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(RESEARCH ARTICLE)

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Production of Bioplastic (Polyhydroxybutyrate) by *Micrococcus luteus* isolated from water sample

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Abstract

Polyhydroxybutyrate (PHB) is a biodegradable polymer produced by various microorganisms as a storage material, offering a sustainable alternative to traditional plastics. Biodegradability of Bioplastics has been widely publicized in society and the demand for packaging is rapidly increasing among retailers and the food industry at large scale. The plastic which is available in market is very dangerous as it is nonbiodegradable. Therefore, it is the demand of the day that biodegradable plastics should be produced and used. This study investigates the production and optimization of PHB using *Micrococcus luteus*, a gram-positive bacterium known for its ability to synthesize PHB under suitable conditions. The research focuses on optimizing culture conditions, including the selection of carbon and nitrogen sources, pH, and temperature, to maximize PHB yield. *Micrococcus luteus* was cultured in a basal medium with different carbon sources (glucose, fructose, sucrose, maltose) and nitrogen sources (ammonium sulphate, peptone, yeast extract, urea). The optimal conditions were determined through systematic variation of pH (5 to 9) and temperature (20°C, 30°C, 40°C and 50°C). This study will also spotlight *Micrococcus luteus* as a potential source for large scale production of PHB with reducing environmental pollutions. Thus, the bacterial mediated PHB synthesis can be used as a better alternative to deal with the currently available practices of plastic use.

Keywords: Polyhydroxybutyrate; *Micrococcus luteus*; Sudan black B stain; Sodium hypochlorite method; Optimization; Growth curve.

1. Introduction

In the modern world, plastics are an integral part of our daily lives, and therefore, the demand for them has increased tremendously, from 1.5 million metric tons in 1950 to approximately 370 million metric tons in 2021. Despite the low cost of these synthetic polymers, they have a negative impact on our environment, and particularly in the form of hazardous waste. Because of their non-degradable nature and the fact that they cannot be recycled, plastics are the main culprits of environmental pollution. Even though a number of recycling efforts have been made, the plastics being disposed of in municipal landfills continue to cause major problems. In response, there has been an increasing number of attempts to find renewable, biodegradable, and recyclable materials (i.e., green materials that are useful for sustainability). Considering that biodegradable plastics are "eco-friendly" in nature, they offer the most feasible solution to protect the environment from petroleum-based plastic hazards. There are different types of biodegradable plastics, such as polylactic acid (PLA), poly (butylene succinate-co-butylene adipate) (PBSA), polycaprolactone (PCL), and poly (hydroxyalkanoates) (PHAs). These types of plastics can be used to replace synthetic plastics, such as polyethylene (PE) and polystyrene (PS), which require hundreds or thousands of years to degrade in the environment (Mohd Adnan et al., 2022). Polyhydroxybutyrate (PHB) is one of the most common group of PHAs found in nature.

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PHBs are carbon and energy reserve polymers produced in bacteria, archaea, and in few eukaryotes, such as yeasts and fungi when carbon source is in plentiful and other nutrients such as nitrogen, phosphorous, oxygen or Sulphur are limited. The storage molecule is then metabolized under unfavorable conditions when other common energy sources are not available. Some bacterial species which naturally produce PHB are *Ralstonia eutrophes*, *Alcaligenes*, *Pseudomonas, Bacillus, Rhodococcus, Staphylococcus* and *Micrococcus* (Christina Thapa et al., 2018).

PHB has been the most promising biodegradable plastics and as an alternative to petrochemical plastics. The molecular weight varies from 2-3 × 103K Da. The weight was depending on the species that actually producing the polymer (Saharan BS, et al., 2012). The main fermentation strategies used to obtain bio-products are batch culture, fed-batch culture, continuous culture and two stage fermentation. Two-stage fermentation is currently the most common method of producing PHAs (fig. 1). In the first stage of the proposed process, biomass is increased to the level needed for PHB production. In the second stage, nutrients are limited in order to stimulate PHB synthesis by bacteria (Neha Sharma, 2019).

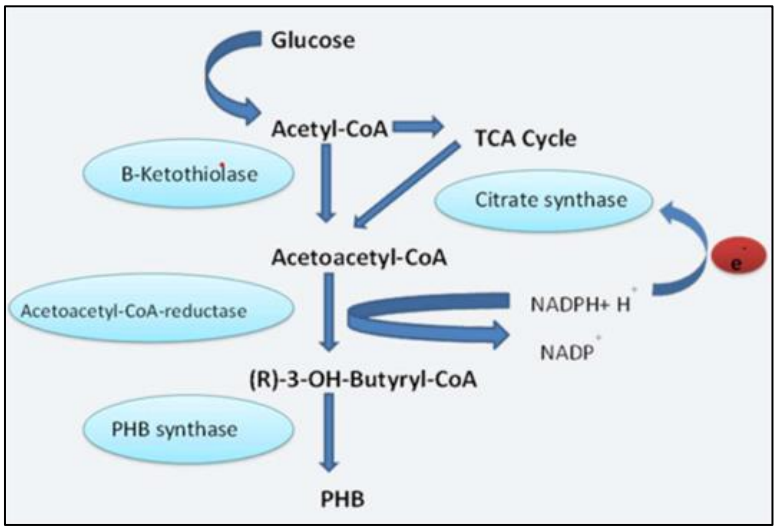

Figure 1 Schematic representation of biosynthetic pathway of catalysed butyrate from acetyl-coA

2. Materials and Methods

2.1. Sample collection

Samples from plastic damped areas were identified and the water samples were collected in a sterile tube (Water sample collected from Sankey tank, Sadashivanagar, Bangalore). The distinctive physio-chemical parameters of the locations might play a role to best suit the growth of PHB synthesizers. Indeed, some of the parameters considered were the presence or absence of water, domestic pollutants, grazing, competitive growth, and geomorphology of the water. All water samples were collected and preserved in vials using sterile conditions, stored at 4 ◦C for 3 weeks for the bacteria isolation.

