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TOWARDS SUSTAINABLE BIOPLASTICS: A COMPARATIVE ANALYSIS OF POLYHYDROXYBUTYRATE (PHB) PRODUCTION BY *ENTEROBACTER HORMAECHEI*

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ABSTRACT

The polyhydroxy -alkanoates (PHAs) are a group of thermoplastic polyesters that possess properties such as thermoplastic characteristics and water resistance similar to the conventional plastics obtained from petrochemical industries. Bacterial cells produce PHAs when essential nutrients such as nitrogen, phosphorous, iron, ammonium or sulphate are limited, and excess carbon is supplied. Due to its biodegradability and biocompatibility properties, PHB holds significant potential for use in various areas such as pharmacological industries, environmental, packaging, veterinary and industrial, agricultural, biomedical, molding products, paper coatings, adhesives, films, and performance additives. In the current study, 13 bacterial strains were isolated from dump yard soil and rhizosphere soil. Among these 3 bacterial strains showed maximum PHB production upon screening and were used for further studies. Out of three strains, *Enterobacter hormaechei*, produced the maximum PHB yield. The maximum PHB production was obtained after an incubation period of 72 hrs. Optimization of environmental and culture parameters were carried out wherein the maximum PHB production was observed at a pH 7.0, temperature 35°, with 1.25% inoculum.. Effect of growth media on the production of PHB was studied, Luria bertani media was found to be more suitable for the production PHB from *Enterobacter hormaechei* Maximum yield of 1.909% of PHB yield was obtained in the current study. The yield can be further enhanced by using agricultural wastes and industrial wastes as a source of carbon and nitrogen for large scale production.

KEYWORDS: Poly hydroxyl butyrate (PHB), *Enterobacter hormaechei*,Sudan black, PHB yield.

1. INTRODUCTION

Plastics have undoubtedly transformed our lifestyles by offering versatile and economic materials for vast array of applications. The versatile qualities of petrochemical based plastic have made them a crucial part of mankind. While plastics have become almost indispensable with widespread applications in various sectors, the unprecedented rhythm of using plastics derived from fossil fuels has led to unmanageable environmental issues. These issues are significantly more serious in the packaging industries. Unfortunately, typical commodity plastics degrade in environment at very slow rates. For instance, Polyethylene terephthalate (PET) has an expected lifespan of at least 100 years. The worldwide productions of plastics reached a staggering 390.7 million metric tons in 2021 (Saikumar *et al.,* 2023). Plastics have a molecular weight ranging from 10-50kDa and this excessive molecular size is one of the reasons for their resistance to chemicals and biodegradation. As a result, continuous research efforts are carried out for developing more sustainable and environmentally friendly plastics derived from renewable feedstocks, including agriculture source, microbial source and biomasses. In this context alternative biodegradable and environmentally sustainable biopolymers such as polyhydroxyalkanoate (PHA) and polyhydroxybutyrate (PHB) holds the potential to replace plastics linear use and dispose practices with a fully circular life-cycle for plastics. (Jansen, 2016).

Mechanically, PHB materials are typically stiff and brittle in nature with low thermal stability and a high degree of crystallinity. Many PHB plastics have properties that are similar to the petroleum polymers. PHB is by far the most intensively studied biopolymer from PHAs' family. (McAdam *et al.,* 2020).

Poly (3-hydroxybutyrate) (PHB) was the first isolated and characterized amongst PHAs. PHB is highly crystalline due to its linear chain structure, containing both amorphous and crystalline phases. It can be found as a polymer or as a part of copolymers and blends. It is

generated as a carbon reserve in a wide variety of production bacterial strains and is produced industrially through bacterial fermentation. PHB also has a number of advantages over synthetic polymers. PHB barrier permeability is superior to both polyethylene (PE) and polypropylene (PP) and they are also found to be more rigid and less flexible than PP. PHB exhibits good barrier properties in comparison to polyethylene terephthalate (PET). Another main characteristic of PHB material is its biodegradability, occurring within a reasonable timescale when the material is in contact with degrading microorganisms in biologically active environments such as fresh water, soil, and aerobic and anaerobic composting, designating them as eco-friendly alternative for synthetic polymers.

