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Production and characterization of Polyhydroxybutyrate using *Achromobacterkerstersii* KUMBNGBT-36 Isolated from dump yard soil Sample

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1. Abstract:
Plastic contamination is fuelling the severe environmental problems presently cause severe treats to humans, animal domain and the world. The recreation of renewable decomposable components started in the 1970s with the need for carbon neutral completely ecological goods driving significant improvement in the modern years. The present research work was aimed to isolate and characterize PHB producing bacterium from scrap yard soil sample collected from Savalanga, Davanagere District, Karnataka. The isolated bacterium was screened by viable staining techniques and solvent extraction method using chloroform. The bacterium was characterized using morphological and biochemical characters. The Achromobacterkerstersii KUMBNGBT-36 was confirmed by molecular characterization using 16s r-RNA sequence and deposited to GenBank, NCBI and assigned with the accession No. OK161008. The production of PHB was optimized using various physico-chemical characters. The cheaper substrates are used for large scale production of PHB in submerged fermentation. The extracted PHB from bacterial cells was quantified and confirmed using Bio-spectrophotometer to get the λ max at 235nm.

Keywords: Polyhydroxy butyrate, Achromobacterkerstersii, viable staining, optimization, agro-industrial wastes and Bio-spectrophotometer

2. Introduction

Plastic constituents have become an integral part of our daily life as a basic need but it causes serious complications as it is non-biodegradable in nature. These components remain as waste for several years in the nature.The mammoth accumulation of non-biodegradable plastics in landfills causes environmental pollution and it is one of the major problems of facing in the current century. Furthermore, to reduce the depletion of fossil fuels there is a need for the development of new alternative i.e., bio-derived or sustainable polymers(Naser *et al*., 2021). The bio based plastic is an idyllic alternative used to reduce the environmental and waste management problems. The Biopolymer is biodegradable in nature and it is produced by wide range microorganisms and these living organisms have the ability to convertbiopolymer into carbon dioxide and humus(Reshma *et al*., 2017). Synthetic polymers are xenobiotic in nature and are intractable to the microbial degradation. The use of petroleum derived plastics causes many environmental problems due this damage caused by synthetic polymer leads to the development of a suitable eco-friendly biodegradable products that can substitute at least some of the commodity of the petroleum derived plastics. The biodegradable plastics are serves as an alternative to reduce the environmental problems caused by profitable plastics(Lopez-Cuellar *et al*., 2011).

Bio-based polymers developed using renewable agricultural or feedstock's showed similar properties of conventional plastics and after disposal the degradation occurs through microbial processes. These bio plastics includesstarch-based plastics, protein-based plastics and celluloseblended plastics. Bio-based plasticsareintermingled with conventional plastics such as polyethylene (PE), polypropylene (PP), and polyvinyl alcohol. The bio-based plastics developed using mixture of conventional plastics are partially biodegradablein the environment (Irsath*et al*., 2015).

Production and commercialization of PHB is still economically inadequatebecause the production cost is very high compared to conventional plastics.The main reason for the increase in the production cost of PHB was utilization of cost expensive products such as pure carbon substrates as a glucose supplements.50 to 60% of the production cost was utilized for these substrates (Khosravi-Darani*et al*. 2013; Kourmentza*et al*. 2015). Characteristic PHB production methods use carbon sources that may contest with food production (Brodin 2017).A less luxurioussubstrates of carbon and high nutrients is required to decrease the production cost of PHB biopolymers. The significant decreases in the production cost were acquired by utilizing the low-cost agricultural waste deposits such as oil wastes, dairy wastes, date seeds, grain crops, bagasse's, starch, rice brans and damaged food grains could significantly reduce the production cost of biopolymers.

In the present study, PHB producing prominent bacteria was isolated from dump yard soil sample collected from Savalanga, Davanagere District, Karnataka.The obtained bacterium was screened by viable staining techniques and secondary screening was performed by solvent extraction method. The bacterium was identified by morphological and biochemical characters. The stain was confirmed by 16s rRNA sequencing method and phylogenetic analysis was performed using neighbour joining method. The PHB production was optimized by using various physico-chemical characters and the low-cost agricultural substrates are used for the large-scale production of PHB in submerged fermentation. The extracted PHB was quantified and confirmed by using Bio-spectrophotometer to get the λ max at 235nm

3. Materials and Methods

3.1. Collection of sample

The scrapyard soil sample was selected for the isolation of PHB producing bacterium. The soil sample was collected from Savalanga, Davanagere District, Karnataka. The sample was stored at 4ºC using polythene bags for furtheranalysis.