2.2. Isolation of PHB producing bacteria

One ml of each sample was dispersed in 10ml of sterile distilled water. Serial dilution of these samples was done up to 10-5, followed by spread plating of 100µl diluted samples on nutrient agar plates. Thereafter, the plates were incubated at 30°C for 48 hours (Christina Thapa et al.,2018).

3. Colony morphology and Biochemical tests

PHB producing bacteria were isolated on nutrient agar by serial dilution method from water sample collected from Sankey tank. Bacterial isolates with different size, shape, color and morphology were further proceeding for PHB screening on nutrient agar by Sudan Black B staining where PHB producers appear bluish black color while non PHB producers remain white. Most proficient PHB bacterial isolates were promote for identification as per Bergey's manual of systemic bacteriology.

3.1. Gram staining

The bacterial smear was prepared on clean slide and heat fixed after drying and the entire slide could be moistened by a low volume of reagent that filled the cuvette in a specific order crystal violet (1-2 min), iodide solution (1 min), decolorant (70% ethanol for 15-20 sec), safranin (2 min), with water washing after each step (Hui Li et al., 2020). Last, the slides were air dried and observed under a bright field microscope using an immersion lens.

3.2. Endospore staining

The bacterial smear was prepared, heat fixed, and covered with malachite green solution (5%). Slides were passed through a flame for 5 min intermittently, making sure to not boil the dye. The slides were then washed with water and stained with safranin (2.5%) for 30 sec. Last, the slides were air dried and observed under a bright field microscope using an immersion lens.

3.3. Indole test

Tryptone broth (Tryptone 10 g, NaCl 0.5 g, $CaCl_3$ 0.03 g in 1000 ml water) was prepared, pH was adjusted to 7, inoculated with test organisms and incubated at 37 °C for 24 h. After incubation kovac's reagent (1 ml) was added and observed for cherry red ring formation.

3.4. Methyl red test

MR-VP broth (buffered peptone 7 g, glucose 5 g, K_2HPO_4 5 g in 1000 ml water) was prepared, pH was adjusted to 7.5, inoculated with test organisms and incubated at 37° C for 24 h. After incubation 2-3 drops of methylene red was added shaken well and left undisturbed for 18-20 min and observed for color change.

3.5. Voges Proskauer test

MR-VP broth (Buffered peptone 7 g, glucose 5 g, K_2HPO_4 5 g in 1000 ml water) was prepared, pH was adjusted to 7.5, inoculated with test organisms and incubated at 37 °C for 24h. After incubation 12 drops of VP reagent I and 2-3 drops of VP reagent II was added and observed for color change.

3.6. Citrate test

Simmon's citrate agar (sodium citrate 2 g, NaCl 5 g, $MgSO_4$ 0.2 g, bromothymol blue 0.8 g, $(NH_4)_2HPO_4$ 1 g, K_2HPO_4 1 g in 1000 ml water) was prepared, pH was adjusted to 7, inoculated with test organisms and incubated at 37°C for 24h. After incubation observed for color change.

3.7. Oxidase test

The loop full of test organisms were smeared on the colorless oxidase disc on clean glass slide and observed for color change.

3.8. Catalase test

The loop full of test organisms were immersed in hydrogen peroxide and observed for bubbling (James G. Cappuccino et al).

4. Screening the isolates for PHB production

The seven colonies are suspected to produce PHB based on morphological and biochemical characteristics.

4.1. Sudan Black B staining on Petri dishes

In order to detect the presence of PHB granules in the isolated strains, Sudan Black B dye was used as the primary screening method. A Zobell marine agar (Peptone 5.0 g, Sodium Chloride 19.45 g, Yeast Extract 1.0 g, Magnesium Chloride 8.8 g, Ferric Citrate 0.1 g, Sodium Sulfate 3.24 g, Calcium Chloride 1.8 g, Potassium Chloride 0.55 g, Sodium Bicarbonate 0.16 g, Potassium Bromide 0.08 g, Strontium Chloride 0.034 g, Disodium Phosphate 0.008 g, Boric Acid 0.022 g, Sodium Silicate 0.004 g, Sodium Fluorate 0.0024 g, Ammonium Nitrate 0.0016 g, Agar 15.0 g for 1000 ml and final pH (at 25°C) 7.6 ± 0.2) supplemented with 1% glucose supplement was added to the nutrient agar medium. The isolated bacterial strain was spread on the Zobell marine agar supplemented with 1% glucose plates, and was allowed

to incubate for 24 h at 30 °C. The Sudan Black B stain was made by dissolving 0.02 g of powder in 100 mL of 70% ethanol. Upon the completion of incubation, Sudan Black B dye was spread over the plates and was allowed to stand for 30 min before being removed. To remove the excess stain, plates were washed with 96% ethanol and left to dry. It has been observed that colonies unable to incorporate the Sudan Black B dye appear white, whereas colonies producing PHB appear bluish-black (Mohd Adnan et al 2022).

4.2. Sudan Black B staining on glass slides

All smears that resulted as positive for the production of PHB granules using the Sudan black B solution (30%) was subjected to slide staining. The dye was prepared by mixing 0.3 mg of the solid dye with 100 mL of 70% ethanol. Smears of the positive bacteria were prepared and heat-fixed on glass slides. Specimens were stained with the Sudan black B solution for 15 min, washed with xylene, and counter stained with Safranin (5% in distilled water) for 1 min before being observed under the microscope with immersion oil, at 100X. Cells containing blue-black cytoplasmic granules were considered PHB producers, preserved in 2% glycerol vials for preservation and further analysis.