PHB is produced in the cells of microorganisms, as product of microbial secondary metabolism, usually in conditions when the cells are subjected to nutrient stress or are in an unfavourable condition such as carbon excessive with limited nutrients, this is possible in both Gram-positive and Gram-negative bacteria. The accumulation of the material is a natural technique used by microorganisms to store carbon and energy when essential nutrient supplies are imbalanced or are depleted. There are number of different species of bacteria which have been known to produce PHBs as intercellular granules. Examples of certain PHB producing strains include *Bacillus* spp., *Alcaligenes*spp., *Pseudomonas* spp., *Azotobacter.*

AIM OF THE STUDY

2. MATERIALS AND METHODS

2.1 Sample collection

Two soil samples were collected aseptically at a depth of 2cm from the dump yard of Yeshwanthpur Industrial Area (latitude: 13°01'22"N, longitude: 77°32'11"E) and front yard of Leprosy Rehabilitation and Training Centre (LRTC), Sumanahalli (latitude: 12°59'07.3"N, longitude: 77°31'08.2"E), Bangalore, India. Both the soil samples were taken to the laboratory in sterile zip locked transparent bags.

2.2 Isolation of Bacteria

Soil sample collected was serially diluted and the sample diluents were inoculated on to nutrient agar (Composition g/lt: Peptone – 5, Yeast Extract – 3, Sodium Chloride – 5– Agar 15, pH 7.0) by spread plate technique 10^{-5} and 10^{-6} diluents were selected for plating.. The inoculated plates were incubated at 37°C for 48 hours. The isolates obtained were streaked onto the nutrient agar slants. The slants were incubated at 37°C for 24 hours. Thereafter the isolates were stored at 4°C until further use. **(Biradar *et al.*, 2015)**

2.3 Screening of PHB producing bacteria (Burdon *et al.*, 1946)

The PHB production was detected by using lipophilic stain Sudan Black. Bacterial smears of isolates were heat fixed on clean grease free, glass slides, followed by staining with SudanBlack (0.3%, w/v). The slides were kept for 20 minutes without allowing the stain to dry, followed by counterstaining with safranin for 1 minute, the slides were allowed to dry and then examined with an oil immersion lens (Burdon *et al.* 1946). Cells appearing black when observed under the compound microscope were characterized as PHB Producer positive strains.

2.4 Morphological and Biochemical characterization of PHB positive isolates

Distinct morphological features of the isolates were recorded on the basis of size, shape and colour. Cellular morphology was studied under microscope using Gram staining and Endospore staining (Aragosa*et al.,* 2021). The biochemical characterization was done by a series of biochemical tests including Indole test , Methyl red (MR) test, Voges- Proskauer Test (VP Test), Citrate utilization, Nitrate reduction, H_2S production, Motility test, Carbohydrate fermentation [Glucose, Sucrose, Lactose and Mannitol], Oxidase and Catalase test. The morphological and physiological characteristics of the bacterial strains were compared with the data from Bergey's Manual of Determinative Bacteriology (Holt *et al.*1994).

2.5 Optimization of various environmental factors

2.5.1 Optimization of Incubation Period

50 ml production media (Composition g/lt: Peptone – 5, Yeast Extract – 3, Sodium Chloride – 5) with pH 7.0 was distributed in each Erlenmeyer conical flask and sterilized by autoclave at 121°C. The production media was 1 % inoculum of positive PHB producers were inoculated to sterile production media and incubated at 37°C for different period of time from 24hours to 72 hours. On Completion of desired incubation period, PHB yield was estimated according to Hahn *et al.*

2.5.2 Optimization of pH

Microorganisms have a minimum, an optimum and a maximum pH for growth. To standardize the optimum pH to produce PHB, 1% of PHB positive bacterial inoculum was inoculated in production media whose pH was varied from 4.0 to 8.0 and incubated at 37°C. After completion of the optimum incubation period PHB yield was evaluated as per Hahn *et al*. **(Nandish *et al.*, 2023).**

2.5.3 Optimization of Temperature

Microorganisms have different range of temperature suitable for their growth. To standardize the optimum temperature to produce PHB, 1% of PHB positive bacterial inoculum was inoculated in production media with optimum pH and incubated at different temperatures ranging from 25°C to 40°C with 5°C variation, later yield of PHB obtained in each variation was examined. Adnan *et al.*, 2023.

