Advances in Bioresearch
Adv. Biores., Vol 15 (1) January 2024: 463-472

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.15.1.463472

Advances in Bioresearch

ORIGINAL ARTICLE

Production and characterization of thermostable Polyhydroxybutyrate synthesized by *Achromobacter marplatensis* KUMBNGBT- 38

Nandish Gurubasajar, Akarsh Subhakar, Manjunatha Dadayya, Jyothi Venkataramanaiah, Prakash Jayaramaiah and Thippeswamy Basaiah*
Department of P. G. Studies and Research in Microbiology, Bioscience Complex, Jnanasahyadri, Kuvempu University, Shankaraghatta – 577 451, Karnataka, INDIA.
Corresponding Author E-mail: thippeswamyb205@gmail.com

ABSTRACT
The Plastic components are very essential products used in daily circumstances as a basic need but they are creating many environmental complications because these plastics did not degrade in the environment. PHB was discovered by Maurice Lemoigne of the Pasteur Institute, Paris in 1925 while studying Bacillus megaterium. The PHB can acts as a carbon reserve under nutrient limiting conditions. The PHB producing potent isolate was selected out of 4 strains isolated from the soil sample collected from Shigandur, Shivamogga district, Karnataka, INDIA. The bacterium was screened by using Sudan black B staining technique and dry cell weight concentration of the isolated Bactria were performed and identified by phenotypic characters. The genomic analysis was carried out using 16s rRNA gene sequence and deposited to GenBank, NCBI and confirmed as Achromobacter marplatensis KUMBNGBT- 38 *and assigned with accession No. OK161021. The cultural conditions required for the PHB production was analysed using different parameters (media-nutrient broth, incubation time-72hrs, temperature-37℃, pH-7.0, carbon source-glucose, nitrogen source-ammonium chloride and carbon to nitrogen ratio is 8:1). The submerged fermentation method was used to produce PHB in a large scale using agro-industrial wastes as a substrate, feed stock and rice bran showed maximum PHB production and the quantity of the PHB present in the cells were quantified using UV- visible spectrophotometer to measure the λ max at 235nm.*
Keywords: Polyhydroxybutyrate**,** *Achromobacter marplatensis,* Agro-industrial wastes and UV- visible spectrophotometer.

Received 03.06.2023 Revised 24.09.2023 Accepted 04.12.2023

How to cite this article:
Nandish G, Akarsh S, Manjunatha D, Jyothi V, Prakash J and Thippeswamy B. Production and characterization of thermostable Polyhydroxybutyrate synthesized by *Achromobacter marplatensis* KUMBNGBT- 38. Adv. Biores. Vol 15 [1] January 2024. 463-472

INTRODUCTION

Petroleum-derived plastics are artificial organic polymers, gained from the naturally obtaining gases or oils and used in modern society in the every part of our daily life [1]. In twentieth century, the urbanization in the locality of shoreline environments, the exploitation of petroleum-based plastic materials in the various applications, especially these plastics are used in medicinal, industrial, commercial and municipal fields have rapidly increased and without knowing these products are released into the nature, which causes significant environmental problems and health hazards [2].

Polyhydroxybutyrate (PHBs) are biologically formed petroleum derived plastic-like material acting as a sink of carbon and reducing comparable for some of the microorganisms [3]. Currently, more than 200 genera of bacteria and some of the haloarchaea species are identified as good PHBs producers and they have the ability to degrade the bio based plastics [4]. The general structure of the PHB molecules consists of a monomer of 3-hydroxy fatty acids, where the residual group length can vary between C_1 to C_{14} [5].

Among the different types of PHAs are produced by microbes, the one of the most prominent PHAs are poly-3-hydroxybutyrate (PHB). The PHB is a short chain polymer and the methyl group was present at the C_3 position, which confers to the polymer with high crystallinity and rigidity. The PHB molecules are

stored within the microbial cytoplasm as granules (the size varies between 0.10 to 0.4 µm), the recovery of the PHB polymer was very difficult and it faces many technological barrier to its application and the production of these polymers are very high, which accounts for 50% of the final polymer price. Therefore, the extraction and purification of the PHB molecules is very difficult, but the solvent-based extraction method is a most employed recovery strategy for obtaining purified cells. The most widely used solvent for PHB extraction was chloroform because it has a good dissolving capacity and binding property. Up to 95% of the recovery can be studied by many researches [6, 7]. Due to their biodegradability and biocompatibility nature of the polymer, it represents the applications in various fields' such as pharmacological, environmental, packaging, veterinary, industrial, agricultural and biomedical [8, 9]. The production cost of the PHB production (generally), its marketable exploitation is significantly lower than expected. The industrial production of PHB was limited by various features, including the high cost of raw materials, low yield of the microbial processes (i.e., 2-3 g/L), the high cost of downstream processing and the separation of PHB from cellular biomass [10].

In the present study, the isolation, screening, characterization, optimization, production and quantification characterization of PHB producing bacterium by using different low cost substrates were analysed. Production of PHB biopolymer using microorganisms i.e. bacteria and use of bio based plastics in the everyday life is one of the very nominal way to counter the serious problems caused by petroleum derived plastics. *Achromobacter marplatensis* produce a good amount of PHB biopolymer and it is easily grown in low cost agricultural waste substrates, so, this entire work is very cost effective and it creates basement to scale up the production of PHB biopolymer in large quantity.

