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ORIGINAL RESEARCH

Production and Characterization of Polyhydroxy butyrate from *Pseudomonas aeruginosa*

Jesvin Pixy, Rakhi Raju and Elcey C. Daniel*

Biotechnology Research Division, Department of Life Sciences,
Kristu Jayanti College, Autonomous, Bengaluru, Karnataka- 560077, India

Abstract

Poly(3-hydroxybutyrate) (PHB) synthesis and accumulation is a mobilizable carbon repository of certain bacteria to thrive the carbon limitation. It is a common carbon- and energy-storage compound, degrades into its monomer 3-hydroxybutyrate (3HB) or D-β-hydroxybutyric acid (DBD) with in the cell under the microaerobic condition. A soil bacterial strain accumulating PHB was isolated and identified as Pseudomonas aeruginosa by 16S rRNA gene sequencing (NCBI accession number MF062071). The organism was subjected to PHB production under varying factors to establish the laboratory scale production with substrate preferences of economic choice. Among the substrates, wheat bran favored a higher PHB production, 0.9 g/l, compared to corn cob powder and coconut husk. The dry cell biomass was also maximum with the substrate, wheat bran. Neutral to near neutral pH (7.0) and an ambient temperature (37°C) showed as ideal under aerobic conditions. The PHB accumulated was extracted, purified and was subjected to FT/IR spectroscopy. A major peak apart from other peaks and a strong absorption band at 1651.83cm-1 corresponding to the C=O thioester bond reveal the functional group which confirm the presence of intracellular PHB.

Keywords

PHB, wheat bran, corn cob powder, coconut husk, FTIR

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Author for Correspondence
elcey@kristujayanti.com

1. Introduction:

The whole of humankind now thrives in a phase where life without plastics and plastic based products seems to be an unimaginable concept and a distant reality. Utilization of plastics in day-to-day life has become a cumbersome task to imagine one devoid of any plastic based products. The convenient low weight and increased durability, of the bio plastics became a promising alternative for the conventional wood, rock and metal based equipments and products and have been used extensively since then. They have the same characteristics of synthetic plastics in addition to biodegradability and biocompatibility. Bio-plastics are derived from renewable resources such as plants, cornstarch, or sugarcane, and they can be designed to break down more easily in natural environments *(Luckachan and Pillai, 2011).* They are the biodegradable materials, also accumulated to store carbon and energy in various microorganismsacts as a reserve carbon and energy source and have the potential to replace petroleum-based plastics as biomedical materials for use in surgical pins, sutures, staples, blood vessel replacements, bone replacements and plates, medical implants and drug delivery devices owing to their superior biodegradability and biocompatibility *(Chen, 2005; Sudesh et al, 2000; Reddy, 2003; Kim et al, 1994; Shivakumar, 2012; Kim et al 2023).* Their accumulation is limited by nutrient deficiency or the presence of surplus carbon.

PHB *(Fig. 1)* exists in the cytoplasmic fluid in the form of crystalline granule about 0.5 m in diameter. Beta hydroxybutyrate is connected by ester linkage and forms PHB, has molecular weight up to 2 million (i.e. 20000 monomers per polymer molecules). It is optically active and It is water insoluble and relatively resistant to hydrolytic degradation.It is optically active

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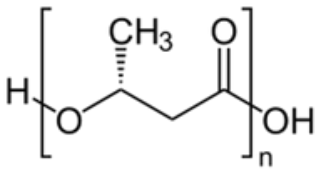


Figure 1. *Poly beta hydroxy butyrate (Lenz and Marchessault, 2004)*

and It is water insoluble and relatively resistant to hydrolytic degradation. Among the several PHA, the pathway for the biosynthesis of PHB has been thoroughly investigated. Starting with Acetyl CoA, PHB is synthesized in three reaction steps *(Sun et al., 1994; Senior et al., 1972; Zhang 2003) (Fig. 2).*