2.5.4 Optimization of Inoculum size

Inoculum size was varied in the study to evaluate the changes in PHB yield, sterilized production media with optimum pH was inoculated with PHB isolates, the

inoculum size was varied from 0.25 % to 1.25% with 0.25% difference and incubated at optimum temperature and optimum period of time. Gayathiri *et al*., 2018.

2.5.5 Optimization of Growth media

To improve the growth of the organisms and increase productivity of PHB study on optimization of growth media was conducted using efficient PHB producer. Growth media like Luria Bertani (LB) Broth media (Composition g/lt: Tryptone – 10, Yeast Extract – 5, Sodium Chloride – 10), Mineral Salts Medium (MSM) Broth (Composition g/lt: K_2HPO_4 – 01, KH_2PO_4 – 0.2, NaCl – 01, $CaCl_2$. $2H_20$ – 0.002, (NH_4) 2 SO4 – 1gm, $MgSO_47H_2O$ – 0.5, $CuSO_45H_2O$ - 0.001, $ZnSO_4$. $7H_2O$ – 0.001, $MnSO_4$. H_2O – 0.001, $FeSO_4$. $7H_20$ – 0.01) and Tryptic Soy Broth (TSB) (Composition g/lt: Tryptone: 17, Soytone – 3, Sodium Chloride – 5gm, Dipotassium phosphate - 2.5, Glucose – 2.5) was used in the study under optimum pH, temperature and incubation conditions.

2.5.6 Optimization of Salinity

Growth rate and productivity rate can be higher when bacteria are exposed to change in salinity, hence study on effect of salinity towards the efficient PHB producer was carried out. Growth media with salt concentration ranging from 1% to 4% was inoculated with the isolate and incubated under optimum conditions, later PHB yield was evaluated in each salt medium. (Thapa *etal.*, 2018).

2.5.7 Optimization of Carbon and Nitrogen source

Carbon Source such as glucose, mannitol, maltose, sucrose and nitrogen source such as peptone, yeast extract, urea and thiourea were used in the study with various concentration ranging from 1 %, 2% and 3%. The PHB yield produced by the efficient PHB producer was evaluated in each source according to Mahitha *etal.*, 2023.

2.6 Identification of PHB producing bacteria by 16S rRNA gene sequencing

The selected PHB producing isolate was identified based on 16S rRNA sequence (Kalaivani *et al.*, 2013). DNA isolation was done by CTAB protocol (Krishnan *et al.*, 2017) also called as hexadecyltrimethylammonium bromide method. 16S rRNA gene amplification was carried out using Taq polymerase. A forward primer (27F): 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer (1492R): 5'-GGTTACCTTGTTACGACTT-3' were used to amplify the gene. The PCR temperature cycling conditions were as follows:Initial denaturation 95°C for 2 minutes, Final denaturation 95°C for 30 seconds, Annealing 50°C for 30 seconds, Termination 60 °C for 4 minutes. The steps were repeated for 30 cycles. The amplification products were purified by adding EDTA and ethanol using multichannel pipette followed by vortex for 10 minutes at 2000 rpm and centrifugation at 3510 rpm for 30 minutes. Denaturation was done at 95°C for 5 minutes and the plate was placed in sequencer. The sequencing data analysis was done using BLAST server or servers related to specific databases. Quality of the obtained sequence can be observed through Electropherogram peaks.

2.7. Production and Extraction of PHB

Growth media suitable for the production of PHB at higher rate was used for the production under optimum conditions, on completion of the incubation period PHB was extracted according to Hahn *et al.*, 1994.