5. Production of PHA by selected isolates

Mineral salts medium (MSM) [composition (g/L): Urea (1.0), Yeast extract (0.16), KH_2PO_4 (1.52), Na_2HPO_4 (4.0), $MgSO_4 \cdot 7H_2O$ (0.52), $CaCl_2$ (0.02), Glucose (40), and trace

element solution 0.1 ml] was used for the production of PHA by the selected isolates. The trace element solution contained (g/L): $ZnSO_4 \cdot 7H_2O$ (0.13), $FeSO_4 \cdot 7H_2O$ (0.02), $(NH_4)_6Mo_7O_{24}$. $4H_2O$ (0.06) and H_3BO_3 (0.06). Both glucose and trace element solution were autoclaved separately, and reconstituted prior to inoculation. The culture was prepared by sub culturing the isolates twice in nutrient broth. Then one ml of a 24 h old culture was inoculated into 100 mL production medium and incubated at 37 °C and 150 rpm for 48 h (Anteneh Getachew et al., 2016).

6. Extraction (by sodium hypochlorite method) and quantification of PHB

Ten mL of culture was centrifuged at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was treated with 10 mL sodium hypochlorite and the mixture was incubated at 30°C for 2 h. The mixture was centrifuged at 5000 rpm for 15 min and then washed with distilled water, acetone and methanol respectively The pellet was dissolved in 5 mL boiling chloroform and evaporated by pouring the solution on sterile glass tray kept at 4 °C and weighed (Singh and parmar, 2011).

Quantification of the percentage PHB accumulation was calculated by applying the following formula:

- PHB accumulation (%) = [Dry weight of extracted PHB (g/L) / Dry Cell weight (DCW) (g/L)] X 100
- Residual Biomass (g/L) = Dry Cell weight (g/L) – Dry weight of extracted PHB (g/L)

7. Molecular identification of bacterial strain

The strain which produce high quantity of PHB was subjected to 16S rRNA sequencing for identification. DNA of the bacterial strain was isolated and the fragment of 16S rRNA gene was amplified by using 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed was constructed using MEGA 11.

8. Estimation of cell growth and PHB production

100 ml of a production medium was prepared and inoculated with 1ml of inoculum containing (4×104 CFU / ml) of bacteria. Flasks were kept at 37°C for 48hrs and the OD of the media was checked for time interval of 4hrs at 600nm and the PHB was extracted and quantified.

9. Optimization of growth medium

The influence of physicochemical factors on PHB production of an identified strain was studied by using different carbon and nitrogen sources, pH, temperature (Fatimah Alshehrie 2019).

9.1. Effect of temperature

1 ml of inoculum containing (4×104 CFU / ml) of bacteria inoculated in a production medium and incubated at different temperature degrees such as (20 °C, 30 °C, 40 °C, and 50 °C) for two days. After growth the PHB was extracted and quantified.

9.2. Effect of pH

pH degrees were tested such as (5, 6, 7, 8, & 9) using 0.1N NaOH and 0.1N HCl. Flasks containing a production medium was inoculated with 1ml of inoculum containing (4×104 CFU/ ml) of bacteria and incubated at 37 °C for two days (Fatimah Alshehrie 2019). After growth the PHB was extracted and quantified.

9.3. Effect of carbon source

The different carbon sources like glucose, maltose, dextrose and sucrose in the concentration of 1% were added to the production media. The specified culture was inoculated into the media with pH 7 and incubated at 37 °C for optimized duration of 72 hours. The produced PHB was then extracted and quantified. From these recordings, the optimum carbon source was elucidated.

9.4. Effect of nitrogen source

The nitrogen sources include yeast extract, peptone, ammonium sulphate, and urea were added to the production medium in the concentration of 0.1 g/100 ml. The bacterial culture was inoculated into the medium with pH 7 and incubated at 37 °C for optimized duration of 72 hours. The PHB produced was then extracted from the medium and subjected to quantification & the optimum nitrogen source determined. (Mahitha S et al., 2023)

10. Result and discussion

10.1. Isolation of PHB Producing bacteria

Isolation of PHB Producing bacteria in the presence study from water sample were collected from Sankey tank, sadashivanagara Bengaluru. The area was based on places with higher probability of PHB producing bacteria due to the high level of water pollution with plastic waste.

A total of 25x10-5 CFU bacterial strains were isolated using nutrient agar media by using serial dilution technique. Out of 25 colonies 7 bacterial colonies was selected based on colony morphology which are tabulated (Table.1) for screening of PHB production. These 7 bacterial strains are referred as B1, B2, B3, B4, B5, B6 and B7 subjected to biochemical characterization (Table.2).

Table 1 Colony morphology of isolated colonies

Strains	Shape	Color	Margin	Elevation	Texture	Appearance	Optical property
B1	Circular	Yellow	Entire	Convex	Smooth	Shiny	Opaque
B2	Circular	White	Undulate	Raised	Smooth	Dull	Opaque
B3	Irregular	White	Entire	Raised	Rough	Dull	Opaque
B4	Irregular	Grayish white	Lobate	Flat	Rough	Dull	Opaque
B5	Circular	Grayish white	Undulate	Flat	Smooth	Dull	Opaque
B6	Circular	White	Entire	Convex	Smooth	Mucoid	Opaque
B7	Circular	Creamy white	Entire	Convex	Smooth	Mucoid	Opaque

Table 2 Biochemical characteristics of the isolated colonies

Strains	Gram staining	Endospore staining	Indole	Methyl red	Voges Proskauer	Citrate	Oxidase	Catalase
B1	+ve cocci in tetrads	-ve	-ve	-ve	-ve	-ve	+ve	+ve
B2	+ve rod	+ve	-ve	-ve	+ve	+ve	+ve	+ve
B3	+ve rod	+ve	-ve	-ve	-ve	-ve	-ve	+ve
B4	+ve rod	+ve	-ve	-ve	+ve	+ve	-ve	+ve
B5	-ve rod	-ve	+ve	+ve	-ve	-ve	-ve	+ve
B6	+ve coccobacilli	-ve	-ve	-ve	+ve	-ve	-ve	+ve
B7	+ve rod	+ve	+ve	+ve	+ve	-ve	+ve	-ve

Out of 7 stains, B1, B2, B4, B6 and B7 are suspected as PHB producing bacteria based on biochemical characterization. These 5 strains were made into pure culture by streak plate method on NA and subjected to screening of PHB production.