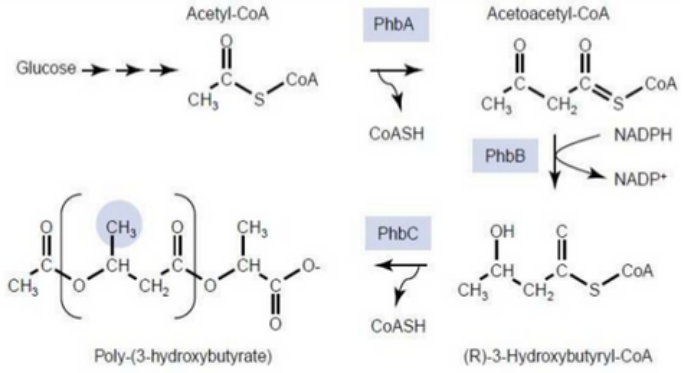


Figure 2: Biosynthesis pathway of PHB

A large number of bacterial species both Gram positive and Gram-negative produce PHB *(Verlinden et al., 2007; Nair, 2013).* Many researchers have explained that several soil bacteria generally produce PHA and PHB. Polyhydroxyalkanoates (PHAs) are synthesized by various microorganisms such as *Cupriavidus necator, Alcaligenes latus, Aeromonas hydrophila, Pseudomonas sp. and Bacillus sp. (Kato et al., 1992)*. The bacterium capable of producing PHB has been identified in more than 20 bacterial genera, including *Azatobacter sp., Bacillus sp., Beijernickia sp., Alcaligenes sp., Pseudomonas sp., Rhizobium sp. and Rhodospirillum sp., Ralstonia eutropha, and Escherichia coli (Mahishi et al., 2003).* Several halophilic microbes including *Haloferax mediterranei, Vibrio sp.,* (*V. natriegens, V. nereis and V. harveyi*) and *Halomonas sp. (Kawata and Aiba 2010).* These microorganisms can accumulate PHA up to 30-80% of their dry weight *(Tombolini et al., 1989)*. PHB has also been isolated from various marine prokaryotes *(Weiner et al, 1997).* The accumulation of PHB serves as a reserve carbon source, which can be oxidized, to carbon dioxide and water, releasing large amount of energy.

PHB is mostly manufactured by batch culture by providing excess supply of carbon source, and limiting some other essential nutrient such as nitrogen, phosphorus or sulphur source *(Darshan et al, 2013; Elsayed et al, 2016).* One of the primary obstacles to the large-scale use of PHB is its relatively high production costs. The processes involved in synthesizing PHB, often through microbial fermentation, can be resource-intensive and costly compared to traditional petrochemical-based polymer production methods. Efforts have been made to reduce the production costs of bacterial PHB exploring the use of inexpensive raw materials as well as the optimum conditions *(Kulpreecha et al., 2009; Sundaramoorthy 2013; Belal 2013; Mona et al, 2001, McAdam, 2020).* Various inexpensive substrates that are used for the production of PHB include whey, starch containing substances like arrowroot, rice water, sago water and biproduct waste glycerol *(Shilpi et al., 2006; Page,1992; Ramadas, 2013; Kawata and Aiba, 2010; Ranjna S, 2021)*. PHA is biodegradable, water insoluble, non-toxic, bio-compatible, piezoelectric, thermoplastic, and elastomeric. These features make them suitable for applications in the packaging industry and a substitute for hydrocarbon-based plastics *(Yu-Hong et al, 2011, Ramkumar et al, 2010).*

Pseudomonas is a diverse genus of bacteria, and *Pseudomonas aeruginosa*, in particular, is of significant clinical importance. *P. aeruginosa* is a ubiquitous bacterium found in various environments, especially in moist ones. It is known for its adaptability and can thrive in diverse conditions. *P. aeruginosa* is often motile due to its flagella, allowing it to move through liquid environments. It is an opportunistic pathogen that can cause infections in humans, particularly in individuals with compromised immune systems or underlying health conditions. The present study has envisaged the production of Polybetahydroxy butyrate granules by *P. aeruginosa* along with the optimization of culture conditions. for the PHB production.