The bacterial culture was centrifuged at 5000 rpm for 15 minutes, supernatant was discarded carefully and the pellet was treated with 10 ml of 6% Sodium hypochlorite. The mixture was then incubated at 50°C for 1 hour and centrifuged at 5000 rpm for 10 minutes. Sodium hypochlorite solution (supernatant) was discarded and the pellet was washed with 2ml of distilled water followed by acetone and ethanol. The pellet was dissolved with 10-15ml of hot chloroform and filtered using a pre-weighed Whatman filter paper in a pre-weighed evaporating dish. The evaporating dish was placed on water bath for the evaporation, Weight of the evaporating dish after evaporation was noted and the percentage of PHB yield was calculated by using the below formula. Biradar *et al.*, 2015. Ratnaningrum*et al.*, 2020):

Percentage (%) of PHB produced

$$= \frac{\text{Final weight – Initial weight X 100}}{\text{Final Weight}}$$

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening

Thirteen morphologically distinct colonies were identified and sub-cultured from the collected soil samples. All the distinct colonies were screened to select PHB producing isolate by Sudan Black Staining. Among the 13 bacterial strains, 3 isolates namely, HA2, HA3 and HA 6 were found to be positive PHB producers which was confirmed by Sudan Black staining. (Figure 1).

3.2 Morphological and Biochemical characterization

Positive PHB producers HA2, HA3 and HA6 isolates were distinguished based on their morphological (Table 1) and biochemical characters, HA 2 and HA6 isolates were confirmed as gram positive bacteria and HA3 was confirmed as gram negative bacteria by gram's staining. Various biochemical tests were performed to identify the bacterial strain to genus level. The isolates HA 2 and HA 6 showed positive result for catalase test, MR test, citrate utilization test, nitrate reductase test, glucose fermentation, sucrose fermentation, mannitol fermentation, motility test, spore forming. The HA3 isolate showed positive results for catalase test, MR test, VP test, citrate utilization test, nitrate reductase test, glucose fermentation, sucrose fermentation, mannitol fermentation, motility test, non spore forming. Thus, from the performed biochemical tests it could be

concluded that the strains HA 2 and HA6 belonged to *Bacillus* sp., whereas the strain HA3 was identified as *Enterobacter* sp. (Table 2, Figure 2).

3.3 Optimization of various environmental factors

Environmental factors such as Incubation period, pH, temperature and inoculum size which play a vital role in the enhancement of productivity yield was primarily studied for all the three PHB producing isolates HA2, HA3 and HA6. From the study, optimum incubation period required for the production of PHB at higher rate was known to be 72 hours by all the three isolates, HA2 strain gave 0.0066% of PHB yield, HA3 strain showed 0.0705 % of PHB yield and HA3 strain showed 0.0310% of PHB yield. The optimum pH was found to be pH7.0 for HA 2, HA3 and HA 6 isolates with 0.0159, 0.0776, 0.0152% of PHB yield respectively and the optimum temperature was found to be 35°C for HA 2, HA3 and HA 6 isolates with 0.0170, 0.0794, 0.0133% of PHB yield respectively. Optimum inoculum size for HA2 and HA6 was 0.75% which resulted in 0.0160 % of PHB yield, whereas HA 3 isolate showed 0.0968 % of PHB yield with 1.25% inoculum size. (Table 3) From the study of these factors HA 3 isolate was better strain compared to other two strains. Further the potential strain capable of giving more productivity yield (HA3) was selected for the continued study, where the effect of growth media, Carbon, nitrogen source and salinity was focused on.

Effect of growth media on HA3 isolates was studied using nutrient broth medium (NB), Luria Bertani broth media (LB), Tryptic soy broth media (TSB) and Mineral salts medium (MSM). HA 3 isolate produced higher PHB yield with 0.0999 % of PHB yield when cultivated in LB media compared to the other media, Effect of carbon source was studied using four different carbon source such as glucose, sucrose, maltose, mannitol in 3 different concentration 1%, 2% & 3%. HA3 isolate showed maximum PHB yield when cultivated in medium supplemented with 1% sucrose having 0.0972 % of PHB yield. Effect of nitrogen source using four different nitrogen source such as yeast extract, urea, thiourea, peptone in 3 different concentration 1%, 2% & 3%. HA3 isolate showed maximum PHB yield when cultivated in medium supplemented with 1% yeast extract having 0.0997 % of PHB yield. Effect of salinity on PHB production by HA3 isolate was studied by supplementing the media with sodium chloride in various concentration ranging from 1% to 4% with 1% increment, HA3 isolate produced higher PHB when cultivated in 1% salt supplemented medium. Percentage of PHB yield was found to 0.0942. (Graph 1 to 8).

