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Isolation, Characterization and Quantification of Polyhydroxybutyrate Producing Bacteria *Achromobacter xylosoxidans* KUMBNGBT-63 from Different Agroresidues

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ABSTRACT

A type of biodegradable polymer called polyhydroxybutyrate (PHB) is created naturally by some bacteria as a means of storing energy. PHB is seen as a possible substitute for plastics made from petroleum because of its biodegradability and renewable status. The current research study uses various agro-industrial residues to isolate, screen, characterize, optimize and quantify bacteria that produces PHB. The powerful bacterium that generates PHB was found in a scrap yard in Rangenahalli, Chikkamagaluru (Dist.), Karnataka, INDIA. The selected bacteria were non-spore-forming, non-motile and Gram-negative. Sudan b black staining indicated the granules' existence in the bacteria. Achromobacter xylosoxidans was recognized and confirmed based on its phenotypic and genotypic characteristics. The 16s rRNA gene's partial sequence was submitted to NCBI, GenBank and given the Accession number OK103997. The growth conditions necessary for the most production of PHB are optimized using a variety of physical and chemical factors, including nutrient broth medium, a 72-hour incubation period, a 37°C temperature, a pH of 7.0, glucose as a source of carbon, ammonium chloride as a source of nitrogen and a carbon-nitrogen ratio of 4:1. The bacterium that produces PHB was grown on several agro-industrial waste products, with feedstock hydrolysate showing the highest level of PHB generation. The 298 nm λ-max is provided by the UV-visible spectrophotometric measurement, which also measures and validates the generation of PHB. All of these results indicate that the isolated Achromobacter xylosoxidans is a productive PHB producer that can be used to make PHB biopolymers.

Keywords: *Polyhydroxybutyrate, Sudan Black B, GenBank, Agro-industrial residues, UV- visible spectrophotometer*

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INTRODUCTION

Petroleum-based plastics, also known as conventional plastics, are synthetic materials made from petroleum or natural gas. These plastics are the most widely used types of plastics and are commonly found in everyday products such as water bottles, food containers and packaging materials. Due to the slow or non-degradable nature of synthetic plastic, which also contributes to air pollution when burned, severe soil and water pollution occurs. To lessen the use of synthetic polymers, governments are searching for alternatives (Mostafa *et al*., 2020; Narayanan *et al*., 2021). A rising number of people are now interested in creating substitute materials that are more environmentally friendly than petroleum-based plastics. Biopolymers created from biological sources are the finest substitutes for polymers made from petrochemicals. Due to their simple synthesis and purifying processes, polymers generated from microbial sources are the most advantageous among the different biological sources (Jiang *et al*., 2018; Yaseen *et al*., 2021).

Polyhydroxybutyrate (PHB) is a biopolymer belonging to the class of polyhydroxyalkanoates (PHA). PHB is a type of polyester produced naturally by various microorganisms as an intracellular energy storage compound. It is a biodegradable and biocompatible material with properties similar to those of conventional petrochemical-based plastics, but with the added benefit of being environmentally friendly. According to many researchers, soil bacteria can produce PHBs are microbial polyesters accumulated inside the bacterial cells under nitrogen limitations and excess carbon supplementation (Alshammari 2021; Fatima *et al*., 2021).

The unfavorable impact of high-level production economics continues to be a substantial obstacle for the bioprocess technology for these polymers. PHB's biodegradability and potential for production from renewable resources have made it a prospective replacement for traditional plastics. Because of its high degree of crystallinity, it has mechanical characteristics that are comparable to some thermoplastics'. PHB is resistant to water and UV rays and has high gas barrier qualities. Under different circumstances, a variety of microorganisms, including bacteria and algae, can create PHB. Recombinant DNA technology can also be used to create it (Kalia *et al*., 2021).

PHB has potential uses in several industries, including biodegradable polymers, packaging and medical implants. Given that biodegradability is a key factor in single-use items like packaging and utensils, its qualities make it a desirable material. Several industries, including pharmaceutical

nanodelivery, food safety and biomedical applications, have strong demand for polyesters (Rofeal *et al*., 2022). PHB effectively replace petrochemical polymers made from fossil fuels. They have been used in biocompatible implants, scaffolds for tissue engineering, smart packaging materials and 3D printing inks (Abd El-malek *et al*., 2020). PHB is an all-purpose biopolymer that can be used in a variety of industries. A desirable replacement for conventional materials, it is non-toxic, biodegradable and biocompatible.