10.2. Screening for PHB producing Bacteria

The isolated bacterial colonies were examined for the accumulation of PHB the bacterial smears directly on petri dish using alcoholic solution of Sudan Black (0.3%) (fig.1).

Out of 5 strains 3 showed positive (B1, B2, & B4) results for Sudan Black staining. Which were examined once again with Sudan Black staining on the glass slide for microscopic investigation (fig.2a & 2b).

Figure 2a Screening of PHB producing bacteria by Sudan black staining in plate

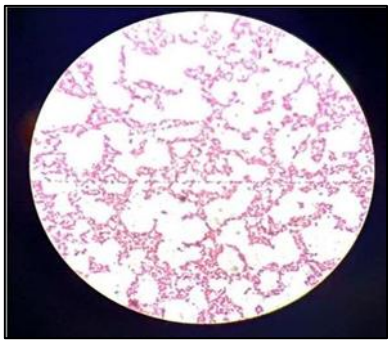

Figure 2b Screening of PHB producing bacteria by Sudan black stating on slide

10.3. Extraction and quantification of PHB

The positive resulted 3 bacterial strains were inoculated into production media and extracted by sodium hypochlorite method and weighed.

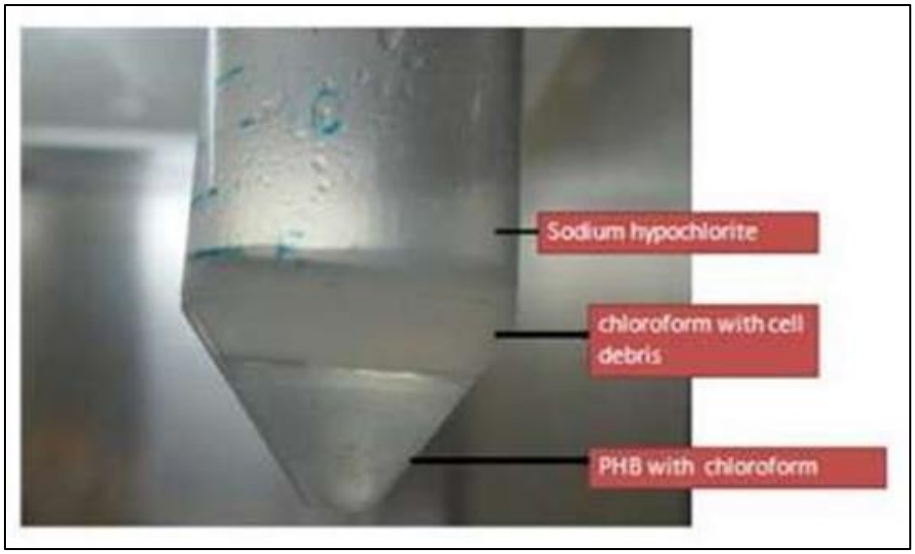

Figure 3 Three layers obtained during extraction of PHB by sodium hypochlorite method

PHB producing strain showed peculiar characteristics upon extraction by sodium hypochlorite. After cell lysis, sodium hypochlorite remains in the upper layer, cell debris are present in the middle phase and the PHB forms precipitate upon addition of chloroform (fig.3).

Quantification of PHB accumulation in percentage was calculated by using the formula and results were tabulated (table 3).

- PHB accumulation (%) = [Dry weight of extracted PHB (g/L) / Dry Cell weight (DCW) (g/L)] X 100
- Residual Biomass (g/L) = Dry Cell weight (g/L) – Dry weight of extracted PHB (g/L)

Table 3 Percentage of PHB accumulation by isolated strains

Bacterial strains	PHB accumulation in %	Residual biomass in g/l
B1	50	1.41
B2	30	0.90
B4	18	0.60

Out of 3 bacterial strains, the B1 strain showed maximum percentage of PHB accumulation which is 50%. Compare to other 2 bacterial strain (B2 &B4) which shows 30% and 18% respectively. So the B1 strain which has high PHB production capacity was subjected molecular characterization.

10.4. Molecular identification of high PHB producing bacteria:

Highest PHB producing bacterial strain (B1) which named as RG SAMPLE were identified based on the 16S rRNA sequence, it was amplified by PCR. The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W (table 4). Distance matrix (table 5) and phylogenetic tree (fig.4) was constructed was constructed using MEGA 11 and the strain was found to be Micrococcus luteus, showed high similarity based on nucleotide homology and phylogenetic analysis.

Table 4 Sequences producing significant alignment

Description	Max score	Total score	Query cover	E value	Per. Ident	Accession
Micrococcus luteus strain 19	2715	2715	100%	0.0	99.93%	OR285839.1
Micrococcus aloeverae strain OAct925	2715	2712	99%	0.0	99.93%	MG661749.1
Micrococcus sp. 3451	2695	2695	99%	0.0	99.93%	KP345947.1
Micrococcus luteus strain CGK112	2686	2686	98%	0.0	99.93%	OM920840.1
Micrococcus sp. Strain S1-V-1	2686	2686	98%	0.0	99.93%	OK326377.1
Micrococcus luteus strain MA3	2684	2684	98%	0.0	99.93%	MT072186.1
Micrococcus luteus strain HPB4	2684	2684	98%	0.0	99.93%	KX817342.1
Micrococcus sp. Strain Mcc28	2708	2708	99%	0.0	99.86%	OP279790.1
Micrococcus luteus strain LAA7	2708	2708	99%	0.0	99.86%	OM585514.1
Micrococcus luteus strain NMRL9	2708	2708	99%	0.0	99.86%	MW788668.1