3.4 16s rRNA sequencing of potential PHB producer

16S rRNA gene sequencing was performed to identify the potential bacterial strain HA 6 to species level. The potential PHB producer bacterial strain was identified as *Enterobacter hormaechei* subsp. xiangfangensis from the 16s rRNA sequencing. (Figure3)

3.4 Production & Extraction

PHB was produced from HA3 isolate keeping the optimized environmental factors constant, later the produced PHB was extracted using sodium hypochlorite gravimetrically and the percentage of PHB yield was calculated. Production of PHB by HA3 *Enterobacter hormaechei* subsp. xiangfangensis using Luria Bertani broth media supplemented with 1% Sucrose, 1% yeast extract and 1% NaCl with pH7.0, inoculated with 1.25% inoculum and incubated at 35°C for 72 hours results in 1.909% of PHB yield. (Figure 4)

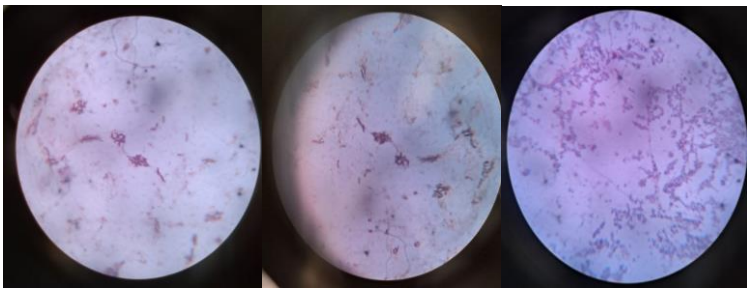
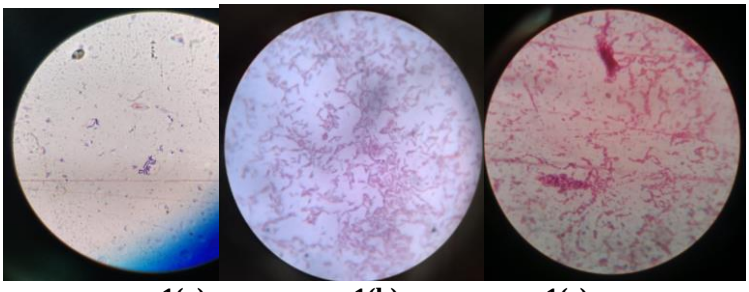


Figure 1: Sudan Black staining of HA2, HA6, and HA 3 respectively



1(a) 1(b) 1(c)

Figure 2: Gram staining of isolated colonies: 1(a): HA2: Gram-positive rods, 1(b): HA 6 Gram-positive rods, 1(c): HA3: Gram-negative rods.

Table 1: Morphological Characterization of positive PHB producing bacterial strains.

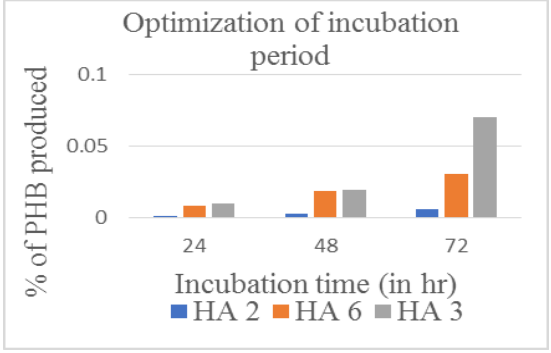
Isolate no.	Margin	Elevation	Opacity	Color	Surface of the colony	Gram staining	Morphology under microscope
HA 3	Irregular	Convex	Opaque	White	Smooth	Positive	Rods
HA 2	Smooth	Convex	Opaque	Off- white	Smooth	Negative	Rods
HA 6	Irregular	Convex	Opaque	White	Smooth	Positive	Rods

Table 2: Biochemical Characterization of positive PHB producing bacterial strains.

