

Research article

Assessment of Polyhydroxybutyrate (PHB) in *Bacillus tequilensis* KUMBNGBT-64a Isolated from Trash Yard Soil

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Abstract

Biodegradable polymers represent a hot topic in current environment. Biodegradable polymers can be decomposed into simple molecules like CO₂, CH₄, H₂O and residual biomass. *Bacillus* sp. KUMBNGBT-64a, isolated from leftover soil that was gathered at Sagar taluk located in Shivamogga, Karnataka, India, was identified as *Bacillus tequilensis* KUMBNGBT-64a. Phenotypic and genotypic characteristics were used to identify the bacteria. Various conditions were employed to maximize the highest production of PHB by the bacteria. The bacterial isolate was grown on affordable agro-industrial wastes to lower production cost and scale up PHB production. The quantity of PHB contained in the isolated bacterial cells was measured using bio-spectrophotometric analysis. *Bacillus* sp. KUMBNGBT-64a, a gram positive, motile and spore forming bacterium, was confirmed as *Bacillus tequilensis* KUMBNGBT-64a by using 16S r-RNA sequence and deposited to GenBank, NCBI. Consent No. PV124813. Additionally, the following conditions of nutritional broth medium, a 72-h incubation period, 37°C temperature, pH 7.0, glucose as a carbon source, ammonium chloride as a nitrogen source, and a 4:1 carbon-nitrogen ratio were shown to be responsible for the greatest generation of PHB. A liquid hydrolysate of feed stock had the highest level of PHB production, and the λ -max of 270 nm was used to quantify and validate PHB production. In the present investigation, *Bacillus tequilensis* KUMBNGBT-64a produced maximum PHB using low cost substrates, which helped to reduce the production cost, and could be used for the large-scale production of the bio plastics.

Keywords: polyhydroxybutyrate; agro-industrial wastes; liquid hydrolysate and bio-spectrophotometer

1. Introduction

The global production and usage of plastics are increasing, with over 380 million metric tons produced annually, causing environmental and health issues due to the release of 80 million tons of plastic waste (Morya et al., 2018). Polyhydroxyalkanoate (PHA) synthesis

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from a variety of biomass types was thoroughly investigated. These biomasses were wastewater, lignocellulosic materials, fats and oils, domestic trash, industrial by-products, sugars, and agricultural waste (Sindhu et al., 2013). The replacement of petrochemicals (obtained from petroleum through refining, are chemical products made from petroleum, coal, natural gas, and renewable sources like maize, palm fruit and sugar cane) is very essential and these petrochemicals were substituted with natural biopolymers like polyhydroxybutyrate (PHB), natural polyesters produced by microorganisms for energy and carbon storage. PHBs have various shapes, high strength, and thermoplastic properties, and are produced under unfavorable growth conditions (Tan et al., 2011).

PHB production in bacteria is influenced by various factors such as carbon sources like glucose, fatty acids, methanol, and organic acids. Further research into genetic engineering and metabolic pathways could enhance yields and make PHB production more economically viable. Renewable resources like agricultural waste and industrial by-products can be used as carbon sources. Carbon dioxide can be utilized by microorganisms through carbon fixation. Wastewater treatment can be sustainable and cost-effective. Optimizing carbon to nitrogen ratios can influence PHB accumulation. Environmental conditions like oxygen limitation, pH, temperature, stress conditions, genetic engineering, and co-culturing can enhance PHB production. These strategies can lead to higher productivity and sustainable practices (Getachew & Woldesenbet, 2016).

There are several uses for recyclable, water-resistant, moldable and non-cytotoxic PHB, including its use as fine chemicals, medical implants, pharmaceuticals, polymers, and drug delivery systems. However, the expensive cost of making conventional plastics (primarily derived from non-renewable fossil fuels like natural gas are widely used in packaging due to their durability, versatility, and cost-effectiveness, including polyethylene, polypropylene, PVC and polystyrene), prevents them from being widely replaced, which delays their commercialization (Maity et al., 2017).

The rod-shaped, aerobic, motile, spore-forming bacteria in the genus *Bacillus* sp. have distinct phenotypic characteristics, such as varying growth conditions, DNA base organization, and dietary sources (Claus & Berkeley, 1986). *Bacillus* sp. produces arginine hydrolases, lysine decarboxylase, ornithine decarboxylase, and rhamnose acid, and is used as a model biofilm-producing bacteria (Gatson et al., 2006).

In this current study, the *Bacillus* strain was isolated from soil for its capacity to produce PHB, a biodegradable polymer that has potential as an environmentally friendly alternative to conventional plastics. PHB's unique properties, including its biocompatibility, biodegradability, and similar processing to petroleum-based plastics, make it suitable for various applications. Unlike traditional plastic production, which relies on fossil fuels, bacterial synthesis of PHB offers sustainability, reduced carbon footprint, and the potential for waste substrate utilization in fermentation processes. Moreover, this research is novel as it explores the optimization of PHB production through specific *Bacillus* strains isolated from soil, which may lead to enhanced yields and broaden the understanding of the metabolic pathways involved. By investigating the potential of local soil bacteria, the aim of this study was to contribute to the development of sustainable bio plastic production methods, ultimately addressing the pressing issue of plastic pollution and paving the way for greener alternatives.