3.2. Isolation of PHB producing bacterium

Onegram of soil sample was weighed and serially diluted up to 10^{-9} dilutions and 10^{-6} to 10^{-8} dilutions were selected for the isolation.Afterwards the diluted samplewasplated on nutrient agar mediaand incubated at 37°C for 24hrs. After incubation the colonies with different morphologicalcharacters were selected and maintained as a pure culture on nutrient agar slants and stored at 4°C for further use [Indira Mikkili*et al*.,2014].

3.3. Qualitative and quantitative screening of PHB producing bacterial strain:

The isolated bacterial strainwasqualitatively analysed by using differential staining and the viability of the stain was identified by Sudan B black staining tequniques.

The amount of PHB present in the selected isolate was determined by quantitative estimation. The selected isolatewascultured on nutrient broth (pH 7) and incubated at 37°C for 96hrs and after incubation the samplewasretrieved for every 24hrs up to 4 days (24-96hrs) to evaluate the PHB production (µg/ml) by solvent extraction method using chloroform as a solvent. The amount PHB biomass was calculated by using the following formula [Murray *et al*., 1994],

$$\text{Yield of PHB } (gL^{-1}) = \frac{W2 - W1}{V}$$

W_1 - Weight of empty petri plate,

W_2 - Weight of dried biomass on the petri plate and

V - Volume of culture broth in litre.

3.4. Morphological, biochemical and molecular identification of PHB producing bacterium:

The PHB producing isolate was identified based on morphological, biochemical and molecular characters. The colony characters were identified by observing shape, size, texture and color of the stainand microscopic charactersof the isolate was identified by using simple and endospore staining technique. Biochemical characters of the strainwasperformed using starch hydrolysis, gelatin hydrolysis, casein hydrolysis, citrate utilization, nitrate reduction, urease, methyl red, vogesproskauer, indole, malonate utilization, H_2S production, KOH, β-galactosidase, lecithinase, lipase, catalase, oxidase and triple iron agar tests (Johri *et al*., 2021) were performed according to the method of Bergey's Manual of Determinative Bacteriology. The genomic DNA of the strain was extracted and

analysed by using 16S rRNA gene sequence by polymerase chain reaction (PCR). The selected sequence of the 16S rRNA gene was associated with related sequences selected from National Centre for Biotechnology Information GenBank (NCBI GenBank) database using Basic Local Alignment Search Tool (BLAST) and the sequence was aligned and submitted to GenBank NCBI and the phylogenetic tree was constructed using neighbour-joining tree method with the bootstrap value 100[MacidNurbas*et al*., 2004].

3.5. Optimization of various culturalparameters for maximum PHB accumulation:

3.5.1. Effect of different broth medium at different incubation periods

Five different broths such as Nutrient broth (NA), Tryptone soya broth (TSB), Minimal salt broth (MSB), Minimal broth (MB) and Luria bertani broths (LB) were selected and the isolate was inoculated into the 100mL of broth medium and incubated at37°C fordifferent time periods ranging from 24 to 96 hrs in optimized conditions. At each time interval (24, 48, 72 and 96hrs), the stainwasscrutinised for growth and accumulation of PHB and yield was recorded [Priyanka lathwal*et al*., 2015].

3.5.2. Effect of Different temperature and pH on PHB production

The pure strain prepared by inoculating the single colony on nutrient agar media was used as an inoculum for the production of PHB. The production of PHB was analysed in 100mL of nutrient broth media at 37°C for 24hrs. To enhance the growth, culture broth was incubated at different temperature viz., 25°C, 30°C, 35°C, 40°C, and 45°C and the pHof the medium was adjusted to 3, 5, 7, 9 and 11to check the acidic and basic nature of the strain (Nisha *et al.,* 2009).

3.5.3. Effect of different carbon source and nitrogen source on PHB production

The media containing PHB strain was amended with different carbon sources such as glucose, fructose, sucrose, maltose and lactose at 2% concentration and different nitrogen sources such as ammonium chloride, peptone, sodium nitrate, urea and yeast extract at 1% concentration was analysedand the PHB accumulation was recorded (Kumar *et al*., 2004).

3.5.4. Effect of different Carbon to Nitrogen ratio (C/N ratio)

To check the different Carbon to Nitrogen ratio (C/N ratio), the media was amended with different carbon sources (2%) such as glucose, fructose, sucrose, maltose and lactose and nitrogen (1%) supplements such as ammonium chloride, peptone, sodium nitrate, urea and yeast extract at different concentrations were used viz., 1:1, 2:1, 4:1, 8:1 and 16:1 ratios were used for the maximum accumulation of PHB [Chandani *et al*., 2018].