The current study uses various low-cost agro-industrial residues to isolate, screen, produce, characterize, optimize and quantify the bacteria that produce PHB. The usage of biopolymers will decrease the use of synthetically created polymers because PHB produced by the bacteria was extremely quickly degraded in the environment. Agro-industrial waste residues are an excellent source for PHB production using *Achromobacter xylosoxidans*, which is also simple to cultivate. Therefore, the total cost of this work is relatively low and the PHB generated by these wastes can be employed in large-scale production, creating the foundation for increasing PHB biopolymer production.

MATERIALS AND METHODS

Study area, sample collection and isolation of PHB-producing bacterium

The soil sample for PHB manufacturing was obtained in the Indian state of Karnataka's Rangenahalli, in the Chikkamagaluru district. The samples were diluted, then using the spread plate method, 10^{-6}, 10^{-7} and 10^{-8} dilutions were inoculated on nutritional agar media. Following incubation, colonies were chosen. The isolates were maintained as pure cultures on nutrient agar slants based on their morphological characteristics. For additional investigation, the chosen colonies were kept in storage at 4°C (Bektasa *et al*., 2023).

Qualitative screening of PHB-producing bacterium

Differential staining was used to identify and screen the isolated bacterial strain and Sudan black B staining was used to identify the PHB granules that were present in the bacterium. (Narayanan *et al*., 2021)

Phenotypic and genotypic characterization of PHB-producing bacterial strains

The bacteria that produces PHB were categorized based on their phenotypic traits. Cultural traits that are incorporated into the morphological characterization include colony size, shape, color and texture. Simple and endospore staining techniques for microscopic characterization were used. Biochemical assays were carried out as per "Bergey's Manual of Determinative Bacteriology". In addition to urease, malonate utilization, Voges Proskauer, indole, methyl red, H2S generation, KOH, -galactosidase, catalase, lipase, lecithinase, oxidase and triple iron agar, these tests also demonstrated the employment of these enzymes. The 16S rRNA gene sequence of the bacteria was examined using the universal primers 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1492R (5′-AAG GAG GTG ATC CAG CCG C-3′) to identify the strain at the species level. The sequence of the 16S rRNA gene was obtained and contrasted with selected comparable sequences from the NCBI GenBank database. Next, an entry number was assigned to the aligned sequence and it was submitted to GenBank, NCBI. The phylogenetic variation of the isolated strain was investigated using the neighbor-joining method (Pol Reshma *et al.,* 2017).

Influence of physico-chemical parameters for PHB production

Effect of different broth medium and different incubation period on PHB production

The chosen isolate was added to 75 mL of five different broths, including Luria Bertani broth (LB), Minimal salt broth (MSB), Tryptone soya broth (TSB), Nutrient broth (NA) and Minimal broth (MB). After sterilization, a bacterial isolate that produces PHB was aggressively cultivated and it was inoculated and incubated under ideal circumstances for various incubation times, ranging from 24 to 96 hours. Cultures were evaluated for PHB production at each interval of time (24, 48, 72 and 96 h), after which the yield of PHB was calculated (Thapa *et al*., 2019).

Effect of temperature on PHB production

To evaluate how the temperature affects the synthesis of PHB, the 100 mL Nutrient broth medium was inoculated into the bacterial culture and incubated at various temperature ranges (4°C, 15°C, 25°C, 37°C and 42°C). After 48 h of incubation at respective temperatures, PHB yield was quantified spectrophotometrically. The best temperature for the greatest production of PHB was established based on the production (Thapa *et al*., 2019).

Effect of pH on PHB production

The optimum pH was determined after the pH of the nutrient broth medium was attuned to 3, 5, 7, 9 and 11 and the bacterial colonies were inoculated into nutrient broth and incubated at 37°C for 48 h. After incubation PHB yield was measured by a spectrophotometer with a suitable wavelength and the yield of the PHB was determined (Lathwal *et al*., 2015).

Effect of different carbon sources on PHB production

Different carbon supplements, such as glucose, fructose, sucrose, maltose and lactose, were added to the 100 mL nutrient broth at a 2% concentration. Freshly developed bacteria were put into 100 mL of nutritional broth that had been supplemented with various carbon sources and they were then left to grow for 48 hours at 37°C. Following incubation, the isolate's PHB production was assessed using a spectrophotometer and the best carbon source was chosen based on the yield (Lathwal *et al*., 2015).

Effect of different nitrogen sources on PHB production

100 mL nutrient broth was prepared by adding different nitrogen supplements like ammonium chloride, urea, sodium nitrate, peptone and yeast extract at 1% concentration. Freshly grown bacterial culture was inoculated and incubated at 37°C for 48 h. After incubation, PHB produced by the isolate was measured by spectrophotometer and based on the yield, the best nitrogen source was determined (Thapa *et al*., 2019).