2. Materials and Methods:

2.1. Sample Collection

Soil samples were collected aseptically from the college campus of Kristu Jayanti College (Lat.- 13° 3' 33.2989" N; Long.- 77° 38' 29.9958" E) and adjacent areas (Lat.- 13° 3' 29.2622" N; Long.- 77° 38' 33.3229" E), Bengaluru. The collected soil samples were subjected to serial dilution *(Booth et al., 2006)*. The samples were diluted serially and 0.1 ml were plated (10-3 to 10-6) onto Cetrimide agar plates, a selective medium for Pseudomonas species, using spread plate technique. The inoculated plates were then incubated at 37°C for 24 hours. In order to confine the PHB accumulating Pseudomonas strain, all the isolated microorganisms were subjected to the conventional staining method, Sudan black staining technique. Few drops of bacterial broth were fixed on a glass slide by applying heat and then stained with a 3% Sudan Black B (w/v in 70% ethanol) solution for 10-15 minutes. The slide was then immersed in xylene until completely decolourized. The sample was counterstained with safranin (5% w/v in distilled water) for 10 seconds, washed again with distilled water, and dried using tissue paper. The slides were examined under microscope. Black granules indicate the presence of PHB *(Schlegel et al., 1970)*

The colonies indicated with the black granules were characterized further to establish its identity by the morphological, microscopic and biochemical analysis. The biochemical assays were carried out to establish the identity of the organisms by analysing the presence of tryptophanase enzyme (indole test), acid production (Methyl Red test), acetoin/ acid pathway (Vogues Proskauer test), Citrate Utilization, Urease production and Nitrate reduction test. In addition, the presence of catalase enzyme and cytochrome oxidase system also were tested. The pigmented colonies obtained on Cetrimide agar plates and positive for the PHB accumulation were maintained as pure culture for further studies. The culture was subjected to 16S rRNA gene sequencing to confirm the identity. The bacterial strain was grown overnight and genomic DNA was isolated *(Green and Sambrook, 2017).* The purified genomic DNA was subjected PCR and 16S rRNA gene was amplified using universal primers. The amplified DNA was sequenced (Applied Biosystems/3730xl/XE3-IOT-2019-1406-029) and the sequences were compared in NCBI database using BLAST and the similarity was established.

2. 2. Production of PHB

The selected microorganism was subjected to study for the production of PHB under different conditions. Initially batch fermentation was carried out in Erlenmeyer flasks containing cetrimide broth as the basal medium. The peptone in the cetrimide broth was replaced by using 3 different inexpensive and easily available substrates like wheat bran *(Fig. 3a)*, corn cob powder *(Fig. 3b)* and coconut husk *(Fig. 3c).* After the addition of each substrate to the basal medium, it was sterilized at 121°C, 15 lbs pressure for 20 minutes, brought to room temperature and were aseptically inoculated with the isolated bacterial culture in triplicate.

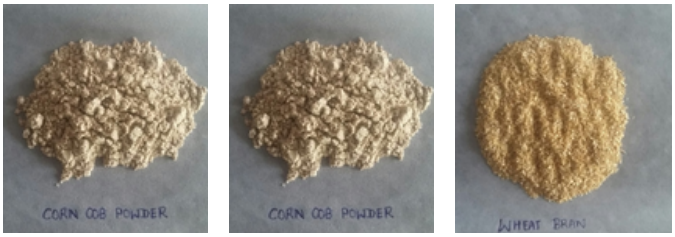


Figure 3: (a) Wheat Bran (b) Corn Cob Powder (c) Coconut Husk

Effect of environmental factors like temperature, pH on the production of PHB was also studied by exposing the microbial isolate in the cetrimide medium. The effect of each of the parameters on PHB production by the isolate was carried out in triplicate parallel with a control ie., other culture conditions constant.

2.3. Effect of temperature on PHB production

The effect of temperature on PHB production by the isolate was determined by growing the isolated culture in the cetrimide broth bearing the three substrates. The pH of the constituted media was adjusted to 7.0, sterilized and then inoculated with the pure culture of the isolate. The three representative ranges of temperatures taken into consideration in this study include 4°C, 25°C, 37°C and 55°C. The inoculated media were incubated at the selected temperatures in this study for 48 hours in a rotary shaker (150rpm). After 48 hours, the PHB granules were extracted and quantified.

2. 4. Effect of pH on PHB production

In order to confirm the effect of pH on PHB production by the isolate, the pH of the cetrimide broth bearing the three substrates was adjusted to the pH ranges taken into consideration. The pH range selected in the present study include 3, 5, 7 and 9. The media were sterilized and inoculated with a pure culture of the isolate and incubated for 48 hours in a rotary shaker (150 rpm) at 37°C. After 48 hours, the produced PHB granules were extracted and quantified.