MATERIAL AND METHODS

Study area and sample collection

Soil sample was collected from dumpsite (garbage and plastic damping) yards of Shigandur, Shivamogga district, Karnataka, INDIA. The soil sample was collected from the different locations of dumpsite (garbage and plastic damping) yards using indiscriminate sampling method. The collected sample was collected and placed in sterile plastic bags and kept at 4°C until use [11].

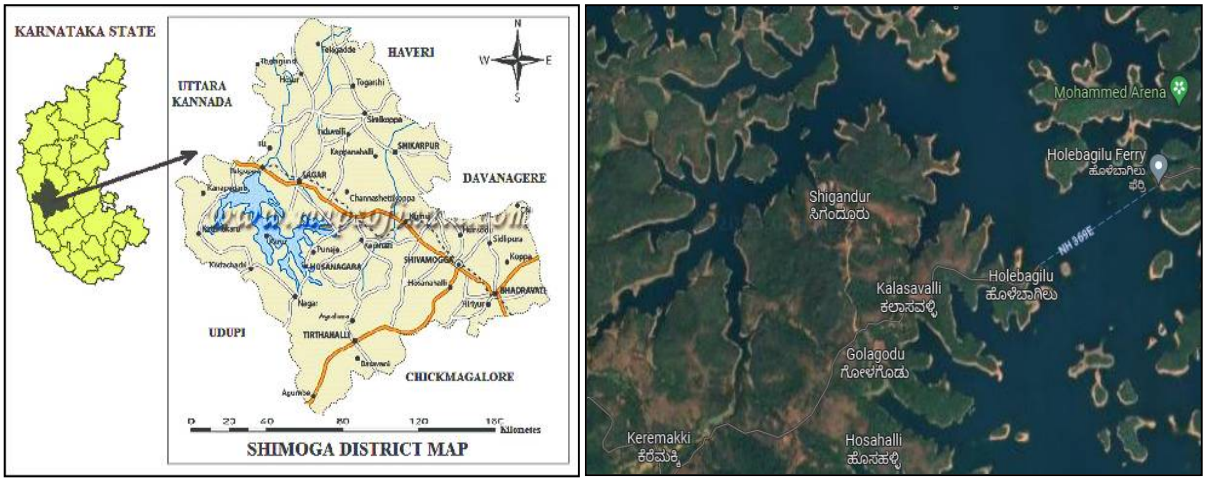

Figure 1; Sample collection site in Shigandur, Shivamogga district.

Isolation of PHB producing bacteria:

One gram of soil sample was serially diluted in sterile distilled water and shaken for 2 min. The diluted sample was heated at 60ºC for 30 min and cooled. The sample was plated onto nutrient agar plates and incubated at 37°C for 24 h. After incubation colonies were selected and sub cultured on nutrient agar slants for further studies [12].

Screening and Production of PHB producing bacteria

The selected bacterial cultures were grown on nutrient agar (Himedia laboratories-India) media supplemented with glucose (2%) as a sole carbon source and incubated at 37ºC for 48 h. After incubation the cultures were grown on Linko media containing 2% sucrose [13]. The PHB producing isolates shows blue color on Linko media. PHB producing isolate was confirmed by using Sudan black B (SBB) staining method. The pure colony of PHB producing bacterium was maintained on nutrient agar plate and stored for further analysis [14].

The carbon and nitrogen present in the production media will enhance the PHB Production by bacterial isolate and PHB accumulation was stimulated by using high carbon containing production media. Thus, the selected media was previously described by Lu et al. (2008) with slight modifications [sucrose -20 g/L;), (NH_4)2SO_4-0.5 g/L); KH_2PO_4-2.3 g/L; Na_2HPO_4-2.3 g/L; $MgSO_4$.7H_2O-0.5 g/L; Na_2HCO_3-0.5 g/L; $CaCl_2$-0.01 g/L with addition to 5ml trace element solution containing ZnSO4.7H_2O-0.01 g/L; $MnCl_2$.4H_2O-0.003 g/L; H_3BO_4-0.003 g/L; $CoCl_2$.7H_2O-0.02 g/L; $CuCl_2$.2H_2O-0.001 g/L; $NICl_2$.6H_2O-0.002 g/L; pH 7] to this medium the bacterium was inoculated and incubated at 37ºC on orbital shaking incubator at 180 rpm.

Characterization of PHB producing potent bacterium

Morphological characterization

The selected strain was identified by various morphological characters such as shape, size, texture and color of the isolate and microscopic characters such as simple staining and endospore staining was studied [15].

Biochemical characterization

The selected bacterium was characterized by various biochemical tests such as starch hydrolysis, gelatin hydrolysis, casein hydrolysis, citrate utilization, nitrate reduction, urease, methyl red, voges proskauer, indole, malonate utilization, H_2S production, KOH, β-Galactosidase, lecithinase, lipase, catalase, oxidase and triple iron agar tests were performed according to Bergey's Manual of Determinative Bacteriology [16].

