Fernández PS, Palop A. Bacillus megaterium spore germination and growth inhibition by a treatment combining heat with natural antimicrobials. Food Technology and Biotechnology. 2006;44(1):17-23.

8. Vary PS, Biedendieck R, Fuerch T, Meinhardt F, Rohde M, Deckwer WD, Jahn D. Bacillus megaterium—from simple soil bacterium to industrial protein production host. Applied microbiology and biotechnology. 2007;76(5):957-67.

9. Borah D, Yadav RN. Optimization of BH medium for efficient biodegradation of diesel, crude oil and used engine oil by a newly isolated Bacillus cereus strain DRDU1 from an automobile engine. Biotechnology. 2014;13(4):181-5.

10. Sathiyanarayanan G, Kiran GS, Selvin J, Saibaba G. Optimization of polyhydroxybutyrate production by marine Bacillus megaterium MSBN04 under solid state culture. International journal of biological macromolecules. 2013;60:253-61.

11. Kumar T, Singh M, Purohit HJ, Kalia VC. Potential of Bacillus sp.

to produce polyhydroxybutyrate from biowaste. Journal of applied microbiology. 2009;106(6):2017-23.

12. Ibrahim MH, Steinbüchel A. Poly (3-hydroxybutyrate) production from glycerol by Zobellella denitrificans MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction. Appl. Environ. Microbiol. 2009;75(19):6222-31.

13. Bhubalan K, Chuah JA, Shozui F, Brigham CJ, Taguchi S, Sinskey AJ, Rha C, Sudesh K. Characterization of the highly active polyhydroxyalkanoate synthase of Chromobacterium sp. strain USM2. Appl. Environ. Microbiol.2011;77(9):2926-33

14. Aly MM, Albureikan MO, El Rabey H, Kabli SA. Effects of culture conditions on growth and poly-β-hydroxybutyric acid production by Bacillus cereus MM7 isolated from soil samples from Saudi Arabia. Life Sci J. 2013;10(4):1884-91.

15. Omar R, Idris A, Yunus R, Khalid K, Isma MA. Characterization of empty fruit bunch for microwave-assisted pyrolysis. Fuel. 2011;90(4):1536-44.

16. Al-Battashi H, Annamalai N, Al-Kindi S, Nair AS, Al-Bahry S, Verma JP, Sivakumar N. Production of bioplastic (poly-3-hydroxybutyrate) using waste paper as a feedstock: Optimization of enzymatic hydrolysis and fermentation employing Burkholderia sacchari. Journal of cleaner production. 2019;214:236-47.

17. Lillo JG, Rodriguez-Valera F. Effects of culture conditions on poly (β-hydroxybutyric acid) production by Haloferax mediterranei. Appl. Environ. Microbiol. 1990;56(8):2517-21.

18. Yüksekdağ ZN, Aslim B, Beyatli Y, Mercan N. Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by Bacillus subtilis 25 and Bacillus megaterium 12. African Journal of Biotechnology. 2004;3(1):63-6.

19. Mahishi LH, Tripathi G, Rawal SK. Poly (3-hydroxybutyrate)(PHB) synthesis by recombinant Escherichia coli harbouring Streptomyces aureofaciens PHB biosynthesis genes: effect of various carbon and nitrogen sources. Microbiological research. 2003;158(1):19-27

20. Getachew A, Woldesenbet F. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. BMC research notes. 2016;9(1):509.

21. Neelamegam A, Al-Battashi H, Al-Bahry S, Nallusamy S. Biorefinery production of poly-3-hydroxybutyrate using waste office paper hydrolysate as feedstock for microbial fermentation. Journal of biotechnology. 2018;265:25-30.

22. Arumugam A, Anudakshaini TS, Shruthi R, Jeyavishnu K, Harini SS, Sharad JS. Low-cost production of PHA using cashew apple (Anacardium occidentale L.) juice as potential substrate: optimization and characterization. Biomass Conversion and Biorefinery. 2019:1-2.

23. Sreekanth MS, Vijayendra SV, Joshi GJ, Shamala TR. Effect of carbon and nitrogen sources on simultaneous production of α-amylase and green food packaging polymer by Bacillus sp. CFR 67. Journal of food science and technology. 2013;50(2):404-8.

24. Gayathiri E, Bharathi B, Siva N, Prabavathi R, Velu S. Production, optimization and characterization of polyhydroxybutryate by Bacillus subtilis isolated from garden soil. Int. J. Res. Pharm. Chem.. 2017;3(2):155-65.

25. Bayarı S, Severcan F. FTIR study of biodegradable biopolymers: P (3HB), P (3HB-co-4HB) and p (3HB-co-3HV). Journal of molecular structure. 2005;744:529-34.

26. Dhangdhariya JH, Dubey S, Trivedi HB, Pancha I, Bhatt JK, Dave BP, Mishra S. Polyhydroxyalkanoate from marine Bacillus megaterium using CSMCRI's Dry Sea Mix as a novel growth medium. International journal of biological macromolecules. 2015 May 1;76:254-61. β-thalassemia by capillary isoelectric focusing. Am J Clin Pathol. 1996;107:88-91.

AUTHORSHIP AND CONTRIBUTION DECLARATION

S.No	Name of Author & Affiliation	Mobile Number & E. Mail	Signature of the Author	Contribution
1	Hussana Hamid Ali Institute of Biochemistry, UoB, Quetta Pakistan	+92 333 0212209 hussanahamid-pices@hotmail.com		Conducted experiments, data analysis, manuscript writing
2	Zia Ur Rehman, Institute of Biochemistry, UoB, Quetta Pakistan	+92 317 8153149 ziaasian08@gmail.com		Research design, Supervision, funding, data analysis, manuscript writing.
3	Sana Basheer, Institute of Biochemistry, UoB, Quetta Pakistan	+92 305 8093701 sanabasheer_aries@hotmail.com		Experimental work
4	Ali Akbar, Department of Microbiology, UoB, Quetta Pakistan	+92 300 7068049 aliakbar.uob@gmail.com		Technical support
5	Muhammad Kamran Taj, CASVAB, UoB, Quetta Pakistan	+92 333 3789889 kamrancasvab@yahoo.com		Technical support