3.6. Mass production and purification of PHB using cheaper substrates:

Different cheaper substrates obtained from the agricultural wastes areused for the maximum production of PHB by using glucose component present in the substrates.Different oil wastes such as feeds, cotton cake, groundnut cake, coconut cake, castor cake, sugarcane bagasse (SCB), rice bran and areca nut husk were analyse for selecting the best substratefor PHB production. The by-products were made into liquid hydrolysate using gelatine and obtained liquid hydrolysate was adjusted to pH 7.0 using NaOH.The cheaper substrate media was sterilized at 121˚C for 15 min and stored at 4°C for further analysis[Nisha *et al*., 2009].

3.7. Estimation of PHB by spectrophotometric method:

The fine PHB powder was taken in test tube and hot chloroform was used to dissolve the compound. After incubation the chloroform get evaporated and 10ml of concentrated sulphuric acid was added to convert the polymer present in the compound into crotonic acid and cooled. The absorbance was measured at 235nm using Con. H_2SO_4 as a blank using Bio-spectrophotometer[Kshamalakshman*et al*., 2004].

4. Results and Discussion

4.1. Collection of sample and isolation of PHB producing bacteria

In this study, Bacterial strains were isolated from dump yard soil samples collected from Savalanga, Davanagere District, Karnataka and the samples were diluted using serial dilution technique and sample was diluted from 10^{-1} to 10^{-8}dilutions. Afterwards the samples were plated on nutrient agar

media and incubated at 37°C for 24 to 48hrs. After incubation bacterial colonies which exhibits different characteristics nature was selected and subculture on nutrient agar media and used for further studies (Figure 1 a&b).

4.2. Qualitative and quantitative screening of PHB producing bacterial strain:

The selected bacterial strainwas characterised using gram staining and screened by Sudan black staining method. After screening the isolate showed positive for both staining methods was selected for the production of PHB (Figure 1c&d).

The pure culture was prepared using positive isolateselected by screening was grown on nutrient broth medium and the chloroform was used as a solvent to know the amount of PHB produced by the isolate using extraction method. After extraction the strain showed maximum amount of PHB i.e., 0.983±0.005g/L after fourth dayincubation (Figure 2). These findings were previously reported by of Monika Sharma *et al*., (2013).

4.3. Morphological and biochemical characterization of PHB producing bacterium

The morphological and biochemical properties of the isolate was determined according to "Bergey's Manual of Systematic Bacteriology" [Kreig*et al*., 1984]. The isolate shows negative for grams' reaction, rod-shaped, non-motile, aerobic, and non-spore forming bacterium and the colony shape of the isolate was circular, white color colony, 1-1.5 mm colony size and have smooth, raised, opaque and sticky surface. Biochemical characters were examined and the isolate was positive for citrate, nitrate, urease, methyl red, indole, melonate, lipase and citrateutilization and it was negative to starch, gelatine, casein hydrolysis, H_2S, KOH, lecithinase, galactosidase and oxidase test. The isolate havethe ability to ferment various sugars such as dextrose, sucrose, lactose and carbohydrate whereas the hydrogen and carbon dioxide were not fermented by the isolate. The morphological and biochemical characters are shown in Table 1 and 2. From these results, the selected bacterial isolate belongs to genus *Achromobacter*according to Bergey's manual of determinative bacteriology [Holt *et al*. 1994] and these findings are compared with the earlier results of Hassan *et al*., (2013).

4.4. Molecular characterization using 16s rRNA gene sequence and phylogenetic analysis

The PHB producing isolate was confirmed by 16S rRNA gene sequence analysis. The sequence of 16S rRNA gene of the isolate was amplified using purified genomic DNA and sequenced. The gene sequence was aligned using pair-wise alignment and it shows highest (100%) similarity with *Achromobacterkerstersii*strain **KUMBNGBT-33**, the nucleotide sequence was deposited to NCBI database and the sequence was allocated with accession no: **MW056185**. The analysis was performed using phylogenetic tree using similar sequence selected from the database by neighbour-joining method is shown in Figure 3 [Benson *et al*., 2014].However, these results were compared with previous findings of Irsath*et al*., (2015).

4.5. Optimization of various cultural parameters for maximum PHB production:

4.5.1. Effect of different broth medium at different incubation periods

The culture was cultivated in different broths by inoculating the pure colony and incubating at different time periods (24hrs, 48hrs, 72hrs and 96hrs). The nutrient broth showed maximum yield i.e., 6.313±0.21 at 72hrs (Figure 4a, b, c and d). After optimum temperature attained the growth decrease was observed. According to Monika Sharma *et al*., 2015, 72hrs is suitable for optimum PHB production.

4.5.2. Effects of different temperature on PHB accumulation

The growth and production of the PHB produced by the isolate shows maximum yield at 37°C after 72hrs of incubation period. Increase in the temperature above 37°C the PHB production will decreases (Figure 5a&b)[Gulab *et al*., 2013]. This result slightly related to earlier reports Hamieh *et al*., 2013.