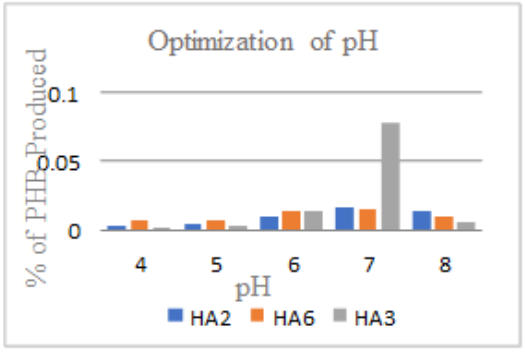
Biochemical test	HA2	HA3	HA6
Catalase Test	Positive	Positive	Positive
Oxidase Test	Negative	Negative	Positive
Indole Test	Negative	Negative	Positive
Methyl red Test	Positive	Positive	Positive
Voges Proskauer Test	Negative	Positive	Positive
Citrate Utilization Test	Positive	Positive	Positive
Nitrate reductase Test	Positive	Positive	Positive
Hydrogen sulphide Test	Positive	Negative	Positive
Carbohydrate Fermentation Glucose	Positive	Positive	Positive
Sucrose	Positive	Positive	Positive
Lactose	Negative	Negative	Positive
Mannitol	Positive	Positive	Positive
Endospore Staining	Positive	Negative	Positive
Motility Test	Motile	Motile	Motile

Table 3: Optimization of environmental parameters.

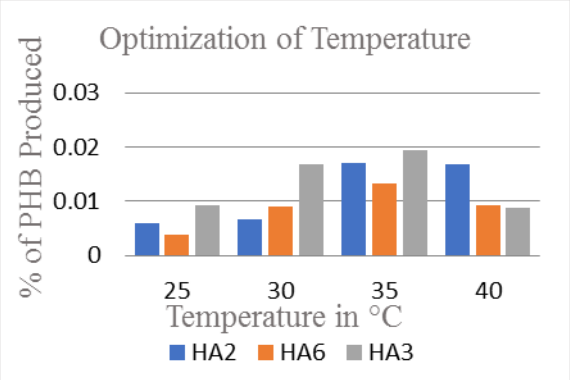
Optimization parameter		HA 2	HA 3	HA 6
		% of PHB yield	% of PHB yield	% of PHB yield
Incubation period (in hrs)	24	0.0017	0.0103	0.0089
	48	0.0033	0.0200	0.0186
	72	0.0066	**0.0705**	0.0310
pH	4.0	0.0035	0.0071	0.0017
	5.0	0.0042	0.0066	0.0033
	6.0	0.0093	0.0138	0.0133
	7.0	0.0159	**0.0776**	0.0152
	8.0	0.0137	0.0091	0.0062
Temperature (in °C)	25	0.0060	0.0093	0.0038
	30	0.0066	0.0167	0.0090
	35	0.017	**0.0794**	0.0133
	40	0.0167	0.0088	0.0093
Inoculum size (in %)	0.25	0.0031	0.0062	0.0042
	0.50	0.0066	0.0066	0.0066
	0.75	0.0167	0.0133	0.0167
	1.00	0.0060	0.0266	0.0124
	1.25	0.0017	**0.0968**	0.0066