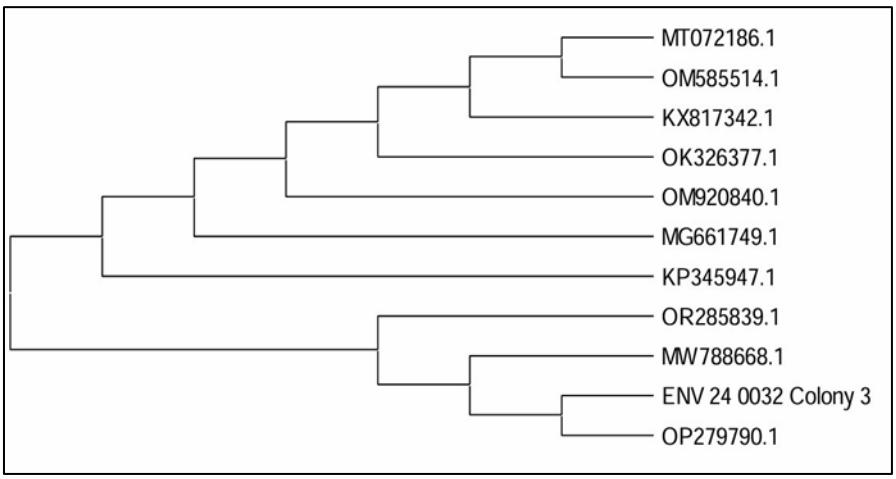

Figure 4 Phylogenetic tree

10.4.1. Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2051.63) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1473 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Table 5 Estimation of evolutionary divergence between sequence (distance matrix)

ENV-24_0032_Colony_3		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
OR285839.1	0.001		0.000	0.000	0.000	0.000	0.000	0.000	**0.001**	**0.001**	**0.001**
MG661749.1	0.001	0.000		0.000	0.000	0.000	0.000	0.000	**0.001**	**0.001**	**0.001**
KP345947.1	0.001	0.000	0.000		0.000	0.000	0.000	0.000	**0.001**	**0.001**	**0.001**
OM920840.1	0.001	0.000	0.000	0.000		0.000	0.000	0.000	**0.001**	0.000	**0.001**
OK326377.1	0.001	0.000	0.000	0.000	0.000		0.000	0.000	**0.001**	0.000	**0.001**
MT072186.1	0.001	0.000	0.000	0.000	0.000	0.000		0.000	**0.001**	0.000	**0.001**
KX817342.1	0.001	0.000	0.000	0.000	0.000	0.000	0.000		**0.001**	0.000	**0.001**
OP279790.1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		**0.001**	**0.001**
OM585514.1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		**0.001**
MW788668.1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal (table 5).

10.5. Estimation of cell growth and PHB production

Growth curve analysis of Micrococcus luteus by taking the OD of the media at 600nm along with the PHB quantity which was tabulated (table 6) and represented (fig.5 and fig.6).

- Lag phase: The first 4 to 8 hours, during incubation of cells, OD at 600nm remains stable indicating minimal growth as cells adapt to their surrounding environment.
- Log phase: These phase approximately extends 8 to 24 hours; which as rapid cell growth or cell division occurs the OD increases. It indicates exponential growth, So PHB accumulation is low as cells are primarily focused on biomass production.
- Stationary phase: After 24 hours, growth rate will be slow down due to nutrient depletion these phase is critical for the PHB production seen maximum accumulation observed between 24 to 36 hours of incubation.
- Death phase: Approximately 36 to 48 hrs, the OD of cells and PHB yield will be observed which is not increased or decreased, it indicates cell death.

Table 6 Growth curve of the isolated stain and PHB accumulation in gram

Time of incubation (hrs)	OD at 600nm	PHB accumulation (in g)
0	0.0	0.0
4	0.0	0.0
18	0.1	0.112
12	0.2	0.156
16	0.4	0.21
20	0.6	0.268
24	0.7	0.32
28	0.8	0.49
32	0.9	0.563
36	0.9	0.566

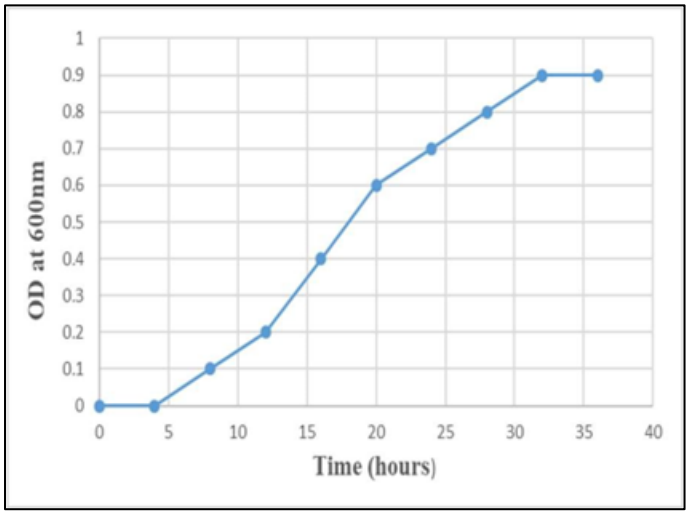

Figure 5 Graphical representation of growth curve of bacteria

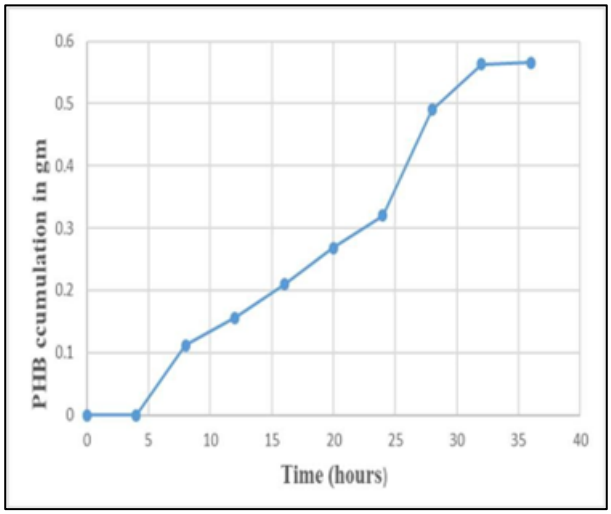

Figure 6 Graphical representation of PHB yield in gram

The optical density increases rapidly in log phase around 24 hrs, As PHB production is maximum at stationary phase around 24 to 36 hours which OD is stable as such it optimal conditions for PHB production. It seen that bacterial growth and PHB production over incubation time.