4.5.3. Effects of pH on PHB accumulation

Figure 5c&d shows that maximum PHB production at pH 7 producing 6.133±0.05g/L yield and increase or decrease in pH, the PHB yield was decreases because the enzymes present in the cells will breakdown into polymeric compounds due to enzymatic action. These results were previously reported by Sindhu *et al*., (2011).

4.5.4. Effects of different carbon source on PHB accumulation

In Figure 6a&b, the glucose as a carbon source shows maximum production of PHB compared to other carbon sources used for the production and it produces 12.033±0.05g/L PHB. Maltose and lactose are also used as acarbon sources for growth and PHB accumulation after glucose [Borah *et al*., 2002]. Similar results were reported by Gavin *et al*., 1992.

4.5.5. Effects of different nitrogen source on PHB accumulation

The nitrogen source i.e., Ammonium chloride showed maximum PHB production followed compared to other nitrogen supplements(Figure 5c&d). Similar results were previouslyreported and from there study, yeast extract produces maximum PHB [Borah *et al*., 2002]. Therefore, the different organismsexploiting different nitrogen supplements are used to produce PHB very efficiently.

4.5.6. Effect of different carbon to nitrogen source

The maximum production of PHB was found at 8:1. The utilization of low or higher carbon to nitrogen ratio will affect the PHBgrowth (Figure 7). The C:N ratio increase up to 16:1and after that decrease in the growth was observed. Similar findings were previously reported by Monika Sharma *et al*., (2013).

4.6. Mass production and purification of PHB using cheaper substrates:

The production and commercialization of bio plasticis very expensive due to the usage of cost expensive products. To reduce the production coat cheaper substrates obtained from the agricultural wastes are acts as a carbon source for PHB production. In several reports, the different bacteria can use various carbon source was reported. Feeds, cotton cake, groundnut cake, coconut cake, castor cake, sugarcane bagasse (SCB), rice bran and areca nut husks were used as a cheaper substratesfor PHB production. Maximum PHB was produced by feed stock compared to other substrates and the agriculture wastesi.e., rice bran showed maximum PHB production compared to other substrates (Figure8a&b). These findings were previously reported by Van-Thuoc*et al*., (2007).

4.7. Estimation of PHB by spectrophotometric method:

The amount of PHB produced by the isolate was estimated using hot chloroform and con H_2SO_4. The acidic nature of the compound produces a crotonic acid and the isolate showed 0.319nm of absorbance measured at 235nm against con H_2SO_4 as a blank using Bio-spectrophotometer(Figure9). These findings were compared with earlier results of (Reshma *et al*., 2017).

5. Conclusion:

A bacterium responsible for the production of PHB was isolated from dump yard sample collected from Savalanga, Shivamogga district and screened by using differential and viable staining techniques. PHB producing potent bacterium was selected and identified by morphological and biochemical characters. The strain was confirmed as *Achromobacterkerstersii*by molecular identification. The growth conditions were optimized by using various parameters such as media, incubation time, temperature, pH, different carbon source, different nitrogen source and different carbon to nitrogen ratio. By utilizing these conditions, the bacterium will produce good growth and production of PHB. CHEAPER substrates obtained from the agricultural derivatives are used to maximise the PHB production. Usage of these low-cost product will reduce the production cost. The λ max was measuredusing spectrophotometric analysis. By this study the cheaper substrates used for the PHB production will reduce cost and can be easily commercialized and used in the agricultural and medical fields.

AUTHORS' CONTRIBUTIONS

NG: Conceptualization, data collection, investigation, writing-original draft and editing. AS: Data rectification, investigation,review and editing. MD: Data editing and phylogenetic analysis. S-HV: Data correction, review and editing. BT: Design and correction, supervision, review & editing.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

This is not applicable. This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

No additional data is available for this paper.