2. 5. Effect of substrate concentration on PHB production

To study the effect of substrate concentration on PHB production, varying concentrations (in grams) of the three substrates were added to the basal cetrimide broth. The substrate concentrations considered in this study include 5g, 10g, 15g and 20g. Prior to inoculation of the media with a pure culture of the isolate, the pH of the medium was adjusted to pH 7 in all the flasks and sterilized. The inoculated media were incubated at 37°C for 48 hours in a rotary shaker (150 rpm). After 48 hrs, the produced PHB granules were extracted and quantified.

2.6. Extraction and estimation of PHB

Cells were collected by centrifugation at 10,000 rpm for 10 minutes from the 48 hours old broth culture and the pellet was re-suspended in 10 ml of sodium hypochlorite reagent *(Williamson and Wilkinson, 1958; Belal, 2013a).* After 1hour incubation at 37°C, the reaction mixture was centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded. The solid pellet was washed successively with 1 ml portions of water, alcohol and acetone. The contents were re-centrifuges at 10,000 rpm for 10 minutes. Following this, the pellet thus obtained was dissolved in chloroform, while the insoluble residue was discarded. Finally, the polymer was retrieved after the evaporation of chloroform at room temperature. The polymer was then dried and weighed. The dry weight of extracted polymer was estimated.

2.7. Measurement of Biomass (Cell dry weight)

After incubation time, the culture medium was collected and centrifuged at 10,000 rpm for 15 minutes. The Supernatant was discarded and the cells were washed in de-ionized water and re-centrifuged for 10 minutes at 10,000 rpm at 4ºC. The cell pellets were dried at 80ºC for 24 hours in a hot air oven. The total bacterial cell dry weight was determined.

Dry weight of the biodegradable polymer and percentage of it against cell dry weight was measured. The estimation of PHB produced was calculated by the following formula:

Yield percentage of PHB = Total Weight of PHB × 100 /Total dry weight of the biomass

2. 8. Analysis of PHB by Fourier Transform Infrared Spectroscopy (FTIR)

The extracted biopolymer (white powder) was analyzed by Fourier Transform Infrared Spectroscopy (FTIR) spectrum to analyze the functional groups in the sample. The characterization was done by employing the model, Perkin Elmer Spectrum Version 10.03.07 NDXUS- 672. The spectrum was taken in a mixed IR 400 -4000 cm-2 with 16 scan speed and was recorded using Attenuated total reflectance (ATR).

3. Results & Discussion:

3.1 Microbial identification

Based on the morphological, microscopic and biochemical analysis, the characteristics of the bacterial isolate was found similar to *Pseudomonas* species. The preliminary phenotypic study of the pigmented colonies obtained on cetrimide agar plates showed that the isolate was Gram negative short rods and motile. It was also non sporing. The isolate synthesized a purple-colored pigment when grown on Cetrimide agar medium *(Fig. 4).* The colonies on the plate also produced a characteristic earthy odor. The bacterial isolate showed the similarity to *Pseudomonas* sp. based on various biochemical tests according to Bergy's manual of systematic bacteriology, the presence and absence of enzymes involved in the biochemical pathways. They also presented a non-fermentative metabolism on a wide range of sugars, fermenting glucose *(Table 1).* Further, it was confirmed that the sequence showed 100% similarity with *Pseudomonas aeruginosa* upon the sequence similarity search in the NCBI nucleotide database. The sequence was deposited in the NCBI database (NCBI accession number MF062071).