Molecular characterization

The genomic DNA of the isolated stain was extracted and the polymerase chain reaction (PCR) was used to amplify the obtained 16s rRNA sequence by using the primers 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG C-3′). After amplification the gene sequence was analysed using alike sequence selected from National Centre for Biotechnology Information GenBank (NCBI GenBank) database using Basic Local Alignment Search Tool (BLAST) and the obtained sequence with 100% similarity was submitted to GenBank NCBI and the accession number was assigned to the submitted sequence. The phylogenetic analysis of the bacterium was constructed using neighbour-joining tree method using maximum bootstrap value [16].

Optimization of PHB producing bacterium

Production PHB in different broth medium at different incubation periods

The pure colony of *Achromobacter marplatensis* responsible for the PHB production was grownup on different culture broth medium such as Nutrient broth (NA), Tryptone soya broth (TSB), Minimal salt broth (MSB), Minimal broth (MB) and Luria Bertani broth (LB) and the strain was inoculated into the different broth medium. The strain was incubated at different time periods i.e., 24 to 96 h in standard conditions. For every 24 h of time interval, the PHB growth and production was scrutinised and the PHB yield was recorded [17].

Accumulation of PHB on different temperature, pH, carbon source, nitrogen source and carbon to nitrogen ratio (C:N ratio)

The culture filtrate was prepared by inoculating the pure strains of *Achromobacter marplatensis* which is responsible for the production of PHB on nutrient agar media and incubated at 37°C for 24 h. The culture filtrate was examined in nutrient broth media at 37°C for 24 h in rotary shaker at 160 rpm. To optimize the different cultural conditions viz. temperature, the culture media was incubated at a range from 4°C, 15°C, 25°C, 37°C and 42°C and the media pH was adjusted to 3, 5, 7, 9 and 11 respectively. For the exploitation of different carbon and nitrogen sources, the culture media was enhanced with different carbon supplements such as glucose, fructose, sucrose, maltose and lactose and different nitrogen supplements such as ammonium chloride, peptone, sodium nitrate, urea and yeast extract were investigated. The amount of carbon and nitrogen utilized by the bacterium was analysed by using Carbon to Nitrogen ratio (C:N ratio). The different carbon (2%) and nitrogen (1%) supplements at different concentrations such as 1:1, 2:1, 4:1 and 8:1 and 16:1 was optimized [18].

Evaluation of agro-industrial residues as substrate for PHB production

The production of PHB was carried out in different low-cost agro-industrial waste substrates by utilizing glucose as a sole carbon source. The media for the PHB production was prepared by using different Agro-industrial wastes such as feed stock, cotton cake, groundnut cake, coconut cake, castor cake, sugarcane bagasse (SCB), rice bran and areca nut husk. 100g of selected agriculture wastes were dissolved in 1200 mL distilled water and heated up to 70°C for 60mins in alliin condenser. The extract was filtered by using muslin cloth and pH of the filtrate was adjusted to 7.0 using NaOH. The selected PHB producing bacterial isolate was inoculated and incubated at 37°C for 72 h. After incubation, the amount of PHB produced by

oil Agro-industrial wastes were recorded and quantified [19, 20]. The amount of PHB produced by the bacterium was quantified by using UV-visible spectrophotometer.

RESULTS AND DISCUSSION

Study area, collection sample and isolation of PHB producing bacterium

The dumpsite contains a compost wastes and plastics wastes, where the microbial load is very rich and it acts as good spring for the isolation of potent bacterium which was responsible for the production of PHB. So the sample was collected from dumpsite of Shigandur, Shivamogga district, Karnataka, INDIA (figure 1). For the production of PHB, the potent isolate was selected out of 4 strains isolated from the collected soil sample (10^{-7} and 10^{-8} dilutions were used for the isolation) (figure 2a, b, c, d) and all the selected isolates were further screened to select the potent PHB producer.

Screening of PHB producing potent bacterium

The selected PHB producing promising bacterium was primarily confirmed for the presence of PHB granules by using Sudan black B (SBB) staining method. In Sudan black B staining, the polymer inclusion bodies were not-observed inside the bacterial cells. So the isolate was negative for the Sudan black B staining and the isolate was further screened by using morphological and biochemical characters (figure 3a). These findings were co-related with Ramachandran and Abdullah et al. [21] , but in their study, the orange color fluorescence colonies were observed on a nutrient-rich medium under ultraviolet light (UV) suggesting the existence of PHB granules.

Characterization of PHB producing potent bacterium

Morphological characters

The morphological properties of the isolated strain was determined according to "Bergey's Manual of Systematic Bacteriology" [22]. The isolate sis a negative, rod-shaped, non-motile, aerobic and non-spore forming bacterium and the shape of the colony was circular, colony is white in color, size of the colony was 1-2 mm and it has smooth, raised, opaque and sticky surface (shown in figure 3b, c, d) and summarized in table 1.