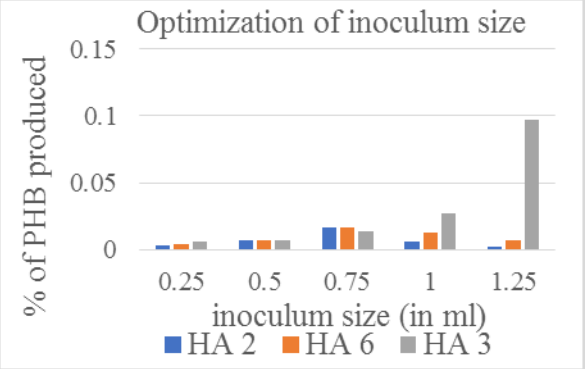
Graph 1



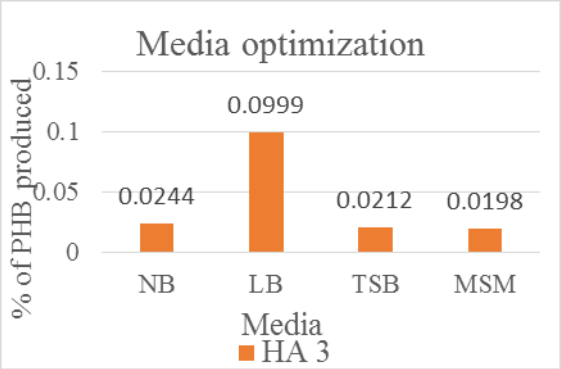
Graph 2



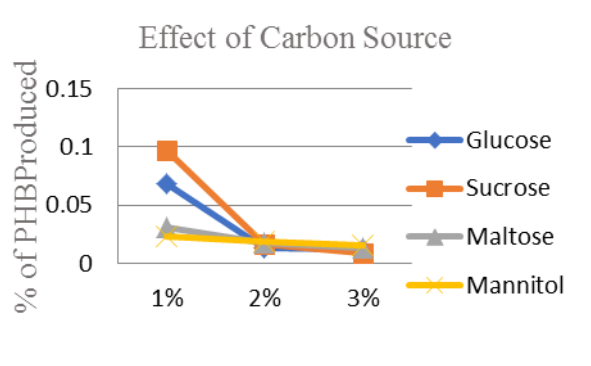
Graph 3



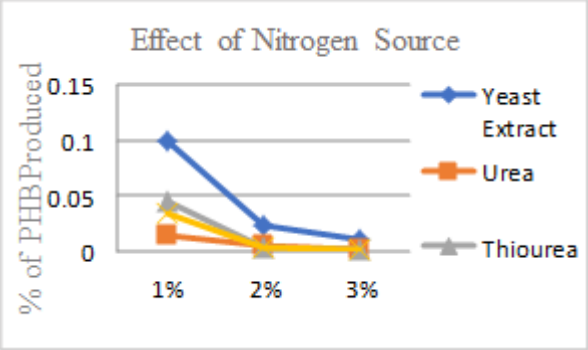
Graph 4



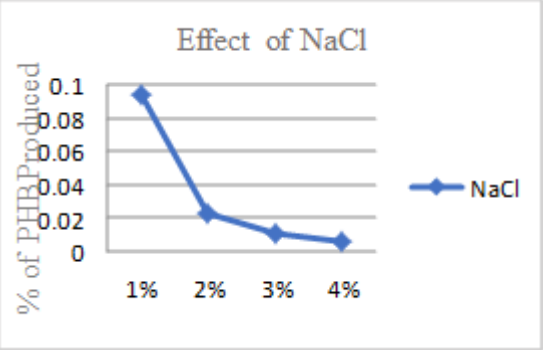
Graph 5



Graph 6



Graph 7



Graph 8

Graph 1 to 8: Graphical representation of Optimum environmental factors.

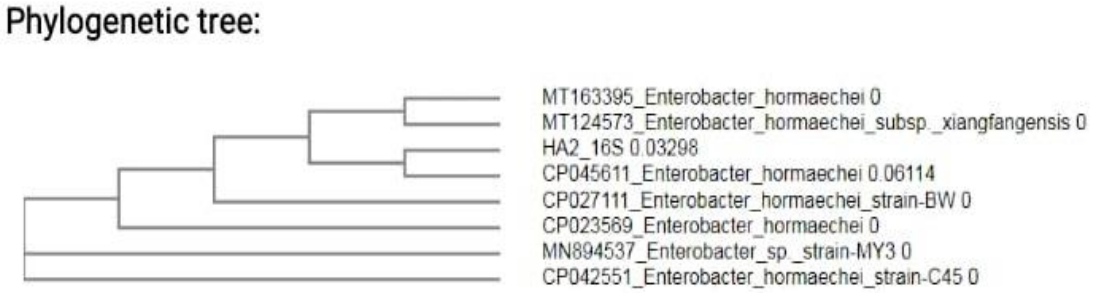


Figure 3: Phylogenetic tree of the identified bacterial strain *Enterobacter hormaechei*