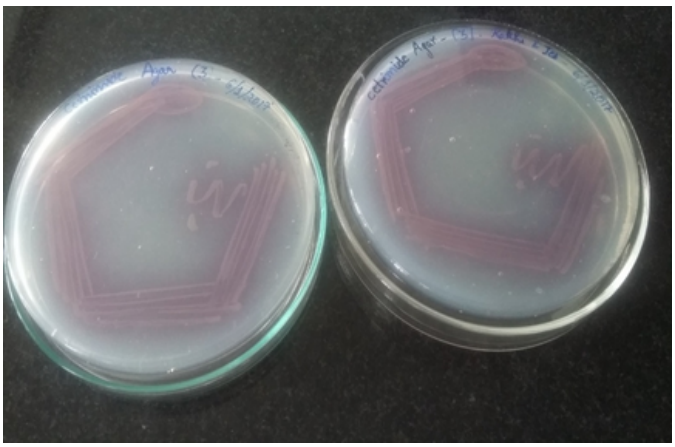


Figure 4: *Pseudomonas* spp. on Cetrimide agar

3. 2 . Detection and analysis of PHB granules

Accumulation of PHB granules in *Pseudomonas aeruginosa* was confirmed by its presence using Sudan black test. PHB granules appeared blackish in color while cell cytoplasmic contents appeared as pink *(Fig. 5).* The accumulated PHB was extracted by the methods carried out by *Williamson and Wilkinson (1958)* which yielded white chalky powder *(Fig. 6).* The identity of the compound was confirmed using FTIR analysis. The analysis revealed that the FTIR spectrum of the extracted white powdery compound from *Pseudomonas aeruginosa* was similar to the spectrum of PHB molecule. The FTIR spectrum of the extracted polymer shows a major peak and a strong absorption band at 1651.83cm-1 corresponding to the C=O thioester bond functional groups *(Fig 7, Table 2)*. Therefore, in the present study, the functional group of the polymer PHB was confirmed as C=O group by FTIR spectroscopy which is in accordance with the reported results *(Colthup et al., 1964; Shah, 2012).*

3. 3. Optimization of culture conditions for PHB production

The effect of different ranges of three parameters i.e., temperature, pH and substrate concentration on the production of polybetahydroxybutyrate by *Pseudomonas aeruginosa* was studied for the optimization of culture conditions. In each case the percentage yield of PHB against the dry cell biomass was estimated.

3.3.1. Effect of temperature on PHB production

The effect of temperature on PHB production by *Pseudomonas aeruginosa,* in the culture broth with the presence of the three substrates is depicted in the figure 8. Among the different temperature ranges, a significant increase in PHB production was observed at a temperature range between 25°C and 37°C rather than at 4°C and 55°C (*Grothea et al., 1999).* A maximum yield of 0.90g/l (75%) was obtained at 37°C using wheat bran as substrate. The PHB content was found to be 0.85g/l (65.38%) using Corn cob powder at 37°C while using coconut husk the yield of PHB was found to be 0.50g/l (45.45%). Therefore, in all the three substrates the highest PHB production was obtained at the temperature of 37°C *(Fig 8).*

3.3.2. Effect of pH on PHB production

For the purpose of studying the effect of pH on PHB production by *Pseudomonas aeruginosa,* the broth with the three substrates prior to inoculation were adjusted to different pH ranges namely 3, 5, 7 and 9. From the results obtained, a maximum yield of 0.96g/l (60.37%) was obtained at pH 7 using wheat bran as substrate. The PHB content was found to be 0.64g/l (50%) using Corn cob powder and 0.28g/l (31.81%) using coconut husk as substrate at pH 7 *(Fig. 9).* Therefore, among the pH ranges considered in this study, *Pseudomonas aeruginosa* showed highest PHB accumulation at pH 7 for all the three substrates (*Fig 4)* since pH 7 is the optimal pH for the enzyme activity involved in PHB

Table 1: Morphological, microscopic and biochemical characteristics of the isolate

Morphological characteristics		**Biochemical Characteristics**	
Colony Features	Observation	Biochemical Test	Observation
Shape	Regular	Indole	Negative
Size	Small	Methyl Red	Negative
Texture	Smooth & Shiny	Vogues Proskauer	Negative
Colour	Purple	Citrate Utilization	Positive
Elevation	Raised	Urease Test	Negative
Density	Slightly opaque	Nitrate reduction	Positive
Margin	Undulate	Catalase	Positive
Microscopic Observation		Oxidase	Positve
		Carbohydrate Fermentation	
Gram's reaction	Gram Negative		
Shape	Rod shaped	Glucose	Positive
Size	Short	Lactose	Negative
Spore	Non-sporing	Sucrose	Negative
Motility	Highly motile	Maltose	Negative