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LIST OF FIGURES

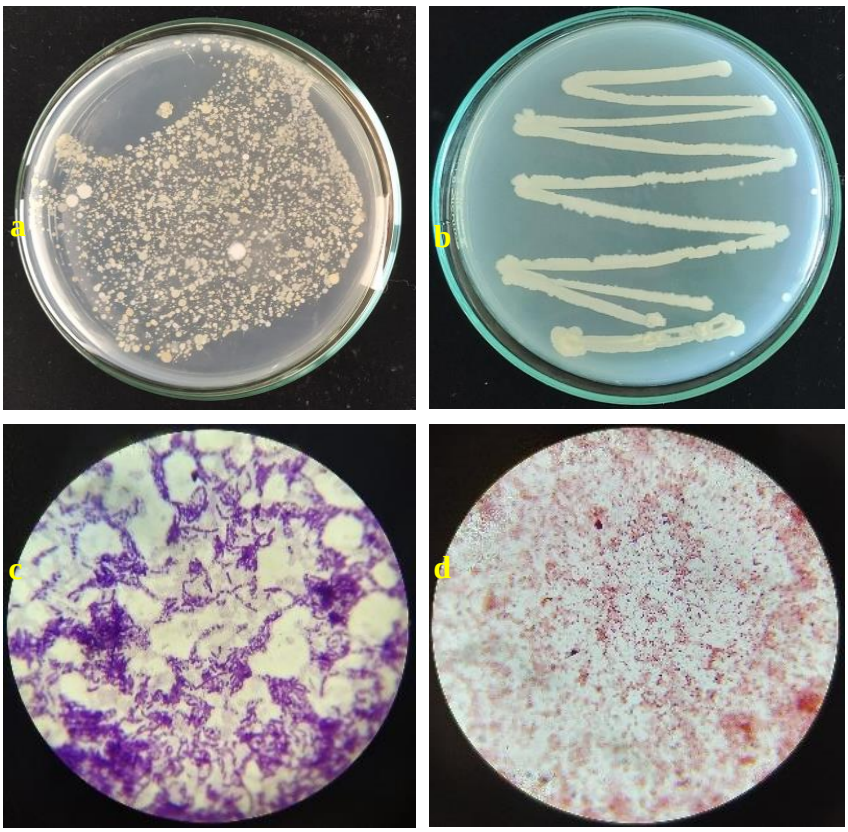

Figure 1: Isolation and screening of bacterial isolate using Gram staining and Suda Bblack staining: 1(a and
b) pure colonies isolated strain: 1(c) shows positive for Grams' reaction and 1(d) shows Sudan B black positive and black colour colonies were observed.

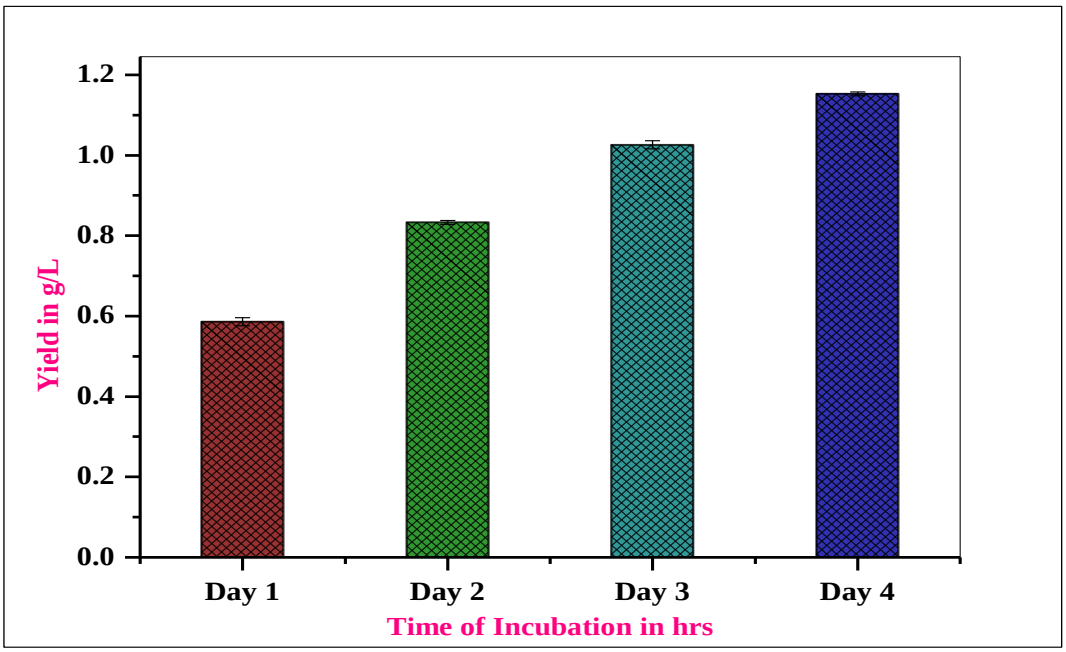

Figure 2: Quantitative estimation of PHB producing bacterial isolate using extraction method.