Effect of different carbon to nitrogen ratio (C/N ratio) on PHB production

Effects of various carbon sources (2%) like glucose, fructose, sucrose, maltose and lactose, as well as nitrogen supplements (1%) like ammonium chloride, urea, sodium nitrate, peptone and yeast extract at various concentrations (1:1, 2:1, 4:1, 8:1 and 16:1). Freshly grown bacterial cultures, was inoculated into 100 mL broth medium supplemented with different carbon and nitrogen sources and incubated at 37°C for 48 h. After incubation, PHB produced by the isolates was measured by spectrophotometer and based on the yield, the best carbon-nitrogen ratio was determined (Lathwal *et al*., 2015).

Production and assessment of PHB using agroresidues

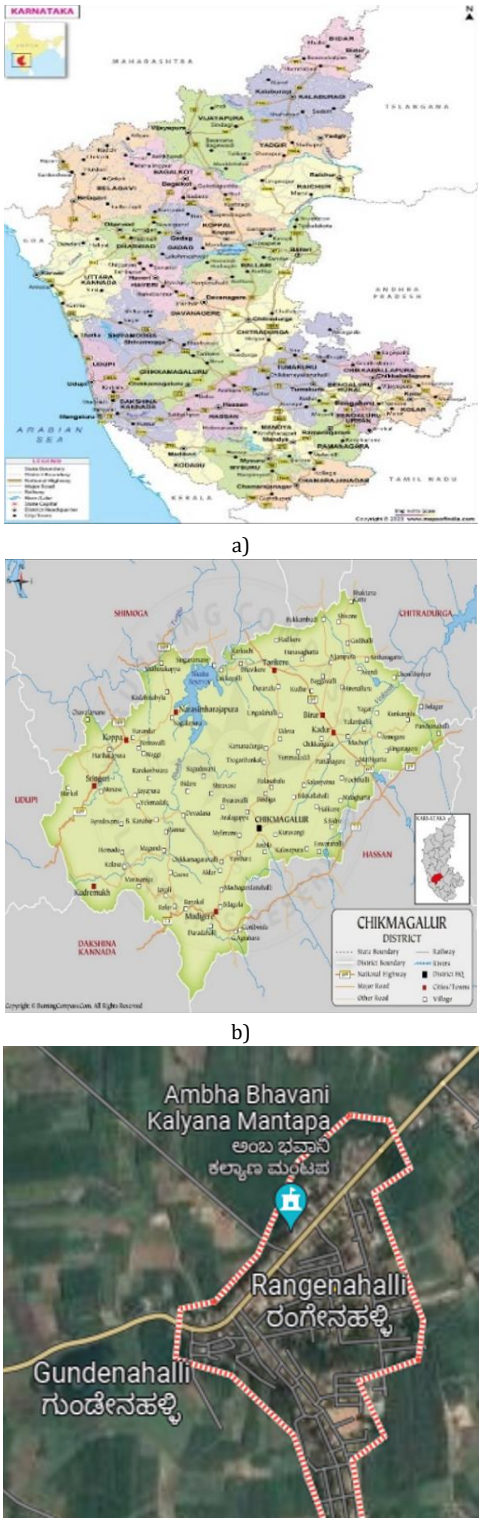
Agroresidues were used to produce PHB based on their availability and production cost. The materials selected residues included rice bran, areca nut husk, castor cake, sugarcane bagasse, coconut cake, cotton cake, groundnut cake and fed stock. According to John *et al*. 2006, these residues were transformed into gelatinous or jelly-like forms by utilizing gelatine and hydrolysate. The sugar molecule was employed as a source of carbon for the manufacture of PHB after the liquid hydrolysate had been filtered through muslin fabric to remove the solid residue. The hydrolysate was sterilized at 121°C for 15 minutes after being neutralized to 7.0 using NaOH. For further analysis the hydrolysate was stored in a refrigerator (John *et al*., 2006).

A liquid hydrolysate of a different substrate was injected with the chosen PHB-producing bacterial culture and it was then incubated at 37°C for 72 h. After incubation, the bacteria's maximal growth was measured spectrophotometrically by reading hydrolysate liquid that had not been infected with any of the substrates. The bacterial cells were isolated and used to measure PHB quantitatively. The bacterial cells that produce PHB were isolated by centrifuging broth culture (a liquid hydrolysate of the source material) at 11,000 rpm for 15 minutes. Following this, they were washed with acetone and ethanol. The pellet was suspended in 4% sodium hypochlorite and incubated at room temperature for 30 minutes. Once more, the suspension was centrifuged for 10 minutes at 10,000 rpm. The particle was rinsed with acetone and ethanol after the supernatant was removed. Hot chloroform was used to dissolve the isolated PHB compound. Following the evaporation of the chloroform, 10 mL of Con. H_2SO_4 (Con. sulphuric acid) was added and the polymer was then transformed into crotonic acid. After that, the molecule was examined at 235 nm for the presence of PHB using a blank of condensed sulphuric acid and λ-max was determined using a bio-spectrophotometer (Pol Reshma *et al*., 2017).

