Identification, Isolation and Characterization of Poly-Hydroxy Butyrate (PHB) Producing Bacteria, and Extraction of the PHB Granules

Ananya V, Pradeek P, Vaishnavi G, Esther Shoba R1*

Department of Life Sciences, Kristu Jayanti College (Autonomous), K. Narayanapura, Kothanur (PO), Bengaluru-560077
Corresponding Author*: esther@kristujayanti.com

Abstract

Plastics, a non-renewable and non-degradable material have become one of the reasons for health crisis. Negative impact of plastic use on wild life and human health looks for an alternate that meets the requirement of human needs and also reduce the nuisance caused to the nature. Plastics that are bio based and degradable and similar to the properties of synthetic plastic is the solution to the problem and are termed as bio-plastics. In the present work, an attempt was made to isolate polyhydroxy butyrate (PHB) producing microorganisms from two different samples, garage soil sample and root nodules of *Trigonella foenum-graecum* leaves. The study also focused on the optimizing cultural conditions for the two isolated and identified PHB producing microorganisms, *Bacillus subtillis* and *Pseudomonas aeruginosa* by changing the parameters, viz., incubation time, pH, carbon source and nitrogen source. Maximum PHB was obtained when the media was supplemented with Glucose as Carbon source for both *Pseudomonas aeruginosa* and *Bacillus subtilis*, Ammonium nitrate as Nitrogen source for *Pseudomonas aeruginosa and* Urea for *Bacillus subtilis*. The optimum pH was found to be pH 7 for both *Pseudomonas aeruginosa* and *Bacillus subtilis and* the optimum temperature was found to be 37˚C for *Pseudomonas aeruginosa* and 30˚C for *Bacillus subtilis.* The study also showed that *Pseudomonas aeruginosa* yields more PHB than *Bacillus subtilis* in comparison.

Keywords: Bioplastics, *Bacillus subtilis*, *Pseudmonas aeruginosa*, optimization

Introduction

The widely used material in modern days is plastic as the world produces more than 380 million tonnes of plastic every year. Since ages, approximately 1600 BC humans have been benefited from the polymers in the form of plates, tubes, fibers, plates, bottles, boxes and more. It is also used in every aspect like food packaging, textile industry, pharmaceutical packaging etc. Chemical nature of plastic is polymers of carbon, hydrogen, oxygen and/or silicon. On the contrary plastic is a non-renewable and non-degradable material. Low tolerance to heat, poor ductility, combustibility and embrittlement are the adverse nature of the plastics. With more use of plastic for day to day activities, accumulation of plastic as waste has led to environmental problems such as air pollution, land pollution and water pollution. As of 2015, of the approximately 6,300 Mt of plastic waste generated, around 9 percent had been recycled, 12 percent incinerated, and 79 percent accumulated in landfills or the natural environment (1). To clear the menace of plastics, it is burnt. The Municipal Solid Waste (MSW) containing about 10-12% of plastic is burnt, releasing toxic gases into the environment which include substances like Dioxins, Furans, Mercury and Polychlorinated biphenyls (2). Buoyancy nature of plastics makes them float on the water surface and about 269,000 tons of plastic are seen on the surface waters across the world (3). Plastic waste affects wild life in the form of entanglement, ingestion and interaction. Plastic ingestion by swallowing from surrounding water or through consumption of organisms that have previously ingested plastics has an intense effect on metabolic rate, reduced reproducibility, reduced growth and development and reduced energy stores. For human health, the micro and nano particles of plastic on ingestion are responsible for cardiovascular, renal, gastrointestinal, neurological, reproductive, and respiratory systems, impacts that include cancers, diabetes, neuro, reproductive, and developmental toxicity. Negative impact of plastic use on wild life and human health looks for an alternate that meets the requirement of human needs and also reduce the nuisance caused to the nature. Plastics obtained from organisms and that can be degraded is the solution to the problem plastic is the solution to the problem and are termed as bio-plastics. Bioplastics are made from a variety of sources like polysaccharides, lipids and also proteins (4, 5, and 6). The main candidates for the large-scale production of PHAs are plants and bacteria. The sources for the bioplastics are plants and microorganisms. The first microbial plastic polyhydroxy butyrate (PHB) is from *Bacillus megaterium* (7).