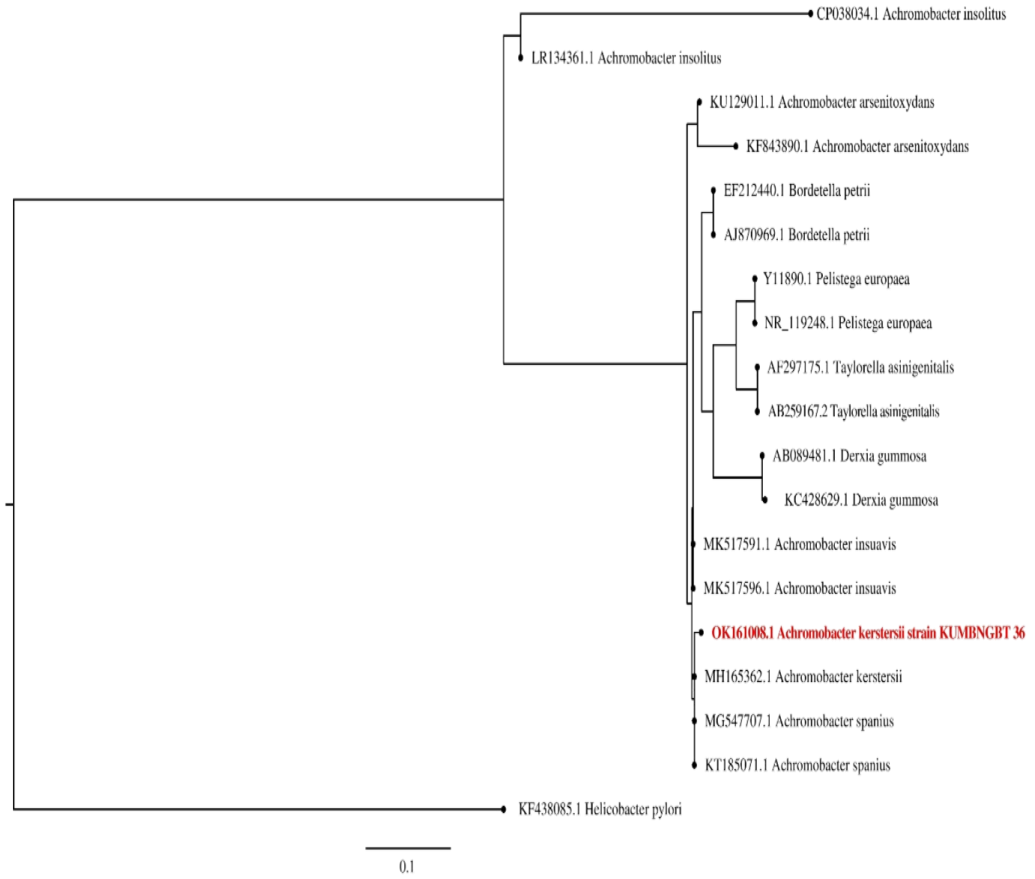

Figure 3: Phylogenetic analysis of *AchromobacterkerstersiiKUMBNGBT*-33 using neighbour joining method at boot strap value 100.

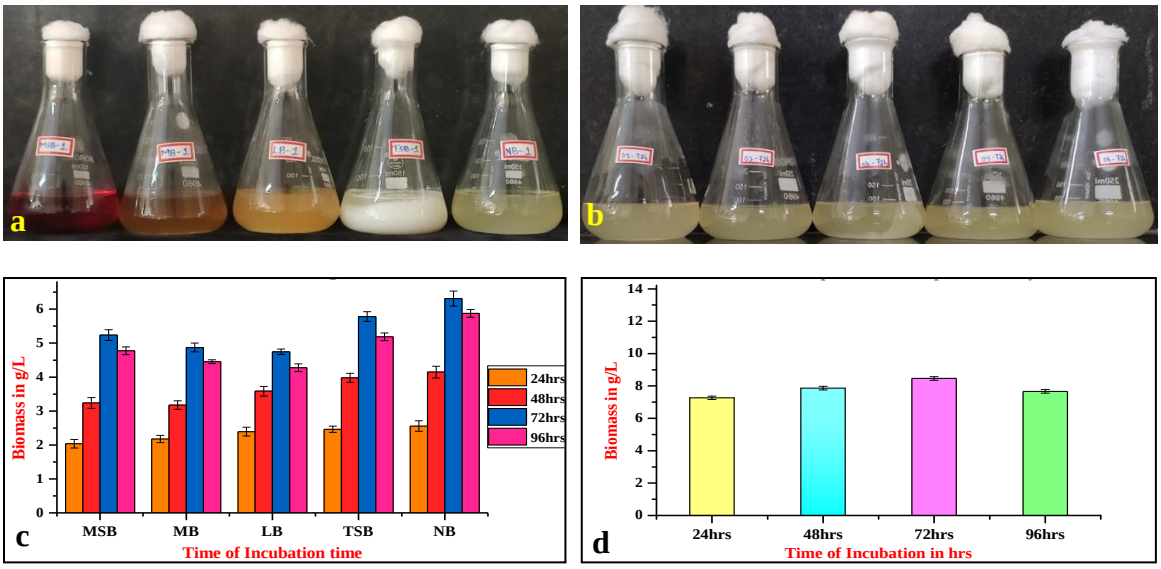

Figure 4: Optimization of isolated Bacterium by using different broth medium at different incubation time. In
figure 4 (a&b) nutrient broth exhibited maximum growth compared to other broths and in figure 4 (c&d) shows 72hrs is good incubation period for maximum production of PHB.