RESULTS AND DISCUSSION

Sample collection and isolation of PHB-producing bacteria

The soil sample was taken from a scrap yard site in Rangenahalli, Chikkamagaluru district, Karnataka, India because it contains a lot of dry and wet waste materials, has a rich microbial load and is a good source for isolating powerful PHB-producing bacteria **(Figure 1)**. From a 10^{-7} dilution on a nutrient agar plate, a total of 16 colonies of a single bacterium were isolated **(Figures 2a and 2b)**.



a)

b)

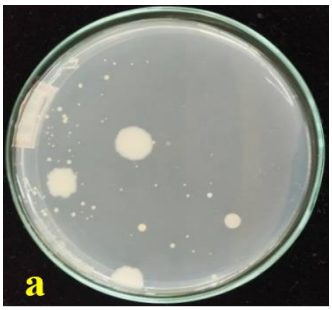
c)

Figure 1. Sample collection site at Rangenahalli, Chikkamagaluru district, Karnataka, INDIA.

Qualitative screening of PHB-producing bacterium

It is necessary to screen bacterial colonies to identify the powerful bacteria that produce PHB. Gram's reaction to the isolated bacterial isolate was negative **(Figure 2c)**. Sudan B

black staining was done to establish the presence of PHB granules. PHB granules can be seen in the bacteria's cells when Sudan black B-stained bacterial slides are examined under 100x magnification **(Figure 2d)**. The Sudan B black staining approach, used in prior investigations by Murray *et al*., 1994, revealed that the black PHB granules presence in the bacterial cells was identical to our results.



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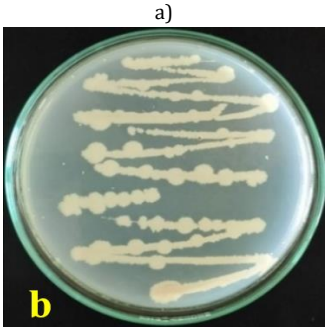

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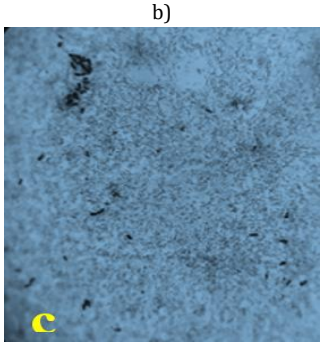

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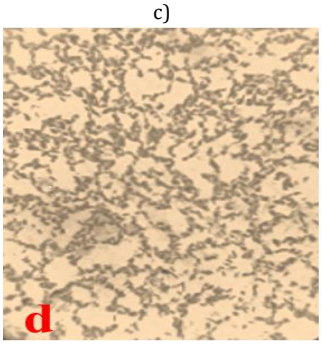

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Figure 2. Isolation and identification of PHB producing bacterial isolate using Gram staining and Suda staining method. a, b) isolated and pure colonies, c) Grams reaction showing negative for isolated colony and d) isolate showing positive for Sudan balck B staining method.

Characterization of PHB-producing bacterium

Morphological and biochemical identification PHB-producing bacterium

The morphological and biochemical characteristics of the isolated strain were determined according to "Bergey's Manual of Systematic Bacteriology" (Krieg & Holt, 1984). The strain is negative for grams' reaction, non-motile, aerobic and non-spore-forming and the shape of the isolate was a circular, white-color colony, 1-1.5 mm colony size and it has smooth, raised, opaque and sticky surface. Biochemical characters were examined and the isolate was positive for citrate, nitrate, urease, methyl red, indole, malonate, lipase and citrate utilization and it was negative for starch, gelatine, casein hydrolysis, H_2S, KOH, lecithinase, galactosidase and oxidase test. The isolate can ferment various sugars such as dextrose, sucrose, lactose and carbohydrate whereas the hydrogen and carbon dioxide were not fermented by the isolate. **Table 1** provide a summary of the morphological and biochemical traits. According to these findings and Bergey's manual of determinative bacteriology (Krieg & Holt, 1984), the chosen bacterial isolate belongs to the genus *Achromobacter* and these findings are compared with the earlier results of Hassan *et al*., (2013).