Accumulating PHAs is a natural way for bacteria to store carbon and energy, when nutrient supplies are imbalanced. These polyesters are accumulated when bacterial growth is limited by depletion of nitrogen, phosphorus or oxygen and an excess amount of a carbon source is still present (8). As PHAs are insoluble in water, the polymers are accumulated in intracellular granules inside the cells. It is advantageous for bacteria to store excess nutrients inside their cells, especially as their general physiological fitness is not affected (9). The increasing damage to the environment due to the

usage of plastics is disturbing and the fact that plastics have become an integral part of our lives. Even though recycling, reusing and alternative usage other than plastic have been adopted, there are certain divisions where plastics are important for packaging and storing. So instead of using synthetic plastics when a group of microorganisms can produce biopolymers interested us to know more about the same. The aim of this study was to identify and isolate PHB producing bacteria from our surrounding environment and extract the granules.

Materials and Methods

SAMPLE COLLECTION

Soil samples were used for the isolation of PHB producing microorganisms. Soil Sample A (SS-A) was collected from a garage near Kristu Jayanti College, Bengaluru on 16thDecember 2019. Sample B (S-B) was from the root nodules of Methi leaves also called as *Trigonella foenum-graecum L.* purchased on 6th January 2020.

ISOLATION OF PURE CULTURES

Isolation of microorganisms was done by serial dilution method and 10^{-5} dilution was used. Dissemination of 100µl diluted samples on nutrient agar plates followed. Afterwards, the plates were incubated for 48 hours at 30°C.Colonies with distinct characteristics were selected and purified by repeated streaking on identical LB agar plates.

PRIMARY SCREENING OF PHB PRODUCING BACTERIA

Primary screening of PHB producing bacteria was done using 2 methods. Lipophilic stain Sudan Black B staining that can be picked up by PHB producing colonies was used to detect PHB development. The second method was staining of the colonial smears with 0.3% Sudan Black B solution staining for 15 minutes followed by immersion in xylene and counterstain with safranin.

SCREENING TECHNIQUES

Twenty four hours old cultures of colonies were gram stained and the slide was observed under microscope for colony characterization and gram reaction.

16s rRNA MICROBIAL IDENTIFICATION

Two colonies were selected based on colony characterization and gram reaction and 16s rRNA partial sequencing was done using applied biosystems in Bangalore.

OPTIMIZATION OF PHYSICAL PARAMETERS FOR MAXIMUM BACTERIAL GROWTH

Effect of pH on bacterial growth

Every microorganism has a minimum, an optimum and a maximum pH for growth. To standardize the optimum pH for the production of PHB, the PHB positive bacterial cultures were inoculated in PHB producing media - Minimal Davis Broth and maintained at different pH (3, 5, 7, 9 and 11) and incubated at 37°C for 48 hours with occasional shaking. After incubation, turbidity of the media due to bacterial growth was measured by spectrophotometer at 640nm.

Effect of temperature on bacterial growth

Bacterial culture was inoculated in Minimal Davis Broth and incubated at different temperatures viz., 25°C, 30°C, 37°C, and 40°C, 45°C for 48 hours days. After incubation the growth of cultures in the media was measured using spectrophotometer at 640nm.

Following optimization all the samples were screened using Sudan stain to confirm the development of PHB.

OPTIMIZATION OF CULTURAL PARAMETERS FOR MAXIMUM PHB PRODUCTION

PHB, a polyester synthesized as energy reserve materials by various microorganisms under unfavorable conditions can accumulate more during stress conditions. In order to standardize the optimum carbon and nitrogen for PHB growth, PHB positive bacterial cultures were inoculated in PHB producing media [Minimal Davis Broth] and incubated for 48 hours at 37°C in shaker.

Effect of carbon sources on PHB production:

Positive bacterial isolates were grown in 250 ml conical flasks containing 100 ml Minimal Davis Broth with different carbon sources like glucose, fructose and maltose (1%). The flasks were incubated at 37˚C on a rotary shaker (150 rpm) for 48 hours. After incubation, PHB produced by the isolates were extracted by HYPOCHLORITE –CHLOROFORM METHOD explained below and quantified.