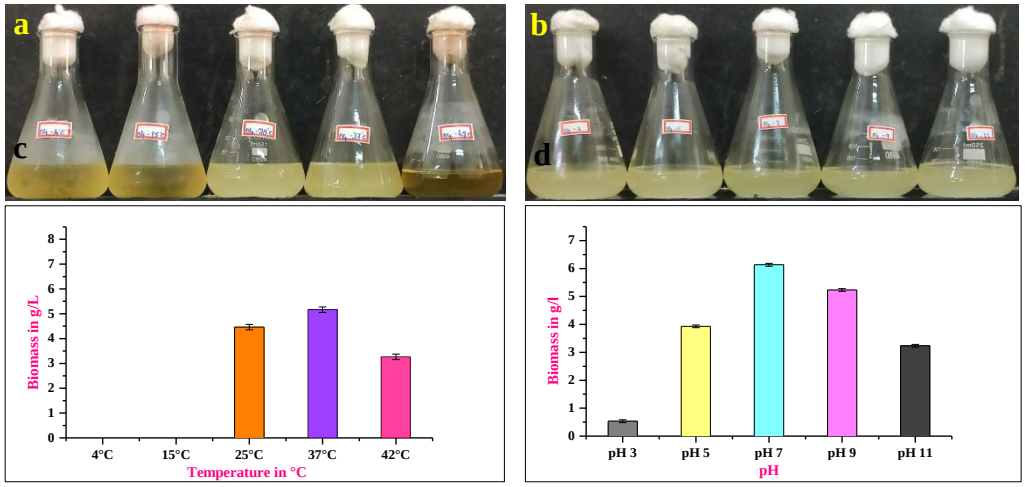

Figure 5. Production of maximum PHB on different temperature and pH; in figure 5(a&b) 37˚C is suitable for PHB production and in figure 5(c&d) maximum PHB was produced at pH 7.

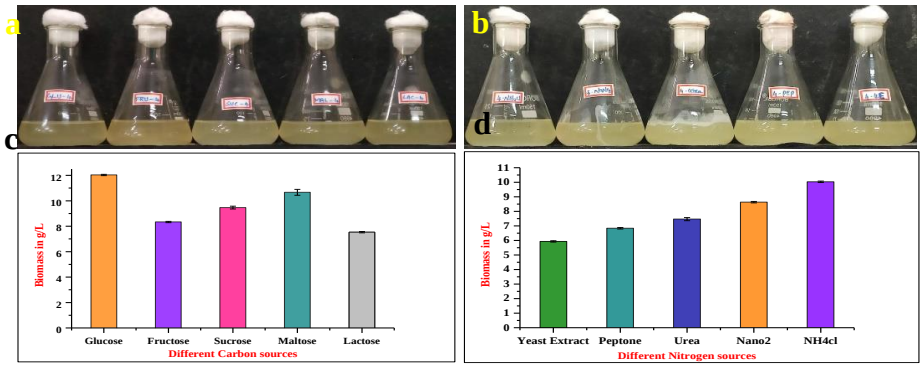

Figure 6: In figure 6 (a&b) the carbon source i.e., glucose showed maximum yield for PHB production and
figure 6 (c&d) shows PHB production was observed in ammonium chloride.

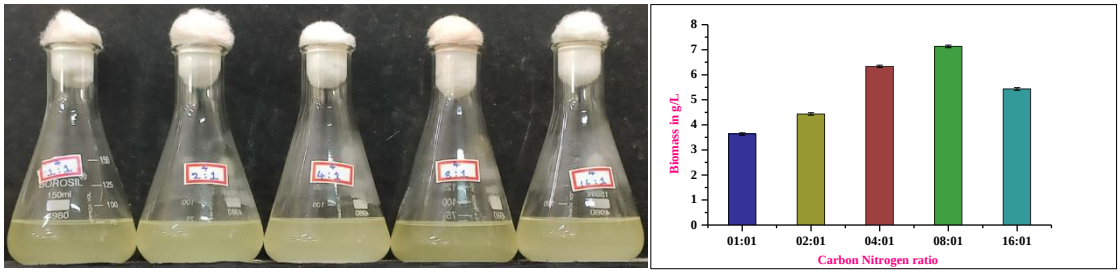

Figure 7: Production of PHB using different carbon to nitrogen ratio. 8:1 ratio shows maximum yield for production of PHB