2. Materials and Methods

2.1 Sample collection and isolation of PHB producing bacterium

The bacterium responsible for the production of PHB was isolated from leftover soil that was gathered at Sagar taluk located in Shivamogga, Karnataka, India. One gram of soil was in succession diluted up to 10^{-9} dilutions and then 10^{-6} to 10^{-8} dilutions were plated on nutrient agar (NA) medium by pour plate technique. The plates were kept at 37°C for 24 h. The colonies grown on nutrient agar plates were chosen based on their phenotypic characteristics and a pure culture was grown on nutrient agar slants and kept in a refrigerator for future research (Mikkili et al., 2014).

2.2 Selection of PHB producing bacteria

The chosen bacterium was analyzed using gram staining and Sudan black B staining method for selection of isolate responsible for PHB (Steinbüchel, 2001).

2.3 Evaluation of PHB producing bacteria

2.3.1 Morphological characterization

The isolated bacterium was identified and characterized according to its morphological characteristics, including shape, size, texture, and color, indicating its cultural condition (Murray, 1994).

2.3.2 Biochemical characterization

Biochemical characterization of PHB producing isolated bacteria was performed by starch hydrolysis, gelatin hydrolysis, casein hydrolysis, citrate utilization, nitrate reduction, urease, methyl red, Voges proskauer, indole, malonate utilization, H_2S production, KOH, β -galactosidase, lecithinase, lipase, catalase, oxidase and triple iron agar tests. The tests were carried out according to Bergey's Manual of Determinative Bacteriology (Claus & Berkeley, 1986).

2.3.3 Genomic characterization

DNA was extracted from the bacterial isolate using a standard phenol/chloroform method, and its quality was checked using electrophoresis in a 0.8% agarose gel and quantified using a Nano-Drop ND-1000 spectrometer. The 16S rRNA gene was amplified using universal oligonucleotide primers, 27F and 1541R, in a thermal cycler. The PCR reaction was performed under the following conditions: 94°C for 10 min, 65°C for 1 min, 72°C for 30 s for 35 cycles, and 72°C for 7 min. The PCR products were detected by agarose gel electrophoresis, stained with ethidium bromide, and visualized using Gel Doc EZ Imager. The amplicon size was verified using a standard 100-base pair DNA ladder. The PCR product was purified using the PEG–NaCl precipitation method. The 16S rRNA gene was sequenced using the Sanger sequencing method, which was carried out in an automated DNA Analyzer. The amplicons were then incubated at room temperature for 15 min, centrifuged at 10,000 rpm for 20 min, washed with 70% ethanol, air-dried, and suspended in 10 μL formamide. The obtained 16S rRNA sequence was deposited in the National

Centre for Biotechnology Information (NCBI) under an accession number "PV124813" and analyzed using the Basic Local Alignment Search Tool (BLAST) provided by the NCBI. Pairwise alignment was conducted using ClustalW and the resulting alignment was used to construct a phylogenetic tree constructed in RAxML (Randomized Axelerated Maximum Likelihood) using maximum likelihood analysis. The phylogenetic tree was analyzed using MEGA7 software with a bootstrapping value of 500 (Kumar et al., 2016).

2.4 Utilization of different factors for optimization of PHB production by *Bacillus* sp. KUMBNGBT-64a

2.4.1 Optimization of different media and incubation period

Bacterial isolate was inoculated into the following different broths such as nutrient broth (peptone-5 g; beef extract-3 g; sodium chloride-5 g; agar -20 g; and distilled water-1000 mL; pH-7.0), tryptone soya broth (pancreatic digest of casein (casein)-17 g; peptone-3 g; sodium chloride-5 g; dextrose-2.5 g; dibasic potassium phosphate-2.5 g; and distilled water-1000 mL; pH-7.3), mannitol salt broth (D-mannitol-10 g; peptone-10 g; sodium chloride-75 g; beef extract-1 g; phenol red-0.025 g; and distilled water-1000 mL; pH-7.4), minimal broth (potassium dihydrogen phosphate-3 g; disodium hydrogen phosphate-6 g; NaCl-5 g; ammonium chloride-2 g; magnesium sulphate-0.1 g; dextrose-8 g; and distilled water-1000 mL; pH-7.0), and Luria broth (tryptone-10 g; yeast extract-5 g; nacl-10 g; and distilled water-1000 mL; pH-7.4). After that, the broths were incubated at different time periods and filtered. The bacterial biomass was washed, purified and treated with hot chloroform. The PHB layer was collected, dried and the extracted PHB yield was assessed (John et al., 2006).