EXTRACTION OF PHB

HYPOCHLORITE –CHLOROFORM METHOD

The optimized pH and carbon source for positive bacterial cultures were used for the extraction of PHB by solvent extraction method (10,13) with slight modifications. Firstly, 1% of PHB positive strain was inoculated in PHB producing media of optimized pH and carbon source and it was incubated at 37°C. 40 ml of 48 hour bacterial culture in Davis broth was taken and pelletized at 6000 rpm for 20 minutes. For the recovery of PHB, 25 ml of 4% sodium hypochlorite was used to resuspend the pellet it was incubated at 37°C for 1 hour. This was followed by centrifugation at 10000 rpm for 10 minute to sediment the lipid granules. The bottom phase obtained was washed with acetone and ethanol (1:1) and 2.5ml of hot chloroform was added. Finally, the precipitate was evaporated in a hot air oven at 37°C kept in watch glass for PHB crystallization.

Esther Shoba R1*   *et al*

Effect of Nitrogen Sources on PHB production:

Positive bacterial isolates were grown in 250 ml conical flasks containing 100 ml Minimal Davis Broth with different nitrogen sources like urea, ammonium nitrate and caesin all at 1% concentration. After incubation, PHB yields were extracted and quantified.

MEDIA FORMULATION

The optimized source of carbon, nitrogen, and physical parameter viz., pH ant temperature was formulated along with the minimal Davis Broth for PHB production using the two isolated, screened and identified microorganism and the PHB was extracted and quantified.

RESULTS:

PRIMARY SCREENING OF PHB PRODUCING BACTERIA

About 40 colonies from sample SS-A and S-B, which were distinct, were chosen based on their shapes and colors. After 24-48 hours culture period, each colony was sub cultured separately and the plates when flooded with Sudan Black B stain the colonies which took up the stain was screened. Among 40 bacteria , 5 were found to be Sudan positive (1 from SS-A and 4 from S-B) (Figure 1)and on the second method of detection by colonial smears with 0.3% Sudan Black B solution staining the Purple to black granules were observed inside the cells with pink background indicating further confirmation (Figure 2).

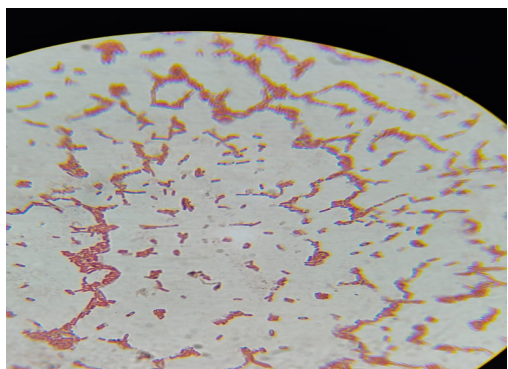Figure 1: Colonies taking Sudan Black staining – PHB production

Figure 2: Observation of Purple to black granules after staining with Sudan Black B staining

SCREENING TECHNIQUES

On gram staining, colony from SS-A was found to be gram negative rods (SS-A-1) and all 4 PHB positive colonies were gram positive. For further studies, one gram positive and gram negative microorganism from each sample was selected. Colony characterizations of the 2 colonies are tabulated in Table 1.

Table1: Colony characterization of the positive colonies SS-A-1 and S-B-2

Characteristics	SS-A-1	S-B -2
Gram Staining	Gram negative	Gram positive
Colony Shape	Circular	Circular
Spore	No	Yes
Margin	Smooth	Rough
Colour	Greenish colour	Slight yellow
Elevation	Flat	Flat
Opacity	Opaque	Opaque

16s rRNA MICROBIAL IDENTIFICATION

Sequence data obtained were aligned and dendrograms were generated using Sequence analysis software version 5.2 from Applied Biosystems. The 16SrRNA sequence of SS-A-1 and S-B-2 was subjected to homology analysis through Basic local alignment search tool (BLAST) at the NCBI server. The sequence showed extensive homologies with the *Pseudomonas aeruginosa* and *Bacillus subtilis.*