Table 1. Morphological and Biochemical characterization of bacteria isolated from dump yard soil sample that were collected from Rangenahalli, Chikmagalur 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
Biochemical tests	
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	
Starch	-ve
Gelatine	-ve
Casein	-ve
Triple sugar iron test	
Dextrose	+ve
Sucrose	+ve

Lactose	+ve
Carbohydrates	+ve
Hydrogen	-ve
Gas	-ve

Genotypic characterization

To determine the species of the bacterium, the primers 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG C-3′) were used to molecularly describe the 16S rRNA gene sequence. Initial denaturation took place at 95 °C for two minutes, final denaturation took place at 95 °C for thirty seconds and annealing took place at 50 °C for a minute. The nucleotide sequence was entered into GenBank NCBI and given a special identification number, namely OK103997. The gene sequence alignment utilizing pair-wise alignment showed the greatest (100%) similarity with *Achromobacter xylosoxidans* isolate KUMBNGBT-63. The PCR amplicon size was 674 base pairs. The neighbour joining approach and a bootstrap value of 500 were used to analyze the isolate's phylogeny **(Figure 3)**. Even though this outcome was connected to earlier data from Irsath *et al.,* (2014).

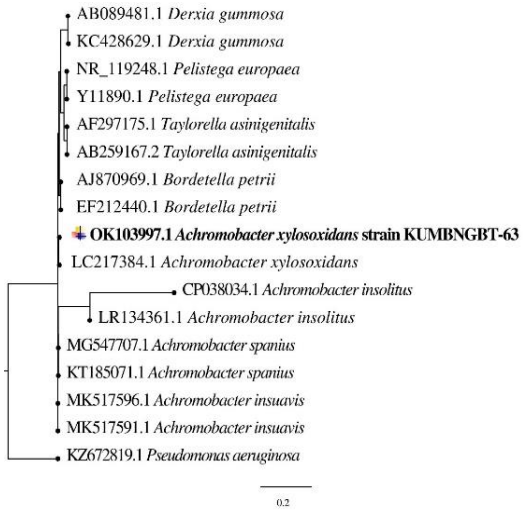
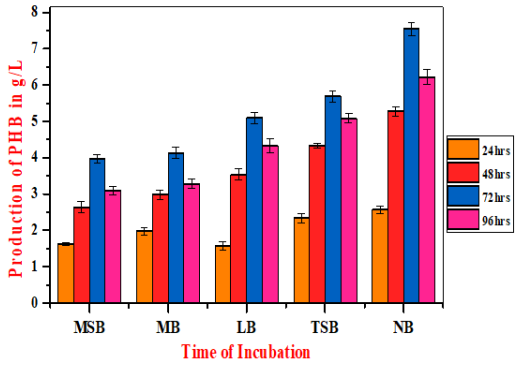


Figure 3. Phylogenetic analysis of *Achromobacter xylosoxidans* KUMBNGBT-63 using the neighbor-joining method and the 100 boot strap value was used for the analysis.

Influence of physico-chemical parameters for PHB production

Effect of different broth mediums and different incubation periods on PHB production

The bacterium that produces PHB was cultivated in five different broth media (Minimal salt broth (MSB), Luria Bertani broth (LB), Nutrient broth (NA), Minimal broth (MB) and Tryptone soya broth (TSB) and incubated at different times. The nutrient broth showed maximum yield i.e., 7.546±0.11 g/L at 72 h time interval **(Figure 4)**. Thapa *et al*., 2019 recorded similar results and discovered that 72 h is enough for optimum PHB production.



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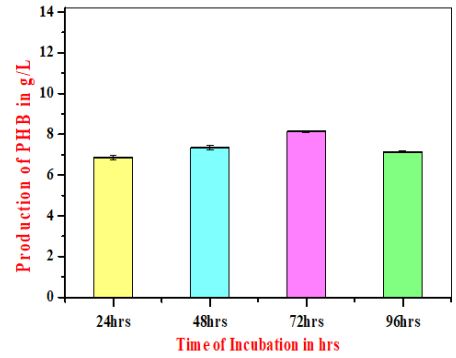

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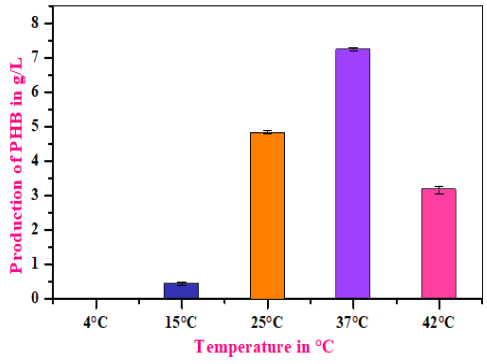
Figure 4. Influence of growth conditions required for the maximum PHB Production using various physic-chemical parameters. Cultivation of PHB-producing bacteria in different broth mediums at different incubation periods.