Biochemical characterization

According to Biochemical properties, the isolate was positive for citrate, nitrate, urease, methyl red, indole, melonate, lipase and citrate utilization and the stain was negative for starch, gelatine, casein hydrolysis, H_2S, KOH, lecithinase, galactosidase and oxidase test. The isolate have ability to ferment various sugars such as dextrose, sucrose, lactose and carbohydrate whereas the hydrogen and carbon dioxide were not fermented by the strain. The biochemical characters are shown in table 1. From the above results, the isolated bacterium belongs to the genus *Achromobacter* according to Bergey's manual of determinative bacteriology [15] and the species level identification was performed using molecular characterization and the obtained results were compared with the earlier findings of Hassan et al. [23].

Molecular characterization and phylogenetic analysis of *Achromobacter marplatensis* KUMBNGBT-38

The isolated bacterium was confirmed by molecular identification using 16S rRNA gene sequence. The genomic DNA was used to amplify the obtained 16S rRNA gene sequence, the sequence was purified using PCR. The gene sequence was aligned by pair-wise alignment exhibited highest (100%) similarity with *Achromobacter marplatensis* KUMBNGBT-38 and the selected nucleotide sequence was deposited to NCBI database and the sequence was allocated with accession no. OK161021. The phylogeny of the sequence was analysed using neighbour-joining method by choosing the related sequence from NCBI GenBank database. (shown in figure 4). However, these results were compared with previous findings of Irsath et al. [24].

Optimization of PHB producing bacterium

Production PHB in different broth medium at different incubation periods

The pure colonies of strain *A. marplatensis* was grown on five different broths to examine the production of PHB. In all the five broths, the maximum PHB yield of 7.073±0.11g/L was shown in nutrient broth compared to all the other broths used for PHB production (figure. 5a, b). For the selection suitable incubation time for PHB production, the strain was incubated at different time intervals (24, 48, 72 and 96 h) and the PHB yield was recorded. (shown in figure 6a) [25]. The optimum time required for the PHB production by *A. marplatensis* was found to be 72 h. The above findings were compared with earlier results of Monika Sharma et al. [25]. According to their study 72 h was found to be optimum incubation period for when studied using *Pseudochrobactrum asscharolyticum.*

Accumulation of PHB on different temperature, pH, carbon source, nitrogen source and carbon to nitrogen ratio (C:N ratio)

To optimize the different temperature range were selected for PHB production by *A. marplatensis*. In all the selected temperatures, in 37°C the strain showed maximum PHB production compared to all the temperatures used for production of PHB. The increase or decrease in the temperature range will affect the PHB production (figure 6b). Similar outcome was observed in previous studies of Grothe et al. [26]. From their data, 33°C appears to be optimum temperature for PHB production. The optimum pH required for the PHB production was found to be pH 7 (figure 6c). At pH 3, 5, 9 and pH 11, the PHB yield was very less when compared with pH 7. According to obtained results the acidic and basic nature of the pH is not suitable for PHB production. The effect of different carbon source (glucose, fructose, sucrose, maltose and lactose) on PHB production was examined. Among all the carbon sources utilizes, the media amended with glucose shows maximum PHB yield by *A. marplatensis* shown in figure 6d [27]. The effect of different nitrogen source (yeast extract, peptone, urea, sodium nitrate and ammonium chloride) on PHB production was revealed in figure 6e. Among the all the nitrogen sources tested, the maximum PHB production was observed in ammonium chloride by *A. marplatensis* [28]. The media was supplemented with 2% carbon source and 1% nitrogen sources tested, 4:1 is a best suitable carbon and nitrogen ratio for maximum PHB production (figure 6f). The above findings were compared with the results of Monika Sharma *et* al. 2013. According their report the 16:1 C:N ratio was found to be optimum for PHB production.

Evaluation of agro-industrial residues as substrate for PHB production

The use of commercially available raw materials are rich in carbon sources and it may reduce the high production cost. The Agro-industrial wastes were obtained after the usage and they are used as a production media for large scale production of PHB by utilizing essential nutrients using selected bacterium i.e., *A. marplatensis*. For the production of PHB, All the selected agro-industrial residues were analysed by UV-Visible spectrophotometer at 540nm. Among all the residues, the maximum PHB was observed in Feed stock compared to all the residues (Figure 7a). According to earlier findings of Naranje et al. [20]. Cotton seed oil cake showed maximum PHB yield. After production the amount of PHB cells present in the compound was analysed and it shows λ *max* of 310nm (shown in figure 7b). The amount of PHB present in the sample was directly associated with amount of UV radiation absorbed by crotonic acid. These findings were compared with earlier results of Pol Reshma *et al*. [16].

Fig 1(a) indicates gram negative for isolated bacterium, **1(b)** positive for Sudan B black and black color colonies were observed, **1(c)** colony morphology of isolated bacterium and **1(d)** the non-spore formation was observed.