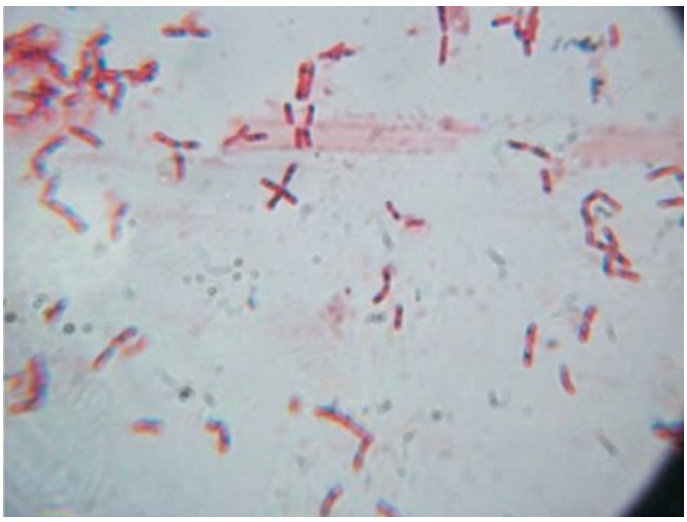


Figure 5: Slide of *Pseudomonas aeruginosa* containing PHB granules as black spots inside the cell

synthesis *(Muralidharan, 2014)*. At pH 3.0 and 9.0 the enzyme activity decreased which resulted in the decreased production rate of PHB accumulation *(Kulpreecha et al., 2009)*.

3.3.3. Effect of substrate concentration on PHB production

Assessment of the substrate concentration on PHB production by *Pseudomonas aeruginosa*, the three different substrates, wheat bran, corn cob powder and coconut husk, were taken in the four different concentrations ie., 5, 10, 15 and 20g respectively. The pH was maintained at 7 and the temperature was kept constant at 37°C for this study. A maximum yield of 0.90g/l (71.42%) was obtained using 20g of wheat bran as substrate. Using 20g of corn cob powder, the PHB yield was found to be 0.7 g/l (59.32%) and for coconut husk (20g) the yield was 0.26g/l (29.88%). The maximum PHB accumulation by *Pseudomonas aeruginosa* was obtained at the highest substrate concentration of 20 g of wheat bran at 37°C and pH 7. However, in all the three substrates used, the higher concentrations enhanced the production of PHB in the medium *(Fig.10).*

The optimum temperature for PHB production using all the three substrates was 37°C and the optimum pH was found to be 7. This have been due to the fact that these are the optimum conditions for the activity of the enzymes involved in the biosynthesis of PHB, 3-ketothiolase and acetoacetyl reductase. It was also deduced that the yield of PHB increased with the increase in the substrate concentration.

In this study a major emphasis was laid on the replacement of expensive substrates for the production of PHB using easily available inexpensive substrates. Excess carbon source in the culture medium with limited nitrogen source triggers bacteria to produce PHB to be used later as carbon source during starvation *(Belal et al., 2016)*. Among the three substrates used, maximum PHB