Accumulation of PHB production on different temperatures, carbon to nitrogen ratio (C: N ratio), carbon sources, nitrogen source and pH

To optimize the production of PHB by *A. xylosoxidans* different temperatures were selected. Among all the selected temperatures, the organism showed maximum PHB production at 37°C. The increase or decrease in the temperature will affect PHB production due to the presence of low polymerase activity **(Figure 5a)**. A similar outcome was observed in previous studies by Grothe *et al*., 1999. From their data, 33°C appears to be the optimum temperature for PHB production. At pH 7 the strain *A. xylosoxidans* showed maximum PHB production compared to other pH ranges **(Figure 5b)**. According to obtained results, the acidic and basic nature of the pH is not suitable for PHB production. The impact of different carbon sources (glucose, fructose, sucrose, maltose and lactose) on PHB production was analyzed **(Figure 5c)**. Among all the carbon sources utilized, the media supplemented with glucose shows maximum PHB yield by *A. xylosoxidans* (Khanna & Ashok, 2005). The effect of different nitrogen sources (yeast extract, peptone, urea, sodium nitrate and ammonium chloride) on PHB production was revealed in **Figure 5d**. Among all the nitrogen sources tested, the maximum PHB production was observed in ammonium chloride by *A. xylosoxidans* (Mahmoud *et al*., 2012). The media was supplemented with a 2% carbon source and nitrogen sources tested, 4:1 is the best suitable carbon and nitrogen ratio for maximum PHB production **(Figure 5e)**. There

was a comparison between these results and the results of Lee *et al.*, (2020). According to their report the 9:1 C: N ratio was found to be optimum for PHB production.



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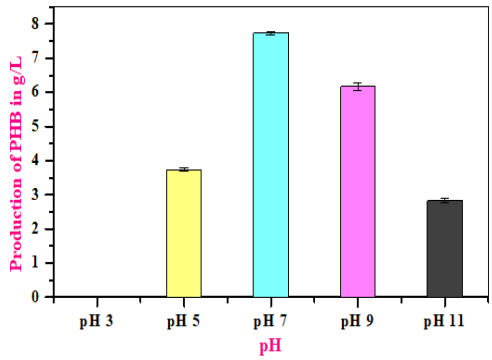

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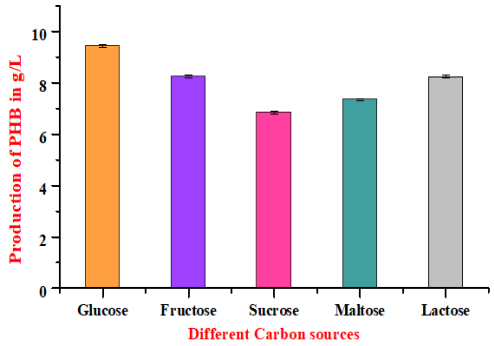

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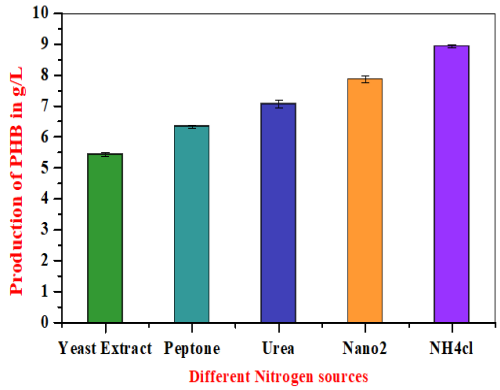

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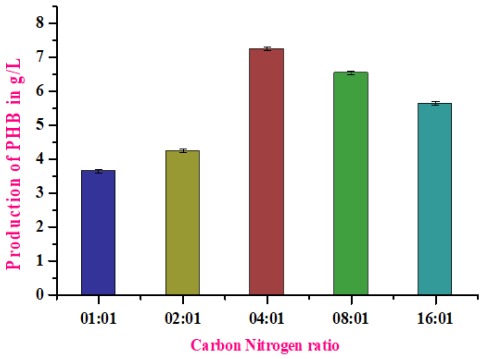


e)

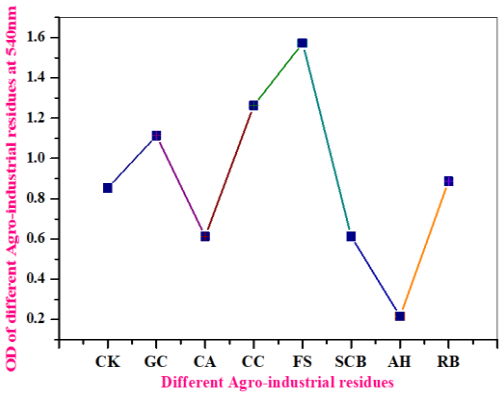
Figure 5. Accumulation of PHB production required for the maximum PHB growth using various physicochemical parameters a) different temperatures effect; b) different pH effect; c) effect of different carbon sources; d) effect of different nitrogen sources and e) different carbon-to-nitrogen ratios (C/N ratio) effect.