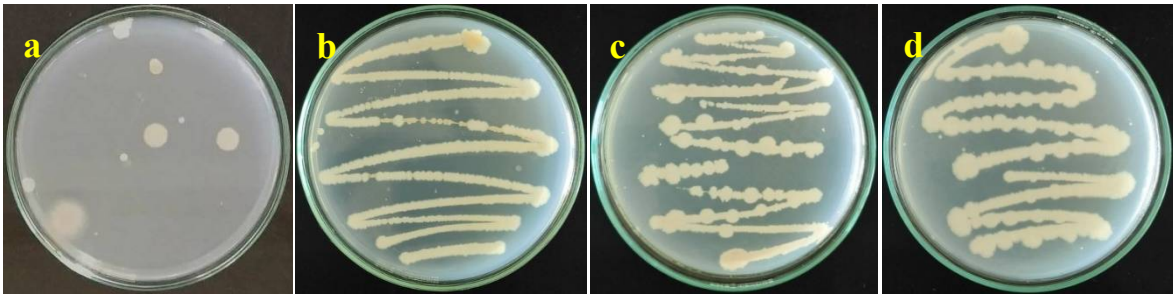

Figure 2; a. colonies of isolated of bacteria on nutrient agar media (10^{-7}) **b.** isolate 1 **c.** isolate 2 and **d.** isolate 3.

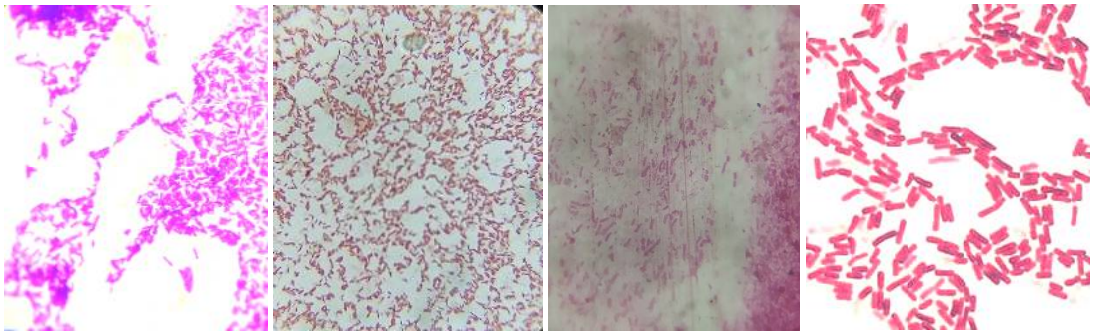

Figure 3; Phenotypic identification of PHB producing bacterial isolate using Gram staining, Suda B black staining, simple staining and endospore staining.

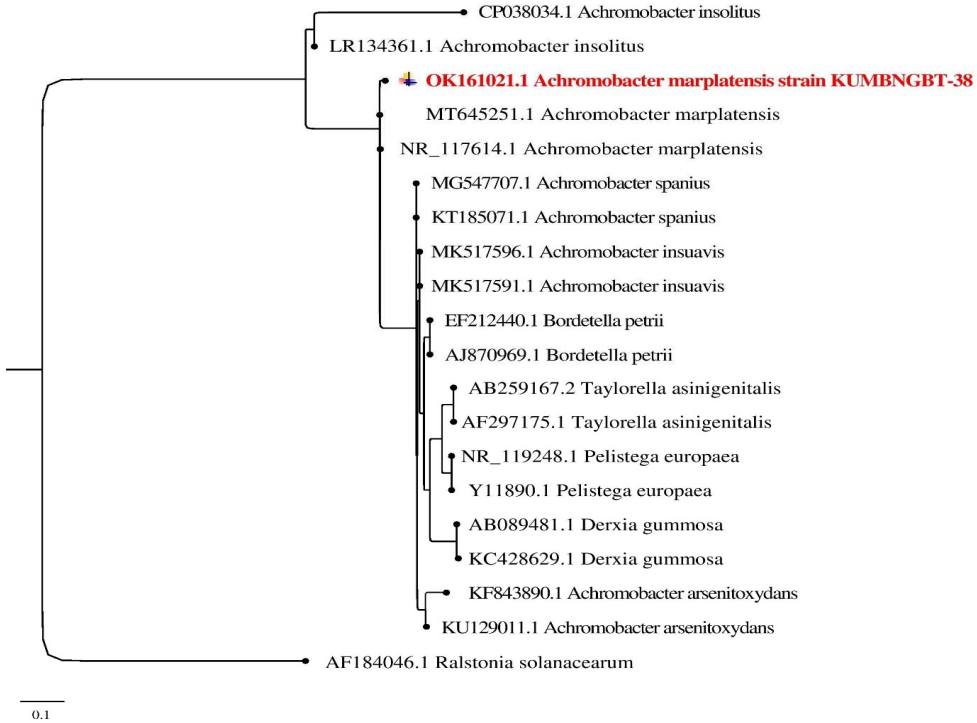

Figure 4; Analysis of Evolutionary relationships of *Achromobacter marplatensis* KUMBNGBT-38 with various entities based upon their similarities and differences in their physical or genetic characteristics.