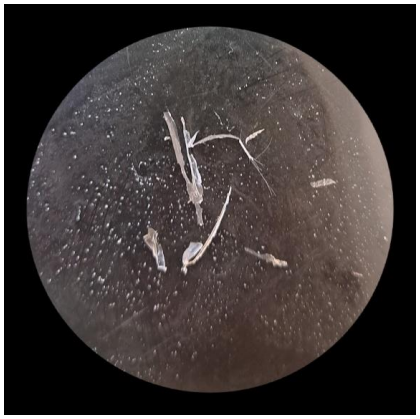
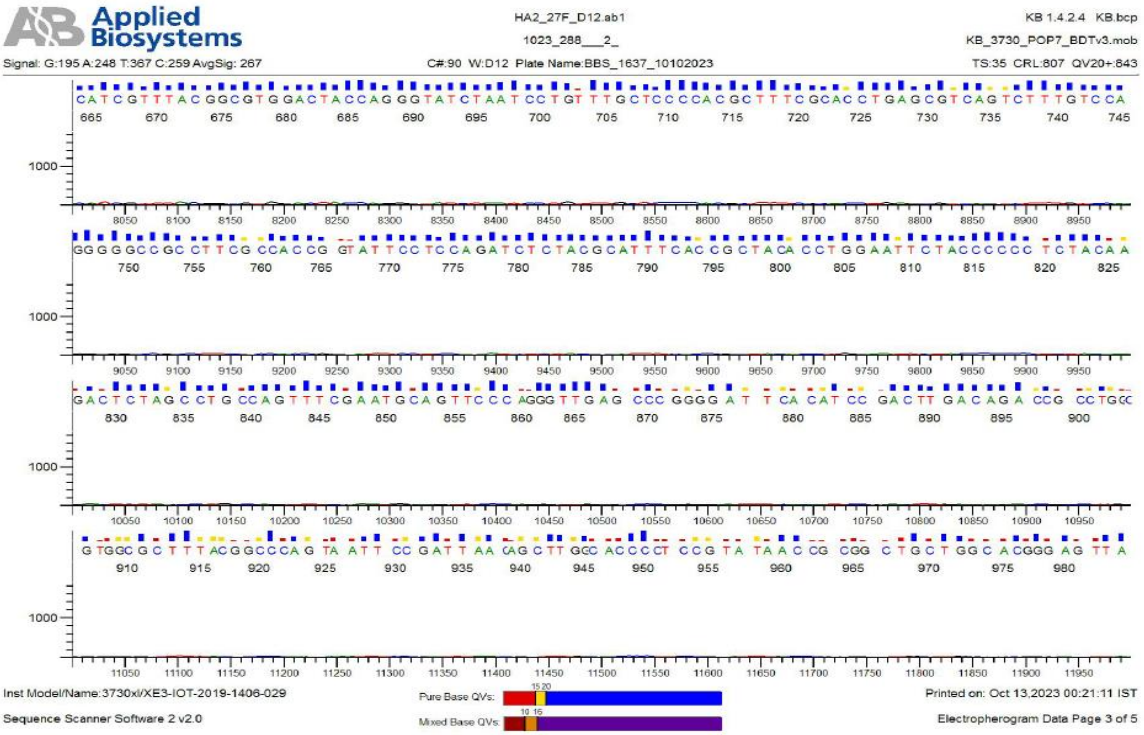


Figure 4: PHB extracted using sodium hypochlorite method.



4. CONCLUSION

Since there are very few papers have been reported on the production of PHB using *Enterobacter* sp. we assume that the bacterial species *Enterobacter hormaechei* could be a potential producer of PHB. Further studies need to be performed to increase PHB production and check for its credibility for commercialization.

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