OPTIMIZATION OF PHYSICAL PARAMETERS FOR MAXIMUM BACTERIAL GROWTH

Effect of pH on bacterial growth

The optical density of the samples grown was spectrophotometrically analysed at 595 nm and pH 7 was found to be optimum for both the organisms (Figure 3).
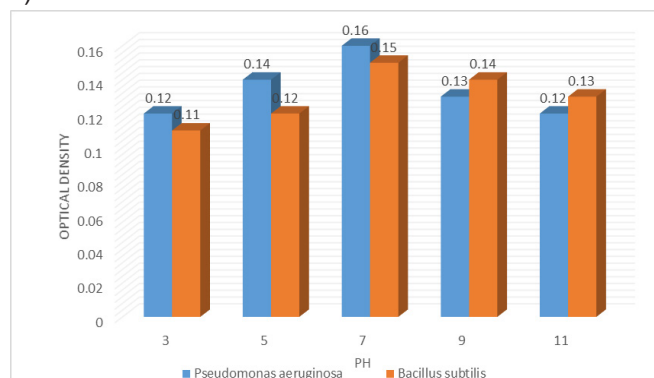

Figure 3: Effect of pH on bacterial growth. Microorganisms' showed an optimum pH of 7.

Effect of temperature on bacterial growth

The optimum temperature for *Bacillus subtilis* was

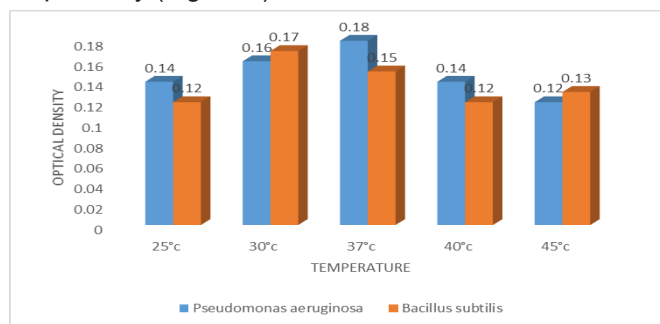found to be 30° C and 37°C for *Pseudomonas aeruginosa* respectively (Figure 4).

Figure 4: Effect of temperature on bacterial growth. Bacillus subtilis was found to be 30° C and

Effect of carbon sources on PHB production:

Glucose, maltose and fructose [1%] were added as carbon sources. Among these three, glucose was found to be the best carbon sources from both *Pseudomonas aeruginosa* and *Bacillus subtilis.* The obtained powder was weighed using weighing balance (Figure 5).
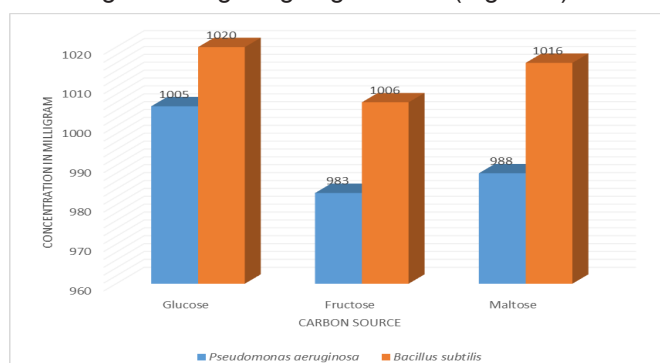

Figure 5 : Effect of carbon sources on PHB production where glucose is found to show optimum PHB production for both Pseudomonas aeruginosa and Bacillus subtilis.

Effect of Nitrogen on PHB production

Urea, Ammonium nitrate and Casein were added as nitrogen sources [1 %] and it was found that Ammonium nitrate was best nitrogen source for *Pseudomonas aeruginosa* to produce PHB and urea for *Bacillus subtilis* (Figure 6).
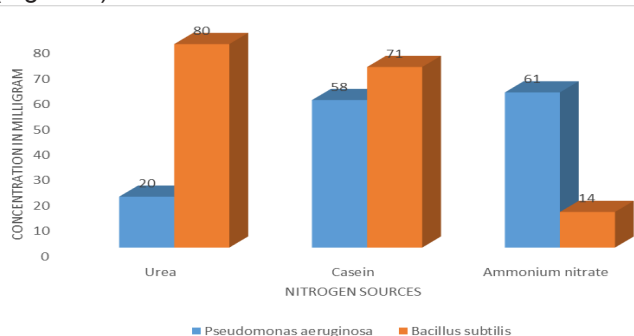

Figure 6: Effect of Nitrogen sources on PHB production where Ammonium nitrate is found to show optimum PHB production for Pseudomonas aeruginosa and Urea for Bacillus subtilis.