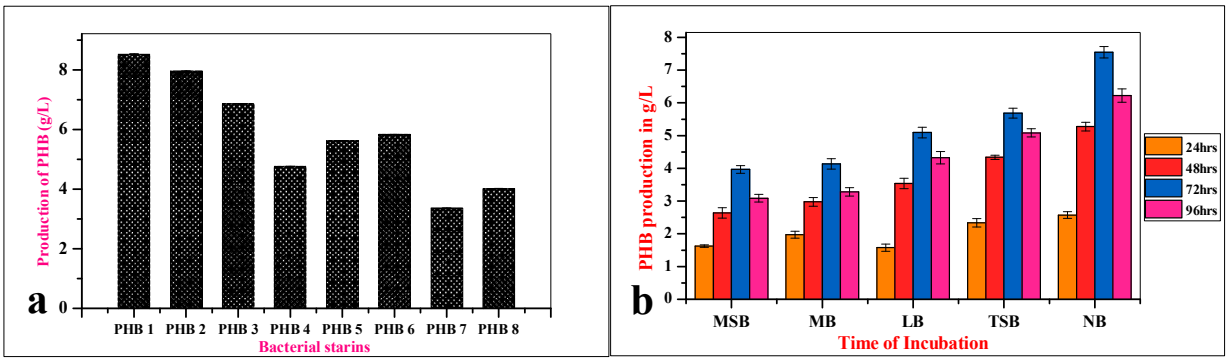

Figure 5; a. production of PHB using solvent extraction method and **b.** effect of different broth medium at different incubation time.

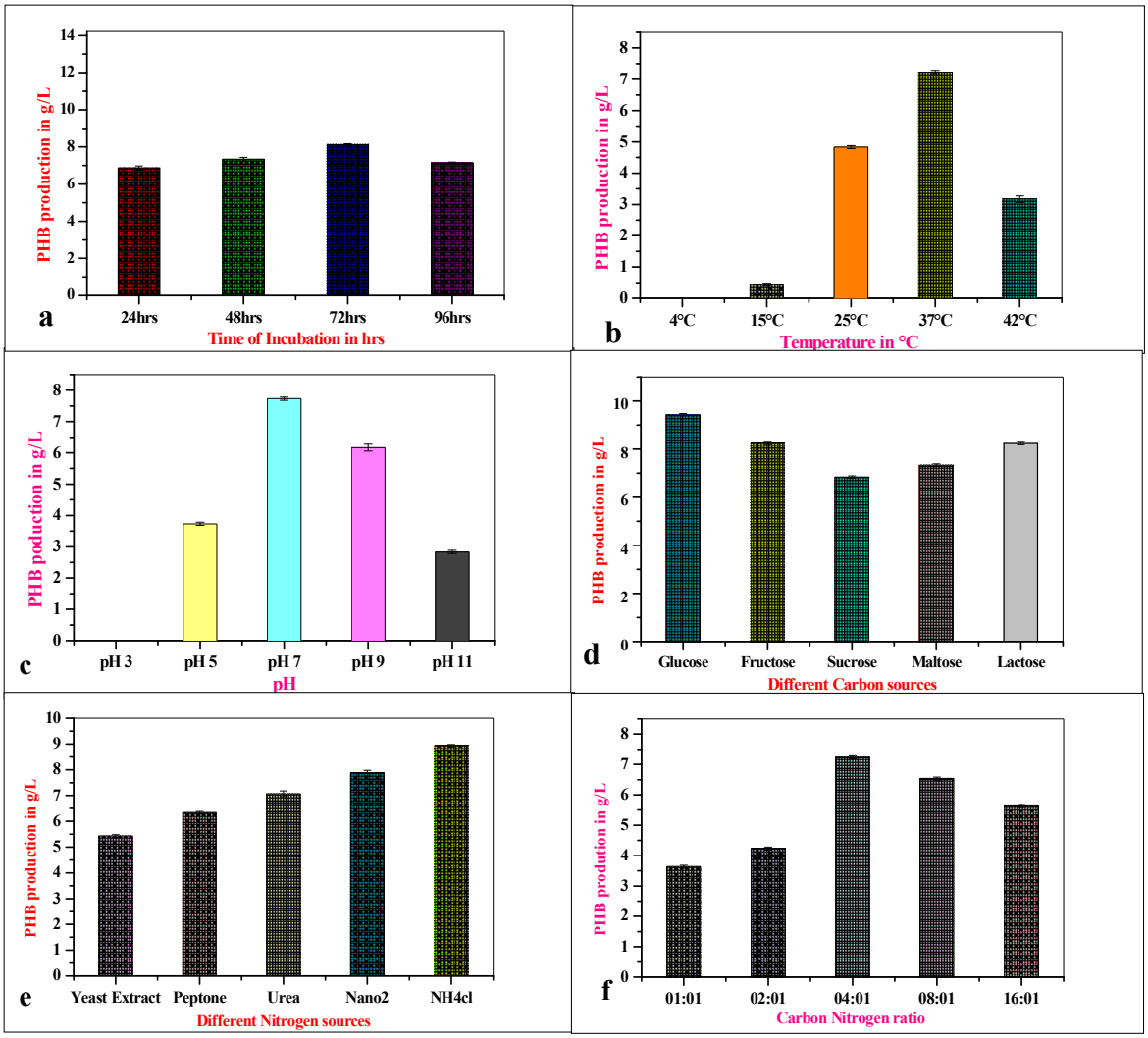

Figure 6; Optimization of *Achromobacter marplatensis* by different optimized conditions using various parameters. **a.** effect of incubation time, **b.** effect of different temperature, **c.** effect of pH, **d.** effect of different carbon source, **e.** effect of different nitrogen source and **f**. effect of carbon-nitrogen ratio.