10.6. Optimization of growth media

The influence of physicochemical factors on PHB production of an identified strain was studied by using different carbon and nitrogen sources, pH, temperature.

10.6.1. Effect of Temperature

To determine the optimum temperature for PHB production, the media was incubated at different temperature 20, 30, 40 & 50 °C. The optimum temperature was 30°C, where the PHB yield and biomass production was seen as 4.38g/L & 9.23g/L respectively. As temperature increases to 40 & 50°C, the growth and the production was decreased drastically to 2.14g/L & 5.01g/L at 40°C and to 1.64g/L & 3.45g/L at 50°C, but as temperature decreases to 20°C no growth and production was seen which was represented (fig.7) respectively.

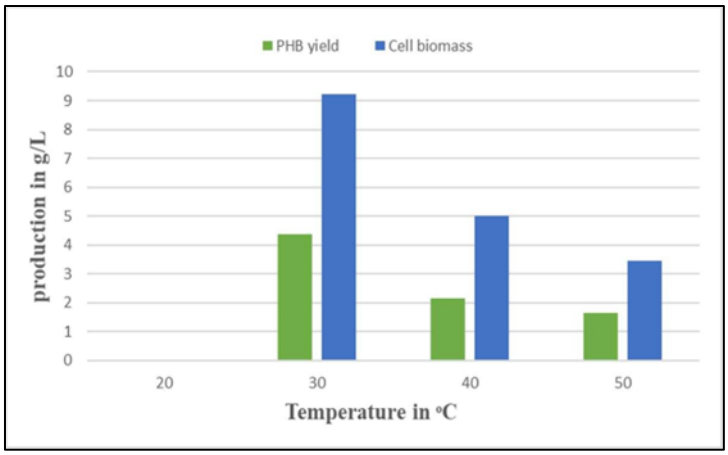

Figure 7 Graphical representation of effect of temperature on PHB and biomass production

10.6.2. Effect of pH

To determine the optimum pH for PHB production, the experiment was performed at different pH values (5, 6, 7, 8, & 9). The highest PHB yield and biomass production was achieved at pH 7 of 4.38g/L & 9.23g/L respectively. While the PHB production was dramatically reduced above and beyond the optimum pH which was represented (fig.8).

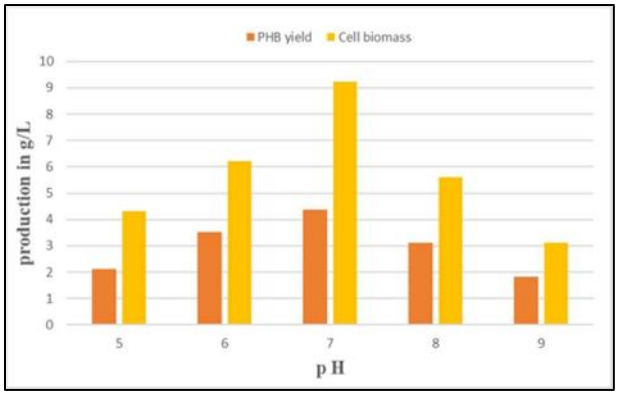

Figure 8 Graphical representation of effect of p H on PHB and biomass production

10.6.3. Effect of Carbon Sources

Carbon sources used during this study were dextrose, sucrose, maltose and glucose. After extraction, the PHB were lyophilized and dry weight was taken (fig.10). Out of the carbon sources used, in the presence of glucose a highest level of PHB production was observed (0.563g), whereas, a lower level of production was obtained with dextrose (0.04g). The details of the amount of PHB produced was represented (fig.9).

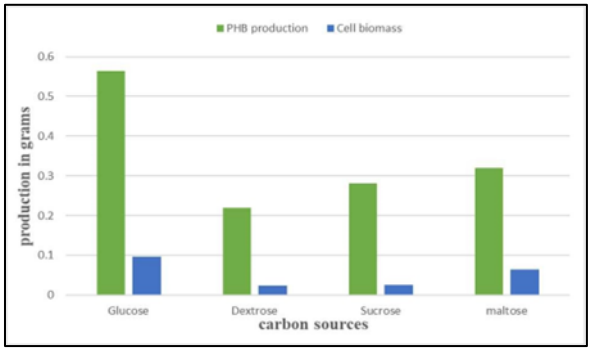

Figure 9 Graphical representation of effect of different carbon sources on PHB and biomass production

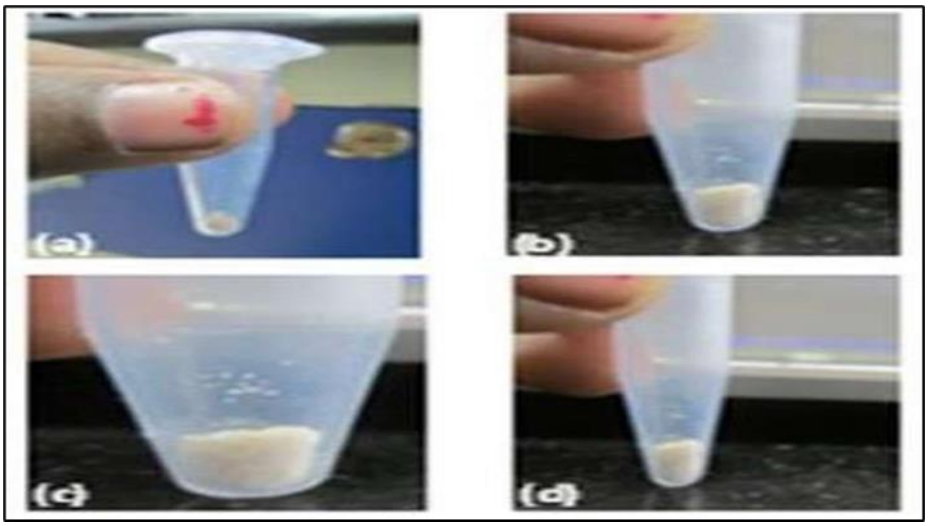

Figure 10 Production of PHB from different carbon source: (a) Dextrose: (b) Sucrose: (c) Glucose: (d)Maltose