MEDIA FORMULATION

Species of Bacillus subtilis and Pseudomonas aeruginosa were able to produce PHB. Pseudomonas aeruginosa when optimized with glucose (carbon source) and Ammonium nitrate (nitrogen source), temperature 37^0C and pH 7 produced 570mg of PHB as compared to Bacillus subtilis optimized with glucose and Urea at temperature 30^0C, pH 7.0 that gave an yield of 340mg of PHB.

Conclusion

The study was conducted in order to isolate novel PHB Producing bacterial strains among the isolates which were screened. Two bacterial strains were screened (*Pseudomonas aeruginosa* and *Bacillus subtilis*) to be positive for PHB production and later the culture parameters were optimized to improve PHB yield. The tests also showed that *Pseudomonas aeruginosa* yields more PHB than *Bacillus subtilis* in comparison. The present study has thus produced useful data on optimized conditions for the production of PHB that can be used as, a rapidly evolving substitute for synthetic plastics based on petroleum.

References

1. Geyer, Jambeck. (2017) Production, use, and fate of all plastics ever made. Law Sci. Adv 3: e1700782.

2. Rinku Verma et al. (2016). Toxic Pollutants from Plastic Waste- A Review. Procedia Environmental Sciences 35 : 701 – 708

3. Eriksen M, Lebreton LCM, Carson HS, Thiel M, Moore CJ, Borerro JC, et al. (2014) Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLoS ONE 9(12): e111913. https://doi.org/10.1371/journal.pone.0111913.

4. Averous, L. (2004). Biodegradable multiphase systems based on plasticized starch: a review. *Journal of Macromolecular Science, Part C: Polymer Reviews*, *44*(3), 231-274.

5. Hernandez-Izquierdo, V. M., & Krochta, J. M. (2008). Thermoplastic processing of proteins for film formation—a review. *Journal of food science*, *73*(2), R30-R39.

6. Siracusa, V., Rocculi, P., Romani, S., & Dalla Rosa, M. (2008). Biodegradable polymers for food packaging: a review. *Trends in Food Science & Technology*, *19*(12), 634-643.

7. Lemoigne, M. (1926). Products of dehydration and of polymerization of β-hydroxybutyric acid. *Bull Soc Chem Biol*, *8*, 770-782.

8. Shang, L., Jiang, M. & Chang, H.N. (2003) Poly (3-hydroxybutyrate) synthesis in fed-batch culture of

Esther Shoba R1*   *et al*

Ralstonia eutropha with phosphate limitation under different glucose concentrations. Biotechnol Lett 25, 1415–1419.

9. Peters, V. & Rehm, B.H.A. (2005) In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS Microbiol Lett 248, 93–100.

10. K.R.Shah (2014) Optimization and production of Polyhydroxybutyrate(PHB) by Bacillus subtilis G1S1from soil, ISSN: 2319-7706 Volume 3 Number 5 (2014) pp. 377-387.

11. Sunitha Panigrahi , Ujwala Badveli. (2013). Screening, Isolation and Quantification of PHB-Producing Soil Bacteria. International Journal of Science Engineering and Invention. Volume 2 Issue 9.

12. Christina Thapa1, Pallavi Shakya1, Rabina Shrestha1, Sushovita Pal1, Prakash Manandhar. (2018). Isolation of Polyhydroxybutyrate (PHB) Producing Bacteria,Optimization of Culture Conditions for PHB production,Extraction and Characterization of PHB. Nepal Journal of Biotechnology. Volume 2.

13. Godale C, Ambarshetti S. (2015) Media optimization for Phb production and its application as precursor for bioplastics. European Journal of Biotechnology and Bioscience. 3(12):49–51.

14. S. Khannan, A.K. Srivastava. (2005). Recent advances in microbial poly-hydroxyalkanoates. Process Biochem., 40, 607–619.