Production and assessment of PHB using agroresidues

Bioplastics have limited commercialization due to their high production costs. Low-cost agro-industrial wastes, which serve as the only carbon source for the manufacturing of PHB, helped to lower the production costs. The numerous bacteria strains that can use diverse carbon sources have been investigated in several papers. The bacteria *Ralstonia Eutropha* reportedly uses glycerol as a carbon source (Taidi *et al.*, 1999). The artificial substrates used to grow the PHB-producing bacteria were being replaced by several low-cost substrates like feeds, coconut cake, rice bran, cotton cake, castor cake, areca nut husk, groundnut cake and sugarcane bagasse (SCB).

In comparison to cotton cake, castor cake, coconut cake, groundnut cake, rice bran, sugar cane bagasse and areca nut husk illustrated in **Figure 6**, the low-cost substrate, or feedstock, demonstrates maximum yield for growth of PHB generated by *A. xylosoxidans*. Van-Thuoc *et al.*, (2008), previously reported these findings. Their research indicated that wheat bran produced the highest yield of PHB.

Because of the compound's acidic nature, the PHB polymer breaks down into its monomers throughout the measurement process. Crotonic acid and con were used to quantify these transformed monomers into compounds. The standard utilized was H2SO4. According to **Figure 6**, the PHB-producing strain's λ-max was 298 nm when measured at 235 nm using a bio-spectrophotometer. The maximum level of PHB in the sample was closely related to how much UV light crotonic acid absorbed. These results were contrasted with those from Pol Reshma *et al.*, 2017, who published previous findings.



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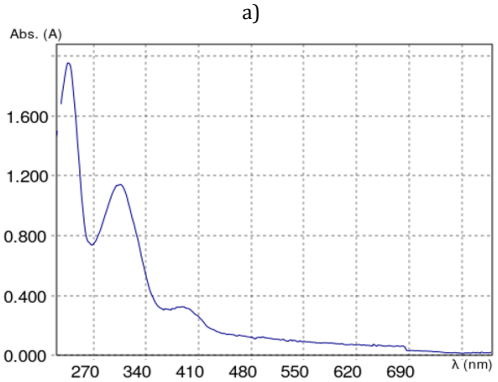

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Figure 6. Production and amount of PHB present in the cells were analyzed using a UV-Visible spectrophotometer using different Agro-industrial residues.

CONCLUSION

The soil sample used to make PHB was obtained from Rangenahalli in the Chikkamagaluru district of Karnataka, INDIA. The isolated strain was largely screened using the Sudan black B staining method and identified by its morphological and biochemical traits. Following species-level identification, the strain's identified as *Achromobacter xylosoxidans*. The influence of growth conditions to optimize the various physicochemical characters using different cultural conditions. For the large-scale production of PHB, low-cost agro-industrial residues were used as cheaper raw materials and the amount of PHB cells present in the isolate was scrutinized using λ-max at 235nm using a UV-visible spectrophotometer. According to the above findings, the bacterium *Achromobacter xylosoxidans* acts as a very good PHB producer and it can be used for the production of biopolymers in commercialized conditions it is very cost-effective compared to other petroleum-derived plastics.

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CONFLICT OF INTEREST: None

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ETHICS STATEMENT: None

REFERENCES

Abd El-malek, F., Khairy, H., Farag, A., & Omar, S. (2020). The sustainability of microbial bioplastics, production and applications. *International journal of Biological Macromolecules*, *157*, 319-328.

Alshammari, E. (2021). The role of diet in bacterial/viral infections: Vegan diet, red meat and the Coronavirus. *Journal of Advanced Pharmacy Education and Research, 11*(2), 71-74.

Bektasa K. I., Kadriye Canb., & Belduzbm A. O. (2023). Isolation and Screening of Polyhydroxybutyrate (PHB) Producing Bacteria from Soils. *Biology Bulletin, 50*(3), 319-328.

Fatima, A., Khaliq, S. A., & Khan, R. (2021). Bacteriological Analysis of Drinking Water from Different Regions of Karachi from February 2016 to August 2016. *Archives of Pharmacy Practice, 12*(4), 25-28.