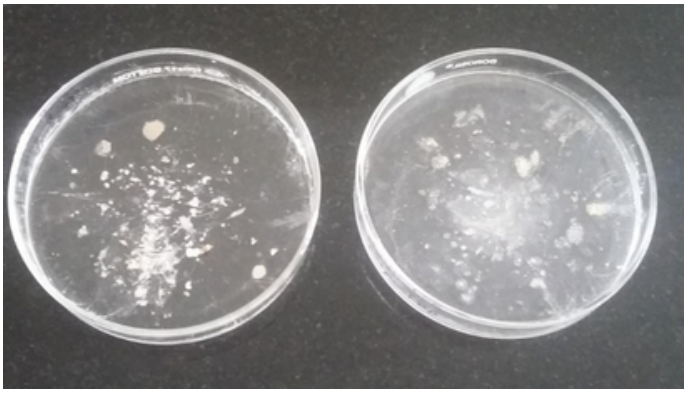


Figure 6. White chalky PHB powder on the Petri plate

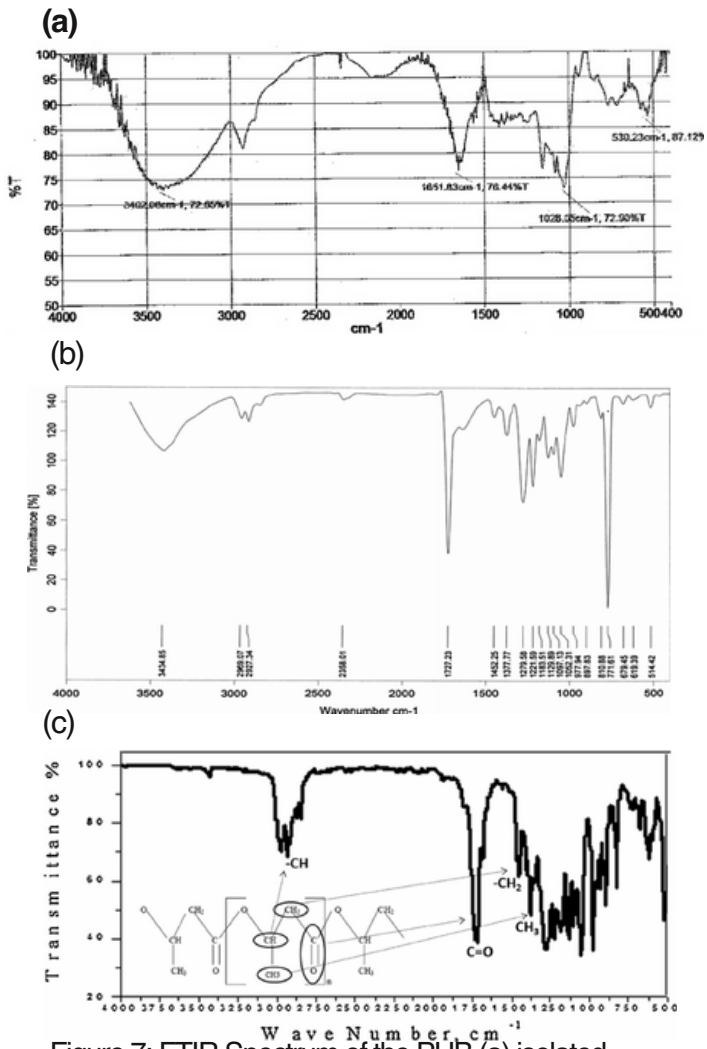


Figure 7: FTIR Spectrum of the PHB (a) isolated from *Pseudomonas aeruginosa* (b) the standard (c) Spectrum of PHB to compare, Courtesy: Raveendran et.al. (2013)

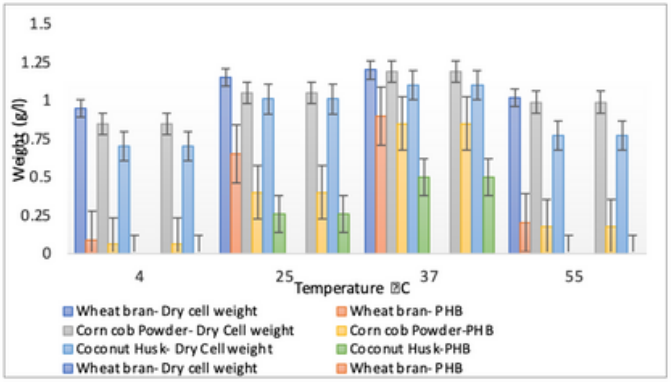
accumulation by the bacterium was detected when wheat bran was used as substrate followed by corn cob powder and coconut husk. This could be attributed to the fact that the nitrogen content in wheat bran and corn cob powder was less compared to the carbon content which provided an ideal condition for the accumulation of PHB since it is formed under conditions of nutritional stress. However, despite the fact that the nitrogen content in coconut husk is negligible, the sources of carbon in coconut husk are highly complex and as such the bacterium may not have been able to degrade and utilize it for its growth and metabolism which resulted in the least biomass production and PHB production.

Table 2: Functinal group at different wavelenth segragated in the FTIR spectrum

Absorbance	Functional Group
3402	-OH
2920	-CH3- Aliphatic
2850	-CH2- Aliphatic
1651	-C=O
1100	-COOH
1028 1200	-C-O-C

The study also revealed that expensive pure chemical substrates can be replaced by using inexpensive substrates for effective PHB production. A similar study by *Murugan et al (2021)* also support the usage of the inexpensive substrate for the production of PHB. *Pseudomonas aeruginosa* is a non-fermentative aerobe that derives its energy from oxidation rather than fermentation of carbohydrates. Although, the organism is an aerobe, it can grow anaerobically, using nitrate as an electron acceptor. This organism grows well at 25° C to 37° C, but can grow slowly or at least survive at higher and lower temperatures. Indeed, the ability to grow at 42° C distinguishes it from many other Pseudomonas species.The information provides valuable insights into the remarkable characteristics of *Pseudomonas aeruginosa*, further emphasizing its adaptability and resilience. It is known for its ability to utilize a wide range of nutrients, showcasing nutritional versatility. This adaptability contributes to its ability to thrive in diverse environments, including clinical settings. *P. aeruginosa* exhibits resistance to high concentrations of salt, dyes, weak antiseptics, and various antibiotics. This resilience contributes to its ability to survive and persist in different conditions, making it a challenging pathogen to control.