10.6.4. Effect of nitrogen sources

Nitrogen sources used during this study were ammonium sulphate, peptone, urea, yeast extract. After extraction, the PHB were lyophilized and dry weight was taken. Out of the nitrogen sources used, in presence of ammonium sulphate

a higher level of PHB production was observed (0.52 g), whereas, a lower level of production was obtained with urea (0.02 g). The details of the amount of PHB produced was represented (fig.11).

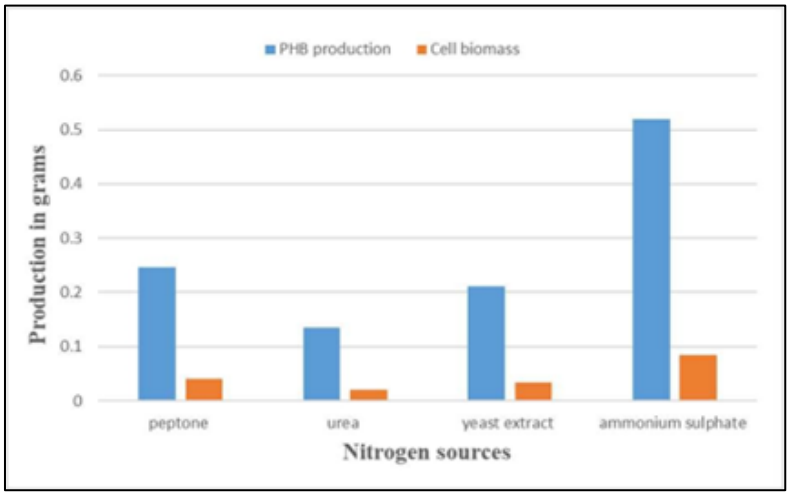

Figure 11 Graphical representation of effect of different nitrogen sources on PHB and biomass production

11. Applications of PHB in various fields

PHB is the most widely and popularly used alternative for non-biodegradable plastics such as polypropylene. PHB has properties such as tensile strength, tensile modulus, and melting temperature, similar to polypropylene. However, PHBs are biodegradable, while polypropylene doesn't have that property.

11.1. Medicine and Drug Delivery

The inflammation caused by macrophages exposes PHB to extracellular liquids and cells, resulting in the degradation of the polymer into monomers and oligomers of 3-hydroxybutyrate. This degradation property has made PHB a good candidate for the delivery of drugs. The PHB was incorporated with an inhibiting agent called ursolic acid against tumor proliferation, producing antitumor PHB nanoparticles. This is done to increase the activity, availability, and delivery of the ursolic acid in PHB nanoparticles against HeLa cells. This study also reveals that the ursolic acid release is more efficient at 96hr, and the number of dead cells was high at that time (Shishatskaya E I, et al., 2008).

11.2. Tissue Engineering:

An extracellular matrix of rabbit chondrocytes was grown on polyhydroxybutyrate-co- hydroxyhexanoate (PHB-co-PHH) scaffolds. Researchers also observed an increased production of collagen in PHB-co-PHH than in normal PHB. For wound dressing and ocular implants, PHB based composites were used, as well as scaffolds for bone implants. Other than these, artificial tissues of retinal, tendon, bone, cartilage, and muscle have been developed using PHB-based composites (B.M. Dakshinamoorthi, et al., 2024).

11.3. Food packaging

When the PHB was successfully evaluated for use in food packaging, it was shown to be less flexible and stiffer than polypropylene. While researching the impact of pasteurization on a pork salad packaged in a PLA or PHB bio-based container and a polyethylene or polypropylene container was examined. PHB films, the researchers discovered, can be utilized to package this kind of food. It has been shown that PHB may completely replace polypropylene in the packaging of fatty goods, such as cream cheese, margarine, and mayonnaise. Additionally, the researchers examined a variety of factors, including dimensional, mechanical, sensory, and physical data. PHB's nanoparticles contribute to the enhanced food packaging's qualities (Sonali Jha, et al., 2024).

11.4. Biosensor in cancer diagnosis

With the addition of acetic acid/chloroform/formaldehyde, the normal mammary epithelial cells (PCS-600-010) and metastatic breast cancer cells (T47D) were bound above the PHB sheet. According to the contact angle image, normal mammary epithelial cells did not show strong adhesion to the PHB sheets, however the breast cancer cells exhibited. The microscopic imaging is an excellent technique for visualizing malignancies. Because normal cells are neutral and

cancer cells emit positive/negative charge, thereby no attraction was visualized between normal cells and the PHB sheet. As a result, the research sheds new insight into the use of PHB sheets for the detection of cancer cells (R. Preethi Rathna, et al., 2023).

11.5. Automotive industry

The bio-based materials are attracting interest from the automotive industry. It has emerged as a novel engine concept using bio-based parts. Here, it is important to remember that industries are becoming more and more in need of biofuel. PHB may be treated to create biodegradable materials including film, fiber, and scratch resistant surfaces (Jhanani S, et al., 2011).

11.6. Agriculture

PHB can be used in the agricultural industry to protect the crops from the soil pests and for encapsulation of fertilizers. It could be done through a "biofeedback" mechanism for agricultural extension. In the biofeedback mechanism, an insecticide is included with PHB granules sown with the plants in autumn. Bacteria start to colonies the PHB and insecticide is released after an induction period of a week. During winters when the temperature of soil decreases, microbial activities also gets reduced causing less release of insecticide and huge wastage of chemicals.