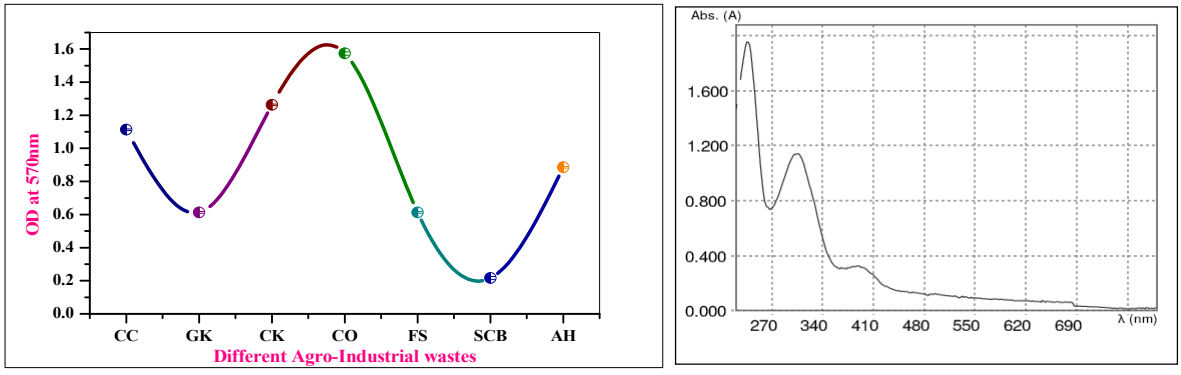

Figure 7; a. production of PHB using different Agro-industrial waste and **b.** estimation of PHB present in the cells using UV-visible spectroscopy.

Table 1: Phenotypic characterization of bacteria isolated from dump yard soil sample collected from Savalanga, Davanagere District, Karnataka.

Morphological character	**Results**
Shape	Circular
Size	1-2 mm
Texture	Sticky
Color	White
Cell Shape	Rods
Cell length	1.4-2.8 µm
Cell width	0.4-1.1 µm
Motility	Non-Motile
Spore formation	Negative
Biochemical characters	**Results**
Citrate utilization	Positive
Nitrate reduction	Positive
Urease	Positive
Methyl red	Positive
Vogues–Proskauer	Negative
Indole production	Positive
Malonate utilization	Positive
H_2S production	Negative
KOH	Negative
Lecithinase	Negative
Lipase	Positive
β-Galactosidase	Negative
Catalase	Positive
Oxidase	Negative
Hydrolysis	
Starch	Negative
Gelatine	Negative
Casein	Negative
Triple sugar iron test	
Dextrose	Positive
Sucrose	Positive
Lactose	Positive
Carbohydrates	Positive
Hydrogen	Negative
Gas	Negative

CONCLUSION

The PHB producing potent strain of *A. marplatensis* was isolated from the soil sample collected from Shigandur, Shivamogga district, and the strain was screened for the PHB production and characterized by using morphological and biochemical characters and confirmed as *Achromobacter marplatensis*. The accumulation of PHB by the isolate was optimized by using different cultural conditions. The low-cost agro-industrial residues were used to produce the large amount of PHB and the amount of PHB present in the cells were examined using λ max at 235nm using UV-visible spectrophotometer. According to above findings the bacterium was selected to be very good PHB producer and it can be used for the production of biopolymer in commercialized conditions and it is very cost effective compared to other plastics.

CONFLICT OF INTERESTS

There aren't any conflicts of interest, according to the writers.

REFERENCES

1. Thodhal Yoganandham Suman, Wei-Guo Li, Shaji Alif, Valappil Rahman Panangala Faris, Duvvuru Joshua Amarnath, Jun-Guo Ma and De-Sheng Pei. (2020). Characterization of petroleum-based plastics and their absorbed trace metals from the sediments of the Marina Beach in Chennai, India. Environ Sci Eur. 32:110. https://doi.org/10.1186/s12302-020-00388-5.