Hassan, M. A., Haroun, B. M., Amara, A. A., & Serour E. A. (2013). Production and characterization of keratinolytic protease from new wool-degrading Bacillus species isolated from Egyptian ecosystem. *Biomed Research International Journal, 2013*, 1-14. doi:10.1155/2013/175012

Irsath, H., Santhosh, S., Hemalatha, V., Vikramathithan, M., Dhanasekar, R., & Dhandapani, R. (2014). Production and Optimization of Polyhydroxy butyrate using Bacillus subtilis BP1 Isolated from Sewage Sample. *International Journal of Pure & Applied Bioscience, 3*(1), 158-166. doi:10.1155/2013/952641

Jiang, Y., Song, X., Gong, L., Li, P., Dai, C., & Shao, W. (2018). High poly (β-hydroxybutyrate) production by Pseudomonas fluorescens A2a5 from inexpensive substrates. *Enzyme and Microbial Technology, 42*(2), 167-172.

John, R. P., Nampoothiri, K. M., & Pandey, A. (2006). Solid-state fermentation for L-lactic acid production from agro wastes using Lactobacillus delbrueckii. *Process Biochemistry, 41*(4), 759-763. doi:10.1016/j.procbio. 2005.09.013

Kalia, V. C., Singh Patel, S. K., Shanmugam, R., & Lee, J. K. (2021). Polyhydroxyalkanoates: Trends and advances toward biotechnological applications. *Bioresource Technology, 326*, 124737.

Khanna, S., & Ashok, K. S. (2005). Statistical media opti¬mization studies for growth and PHB production by Ralstonia eutropha. *Process of Biochemistry, 4*, 2173-2183. doi:10.1016/j.procbio.2004.08.011

Krieg, N. R., & Holt, J. G. (1984). *Bergey's Manual of Systematic Bacteriology*. 1st ed. vol. 1. Williams and Wilkins: Baltimore.

Lathwal, P., Nehra, K., Singh, M., Jamdagni, P., & Rana, J. S. (2015). Optimization of Culture Parameters for Maximum Polyhydroxy butyrate Production by Selected Bacterial Strains Isolated from Rhizospheric Soils. *Polish Journal of Microbiology, 64*(3), 227-239.

Lee, Y. R., Fitriana, H. N., Lee, S. Y., Kim, M. S., Moon, M., Lee, W. H., Lee, J. S., & Lee, S. (2020). Molecular profiling and optimization studies for growth and PHB production conditions in Rhodobacter sphaeroides. *Energies*, *13*(23), 64-71. doi:10.3390/en13236471

Berekaa, M. M., & Al Thawadi, A. M. (2012). Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by Bacillus megaterium SW1-2: Application of Box-Behnkenesign for optimization of process parameters. *African Journal of Microbiology Research, 6*(4), 838-845.

Mostafa, Y. S., Alrumman, S. A., Otaif, K. A., Alamri, S. A., Mostafa, M. S., & Sahlabji, T. (2020). Production and characterization of bioplastic by polyhydroxybutyrate accumulating Erythrobacter aquimaris isolated from mangrove rhizosphere. *Molecules*, *25*(1), 179.

Narayanan, M., Kandasamy, G., Murali, P., Kandasamy, S., Ashokkumar, V., & Massif, O. (2021). Optimization and production of polyhydroxybutyrate from sludge by Bacillus cereus categorized through FT-IR and NMR analyses. *Journal of Environmental Chemical Engineering*, *9*(1), 104-108.

Reshma, P., Sonal, S., & Vatsala, S. (2017). Screening and Isolation of Polyhydroxy butyrate Producers. *International Journal for Research in Applied Science & Engineering Technology (IJRASET), 5*(12), 1259-1264.

Rofeal, M., Abdelmalek, F., & Steinbuchel, A. (2022). Naturally-Sourced Antibacterial Polymeric Nanomaterials with Special Reference to Modified Polymer Variants. *International Journal of Molecular Sciences*, *23*(8), 4101.

Taidi, B., Anderson, A. J., Dawes, E. A., & Byrome, D. (1994). Effect of carbon source and concentration on the molecular mass of poly (3-hydroxybutyrate) produced by Methylobacterium extorquens and Alcaligenes eutrophus. *Applied Microbiology and Biotechnology, 40*, 786-790.

Thapa, C., Shakya, P., Shrestha, R., Pal, S., & Manandhar, P. (2019). 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. doi:10.3126/njb.v6i1.22339

Van-Thuoc, D., Quillaguaman, J., Mamo, G., & Mattiasson, B. (2008). Utilization of agricultural residues for poly (3-hydroxybutyrate) production by Halomonas boliviensis LC1. *Journal of Applied Microbiology, 104*(2), 420-428. doi:10.1111/j.1365-2672.2007.03553. x

Yaseen, S. S., Mohamed, A. H., Salih, S. M., & Abass, K. S. (2021). Prevalence of bacterial infection among narghile smokers complaining of respiratory problems in Kirkuk city, Iraq. *Journal of Advanced Pharmacy Education and Research, 11*(4), 91-94.