(a)



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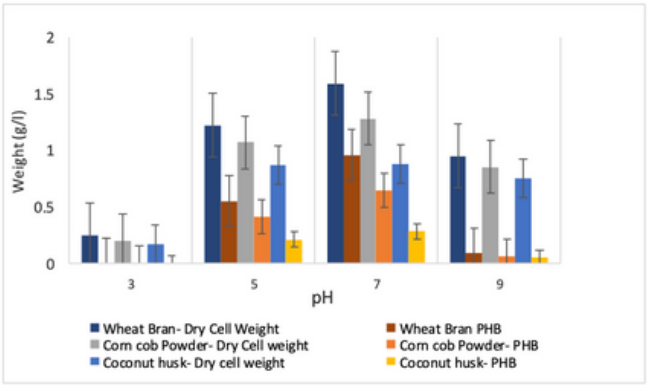

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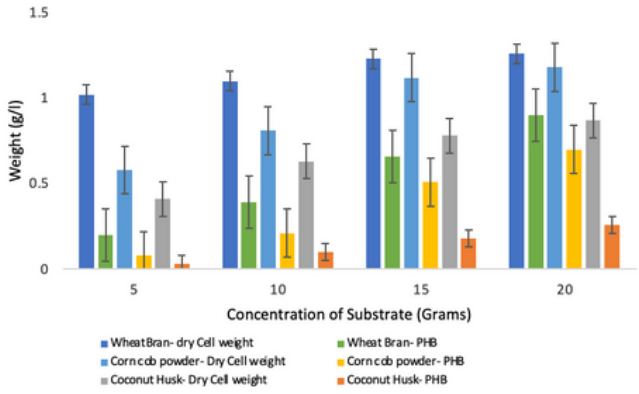

Figure 8: (a) Effect of Temperature and Substrate on Dry cell weight and PHB production. (b) Effect of pH on the production of PHB (c) Effect of pH on the production of PHB

Pseudomonas species, including *P. aeruginosa,* are known to actively accumulate polyhydroxybutyrate (PHB) granules as reserve materials under stressful conditions. This is a strategy for surviving nutrient exhaustion, where the bacteria enter a polymer accumulation phase after the growth phase. The two distinct phases, a growth phase and a polymer accumulation phase, highlight the dynamic response of *P. aeruginosa* to changing environmental conditions. When nutrients become limited, the bacterium shifts its focus to synthesizing PHB granules as a survival strategy

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4. Conclusion

The use of petrochemical plastics has led to serious cases of environmental pollution. This has created a renewed interest in polymers of biological origin in the recent past and Polyhydroxy butyrate has proven to be an efficient candidate to combat the issues caused by petroleum based non-degradable polymers. PHB can be degraded by various microorganisms and thus are eco-friendly and biocompatible, thereby finding its place in drug delivery and other medical purposes. Degradation of PHB by microorganisms expose during the microaerophilic condition was reported by Hannya, et al (2017).

In the present study three easily available inexpensive substrates were used for the production of PHB by the isolated soil bacterium, *Pseudomonas aeruginosa*. These substrates were wheat bran, corn cob powder and coconut husk. The investigation revealed that the highest yield percentage of PHB was achieved by using wheat bran as substrate. This was followed by corn cob powder and coconut husk. This study also included the optimization of culture condition and substrate concentration for the maximum production of PHB. From the study it can be concluded that the optimum temperature for PHB production by Pseudomonas aeruginosa using all the three substrates was 37°C and the optimum pH was found to be 7. Thus, the soil isolate, *Pseudomonas aeruginosa* can be used as an efficient organism for the production of PHB under these optimal culture conditions.

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