2. Andrady AL, Neal MA. (2009). Applications and societal benefits of plastics. Philos Trans R Soc B Biol Sci. 364:1977–1984.
3. Anderson AJ, Dawes EA. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial Polyhydroxyalkanoates, microbiological reviews, vol. 54. Washington: American Society for Microbiology (ASM). pp-450.
4. Han J, Hou J, Liu H, Cai S, Feng B and Zhou J. (2010). Wide distribution among halophilic archaea of a novel polyhydroxyalkanoate synthase subtype with homology to bacterial type III synthases. Appl Environ Microbiol. 76(23): 1-9.
5. Kynadi AS and Suchithra TV. Polyhydroxyalkanoates: biodegradable plastics for environmental conservation industrial environmental biotechnology. In: Patra JK, editors. Studium Press (India) Pvt. Ltd.; 2014.
6. Manangan T and Shawaphun S. (2010). Quantitative extraction and determination of polyhydroxyalkanoate accumulated in *Alcaligenes latus* dry cells. Science Asia. 36(3):199–203.
7. Anis SNS, Md Iqbal N, Kumar S and Amirul AA. (2013). Effect of different recovery strategies of P (3HB-co-3HHx) copolymer from *Cupriavidus necator* recombinant harboring the PHA synthase of *Chromobacterium* sp. USM2. Sep Purif Technol. 102:111–7.
8. Ke Y, Zhang XY, Ramakrishna S, He LM, and Wu G. (2017). "Reactive blends based on polyhydroxyalkanoates: Preparation and biomedical application," Materials Science and Engineering: C. 70(2):1107–1119.
9. Ho MH, Li SY, Ciou CY and Wu TM. (2014). "The morphology and degradation behaviour of electrospun poly (3-hydroxybutyrate)/magnetite and poly (3-hydroxybutyrate-co-3-hydroxyvalerate)/magnetite composites," Journal of Applied Polymer Science. 131(22):1–9.
10. Chatzidouka C, Penloglou G, and Kiparissides, C. (2013). "Development of a structured dynamic model for the production of polyhydroxybutyrate (PHB) in Azohydromonas lata cultures," Biochemical Engineering Journal. 71:72–80.
11. Yilmaz M, Soran H, Beyatli Y. (2005). Determination of Poly-hydroxybutyrate (PHB) production by some Bacillus spp. World Journal of Microbiology and Biotechnology 21:565-566. 10.1007/s11274-004-3274-1.
12. Preethi R, Sasikala P, Aravind, J. (2012). Microbial production of Polyhydroxyalkanoate (PHA) utilizing fruit waste as a substrate. Research in Biotechnology. 3(1):61-69.
13. Linko M, Haikara A, Ritala A and Penttila M. (1998). Recent advances in the malting and brewing industry. J. Biotechnol. 65(2-3):85-98.
14. Steinbuchel A, Fründ C, Jendrossek, D and Schlegel H. (1987). Isolation of mutants of *Alcaligenes eutrophus* unable to derepress the fermentative alcohol dehydrogenase. Arch. Microbiol. 148(3):178-186.
15. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. (1994). Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins, Baltimore. http://dx.doi.org/10.1080/17518253.2015.1109715.
16. Pol Reshma, Shinde Sonal, Singh Vatsala. (2017). Screening and Isolation of Polyhydroxy butyrate Producers. International Journal for Research in Applied Science & Engineering Technology (IJRASET). 5:1259-1264.
17. Lathwal P, Kiran Nehra, Manpreet Singh, Pragati Jamdagni, Jogender S. Rana. (2015). Optimization of Culture Parameters for Maximum Polyhydroxy butyrate Production by Selected Bacterial Strains Isolated from Rhizospheric Soil. Polish Journal of Microbiology. 64(3):227–239.
18. Bhuwal AK, Gulab Singh, Neeraj Kumar Aggarwal, Varsha Goyal, Anita Yadav. (2014). Poly-β-hydroxybutyrate production and management of cardboard industry effluent by new Bacillus sp. NA10. Bioresources and Bioprocessing. 1(9):1-11.
19. Mekala M, Rajendran R, Suganya K. (2013). Comparative studies on cell growth and production of PHB (Poly Hydroxy Butyrate). International Journal of Environmental Sciences. 3(6):2261-2276. 10.6088/ijes.2013030600041.
20. Naranje NK, Bharat J. Wadher, Manohar G. Muddeshwar. (2016). Utilization of Low Cost Carbon Substrates and Optimization Studies for Maximum Yield of Polyhydroxybutyrate from B. megaterium. International Journal of Current Microbiology and Applied Sciences. 5(11):623-630
21. Ramachandran H and Abdullah A. (2010). Isolation of PHA-producing bacteria from Malaysian Environment. Proceedings of the 7th IMT-GT UNINET and the 3rd International PSUUNS Conferences on Bioscience. 178-179.
22. Irsath H, Santhosh S, Hemalatha V, Vikramathithan M, Dhanasekar R, Dhandapani R. (2015). Production and Optimization of Polyhydroxy butyrate using *Bacillus subtilis* BP1 Isolated from Sewage Sample. International Journal of Pure & Applied Bioscience. 3(1):158-166. http://dx.doi.org/10.1155/2013/952641.
23. Monika Sharma, and Harish Kumar Dhingra. (2013). Isolation and Culture Conditions Optimization for PHB Production by *Pseudochrobactrum asaccharolyticum*. International Journal of Science and Research (IJSR). 4:1895-1901.
24. Grothe EM, Moo-Young, Chisti Y. (1999). Fermentation optimization for the production of poly- (B-hydroxybutyric acid) microbial thermoplastic. Enzym. Microbial. Tech. 25:132–141. https://doi.org/10.1016/S0141-0229(99)00023-X.
25. Khanna S, Ashok K, Srivastava (2005) Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. Process of Biochemistry 4:2173-2183. https://doi.org/10.1016/j.procbio.2004.08.011.
26. Krieg N. R and Holt J. G. (1984). Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, MD.

27. Hassan MA, Haroun BM, Amara AA and Serour EA. (2013). Production and characterization of keratinolytic protease from new wool-degrading *Bacillus* species isolated from Egyptian ecosystem. Biomed Research International Journal. doi.org/10.1155/2013/175012
28. Mahmoud M, Berekaa, Ali M, Al Thawadi. (2012). Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by *Bacillus megaterium* SW1-2: Application of Box-Behnkenesign for optimization of process parameters. Afr J Microbiol Res. 6: 838-845.