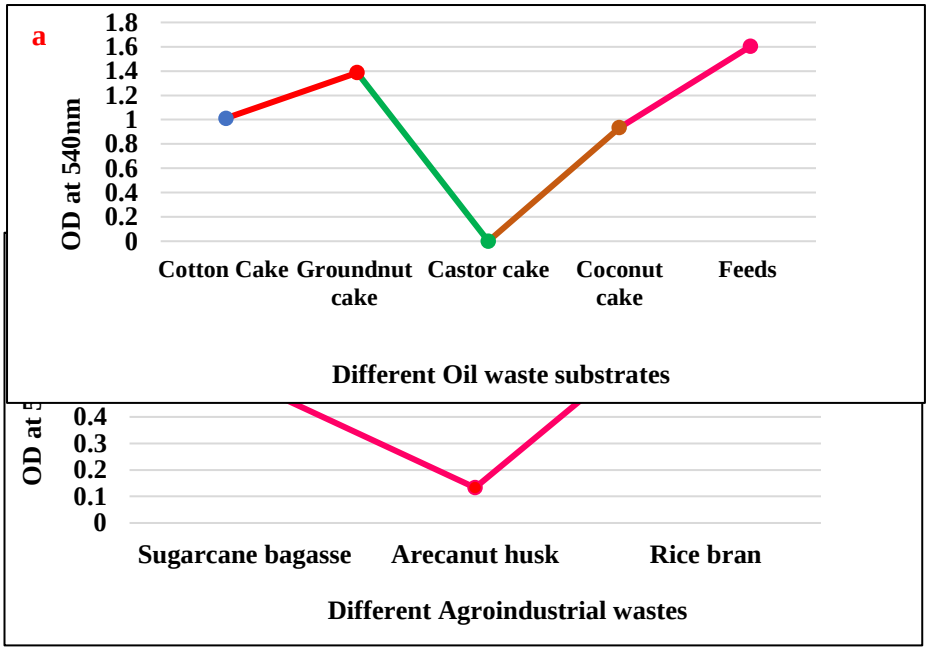

Figure 8: Production of PHB using cheaper substrates. In fig 8 (a&b) the production of PHB using low-cost agricultural wastes, the feed stock and rice bran showed good yield compared to other wastes used for PHB production.

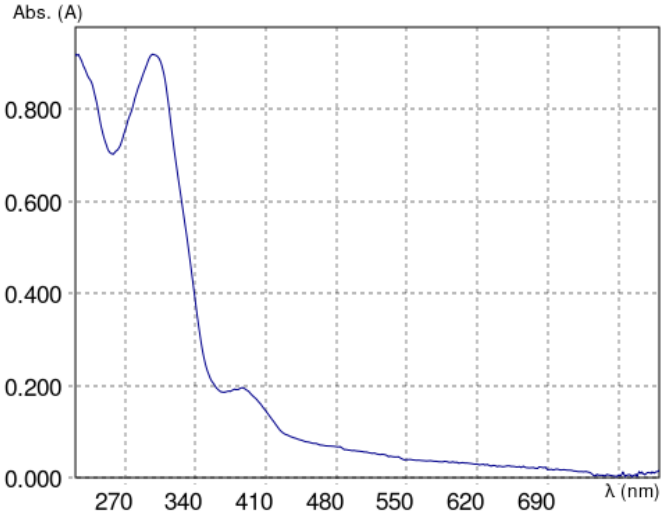

Figure 9: Estimation of amount of PHB present in the compound using Bio-spectrophotometer at 235 nm

LIST OF TABLES

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

Colony Morphology	**Results**
Shape	Circular
Size	2-3 mm
Texture	Sticky
Color	White
Microscopic Characters	
Cell Shape	Rods
Cell length	2.8-3.2 µm
Cell width	1.3-1.5 µm
Motility	Non-Motile
Spore formation	-ve

Table 2: Biochemical characterization of bacteria isolated from dump yard soil sample collected from Savalanga, Davanagere District, Karnataka.

Biochemical tests	**Results**
Citrate utilization	+ve
Nitrate reduction	+ve
Urease	+ve
Methyl red	+ve
Vogues–Proskauer	-ve
Indole production	+ve.
Malonate utilization	+ve

H_2S production	-ve
KOH	-ve
Lecithinase	-ve
Lipase	+ve
β-Galactosidase	-ve
Catalase	+ve
Oxidase	-ve
Hydrolysis	-ve
Starch	-ve
Gelatine	-ve
Casein	
Triple sugar iron test	
Dextrose	+ve
Sucrose	+ve
Lactose	+ve
Carbohydrates	+ve
Hydrogen	-ve
Gas	-ve