11.7. Environmental applications

The use of normal non- biodegradable plastics has created several environmental issues like soil pollution, water pollution, etc. that have led to various harmful hazards. To handle this huge problem and reduce the use of harmful and regular synthetic plastic in an ecofriendly way to protect the environment, PHB (Poly- hydroxy-butyrate) a bio-degradable bioplastic has emerged. Due to their unique properties such as fast degradability in the natural environment, high melting temperature, high crystallinity, and absence of residues unlike synthetic polymers, they are best for biodegradable plastics (Bidisha Kumari, et al., 2023).

12. Conclusion

The amount of plastic waste increases every year and the exact time needed for its biodegradation is unknown. Ecological awareness has impelled the development of new biodegradable materials. Bio-plastics can be defined as plastics made of biomass such as corn and sugarcane. These substances have been increasingly highlighted as means for saving fossil fuels, reducing CO2 emission and plastic wastes. Biodegradability of bio plastics has been widely publicized in society and the demand for packaging is rapidly increasing among retailers and the food industry at large scale.

Using conventional plastics comes with a multitude of drawbacks: the large amount of energy that is required to produce the plastic, the waste that is a result of plastic production, and the use of materials that do not biodegrade readily. In order to shift the production of plastics towards a more sustainable path, research is being conducted to determine the types of renewable bioplastic resources that could be converted into plastic form.

Water samples were collected and bacterial isolate were isolated and screening test was done for the identification of PHB producing bacteria.16s RNA sequencing was done to identify the organism and its characteristics. PHB was extracted from different carbon sources (e.g. dextrose, lactose, fructose, maltose, galactose), where glucose produce more amount of PHB (0.562 g /100 ml culture). Similarly, PHB is extracted from different nitrogen sources (e.g. Ammonium sulphate, urea, peptone, yeast extract), where ammonium sulphate produce more amount of PHB (0.52g /100ml culture).

Compliance with ethical standards

Acknowledgment

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Disclosure of conflict of interest

The authors declared, there is no conflict of interest.

References

[1] Mohd Adnan, Arif Jamal Siddiqui, Syed Amir Ashraf, Mejdi Snoussi, Riadh Badraoui, Mousa Alreshidi, Abdelbaset Mohamed Elasbali, Waleed Abu Al-Soud, Salem Hussain Alharethi, Manojkumar Sachidanandan, Mitesh Patel, (2022). Polyhydroxybutyrate (PHB)-Based Biodegradable Polymer from Agromyces indicus: Enhanced Production, Characterization, and Optimization. Polymers (Basel)., 14(19):3982.

[2] Christina Thapa, Pallavi Shakya, Rabina Shrestha, Sushovita Pal, and 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., 6(1):62-68.

[3] Mahitha S, Sasi Premila J.M. and Abiya Chelliah D, (2023). Optimization of Culture Conditions for Polyhydroxybutyrate (PHB) Production in the Positive Bacterial Isolates of the Garden Soil. Biological Forum – An International Journal., 15(3): 192-198.

[4] Fatimah Alshehrei, (2019). Production of Polyhydroxybutyrate (PHB) by Bacteria Isolated from Soil of Saudi Arabia. J Pure Appl Microbiol., 13 (2): 897-904.

[5] Shishatskaya E I, Voinova O N, Goreva A V, Mogilnaya O A, Volova T G. Biocompatibility of polyhydroxybutyrate microspheres: In vitro and in vivo evaluation. Mat. Sci., 19; 2493–2502.

[6] Saharan BS, Sharma AS, (2012). Bioplastics-For sustainable development: A Review. Interntional Journal of Microbial Resource Technology 1:11-23.

[7] Jhanani, S., & Joseph, K. (2011). Used oil generation and management in the automotive industries. International journal of environmental sciences, 2(2); 638-648.

[8] Balakumaran Manickam Dakshinamoorthi, Uma Adaikalavan, Ananth Chinnarasu, Nithya Krishna and Swetha Jothiraman. (2024). Polyhydroxybutyrates: A Sustainable Alternative for Synthetic Polymers. Biosciences, Biotechnology Research Asia., 21(3); 851-862.

[9] Sonali Jha and Trupti Pandya. (2024). A review on Polyhydroxybutyrate (PHB): Production, Properties, Applications, and Environmental Implications. International Journal of Novel Research and Development., 9(4); 277-287.

[10] Neha Sharma. (2019). Polyhydroxybutyrate (PHB) Production by Bacteria and its Application as Biodegradable Plastic in Various Industries. Academic Journal of Polymer science., 2(3); 555586.

[11] R. Preethi Rathna and M. Kulandhaivel. (2023). Microbial Production of Biopolymer Polyhydroxybutyrate (PHB): Current Challenges and its Application. Asian Journal of Chemistry., 35(10); 2289-2300.

[12] Bidisha Kumari and Anisha. (2022). Polyhydroxybutyrate Production by various Substrates: Optimization and Application Biological Forum – An International Journal., 15(1): 463-474.

[13] P Singh and N Parmar. (2011). Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria. African Journal of Biotechnology., 10(24); 4907-4919.

[14] Anteneh Getachew and Fantahun Woldesenbet. (2016). Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. BMC Research Notes., 9:509.

[15] Hui Li, Lele Li, Yuan Chi, Qingwu Tian, Tingting Zhou, Chunhua Han, Yuanqi Zhu, and Yusun Zhou. (2020). Development of a standardized Gram stain procedure for bacteria and inflammatory cells using an automated staining instrument. MicrobiologyOpen., 9(9): 1.

[16] James G. Cappuccino, and Natalie Sherman. Microbiology: A Laboratory Manual. (10th edition)., Pearson Education, Inc., Lake Ave., Glenview, IL, 60025. pp. 171-205.