2.4.2 Effect of temperature, pH, carbon source, nitrogen source and carbon-nitrogen ratio (C/N ratio) on PHB production by *Bacillus* sp. KUMBNGBT-64a

The pure cultures of *Bacillus* sp. KUMBNGBT-64a, which were able to produce PHB, were inoculated on nutritional agar media and incubated at 37°C for 24 h to produce the culture filtrate. The culture filtrate was tested in nutrient broth media at 37°C for 48 h at a speed of 200 rpm in a rotary shaker. The culture media were incubated at a range of 4°C, 15°C, 25°C, 37°C and 42°C. The media pH was altered to 3, 5, 7 and 11 in order to maximize the various cultural parameters, including temperature. The media was supplemented with various carbon sources, including glucose, fructose, sucrose, maltose, and lactose, and various nitrogen sources, including ammonium chloride, peptone, sodium nitrate, urea, and yeast extract, for the purpose of testing the utilization of various carbon and nitrogen sources. The carbon to nitrogen ratio (C:N ratio) was varied to analyze how much carbon and nitrogen the bacterial strain used. Carbon (2%) and nitrogen (1%) supplements were optimized at various concentrations: 1:1, 2:1, 4:1, 8:1 and 16:1 (Bhuwal et al., 2014).

2.5 Development of PHB-producing bacteria on various agro-industrial wastes

The study sought to identify the most cost-effective raw material for generating PHB by hydrolysis of several agro-industrial wastes: feedstock, cotton cake, groundnut cake, coconut cake, castor cake, sugarcane bagasse, rice bran, and areca nut husk. The wastes were gelatinized using gelatine, and a basal medium was created by suspending 150 g of

each waste in 1200 mL of distilled water (12.5 % w/v). The mixture was boiled at 100°C for 30 min. The residual substances present in the waste substrate were extracted in boiling water with subsequent filtering of the liquid hydrolysate. The obtained liquid hydrolysate from the substrate was supplemented with glucose as a carbon source (2 % w/v) and ammonium chloride as a nitrogen source (1 % w/v) and then the media were heated up to 70°C for around 30-60 min in an allium condenser and sterilized. After sterilization, the prepared media were used for the cultivation of PHB producing bacteria. The bacteria responsible for the production PHB was inoculated into 1000 mL broth obtained from the cheaper substrate (pH 7.0) and cultivated at 37°C for 3 days. The amount of PHB produced by the bacterium using different agro-industrial by-product liquid hydrolysates was recorded spectrophotometrically by measuring the optical density at 540 nm (Reinecke & Steinbüchel, 2009; Mekala et al., 2013).

2.6 Quantitative assessment of PHB

Bacterial cells producing PHB were collected and analyzed using a broth culture and sodium hypochlorite solution. The developed PHB extract was liquefied in hot chloroform, and concentrated sulfuric acid was added to convert it into crotonic acid. The PHB present in the extract was measured using bio-spectrophotometer at 235 nm using conc. sulfuric acid as the blank (Reshma et al., 2017).

3. Results and Discussion

3.1 Sample collection and isolation of PHB producing bacterium

The soil sample collected from the leftover waste yard located in Sagar taluk, Shivamogga was rich in microbial load and served as a valuable source for the isolation of effective PHB-producing bacteria (Figure 1). Total 5 potent PHB producing colonies of single bacterium were isolated from 10^{-8} dilution on nutrient agar plate and the bacterial strains were sub-cultured on nutrient agar slants and used for further analysis.

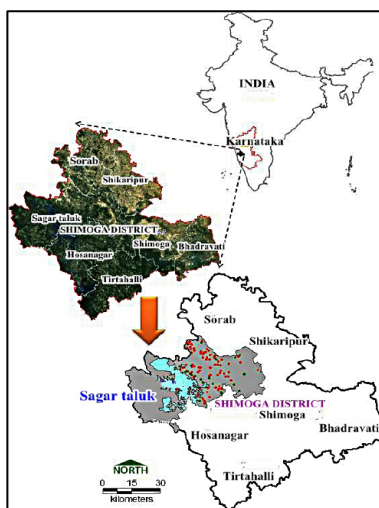


Figure 1. Sample collection site at Sagar, Shivamogga district, Karnataka, India

3.2 Screening of PHB producing bacterial strain

The isolated bacterium was primarily screened using different staining techniques. The bacterial strain showed positive for gram reaction and the presence of PHB granules inside the colonies were observed by Sudan black B staining method (Figure 2).

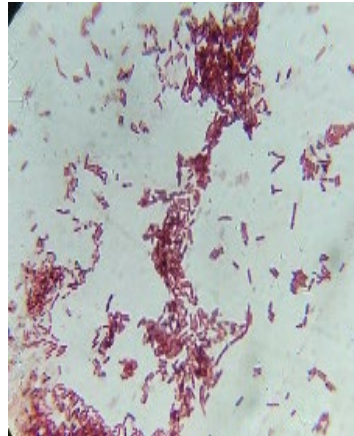


Figure 2. Microscopic observation of PHB producing bacterial isolate using Sudan black B staining technique

3.3 Characterization of PHB producing bacterium

3.3.1 Morphological characterization

According to “Bergey’s Manual of Determinative Bacteriology”, the isolate showed positive result for both gram staining and endospore staining method (Kumar et al., 2016). The isolate was aerobic, motile and showed spore formation inside the cells. The shape of the isolate was circular and yellow in color. It also has smooth, raised, opaque and sticky surface, as shown in Figure 3. The cony characteristics are described in Table 1.

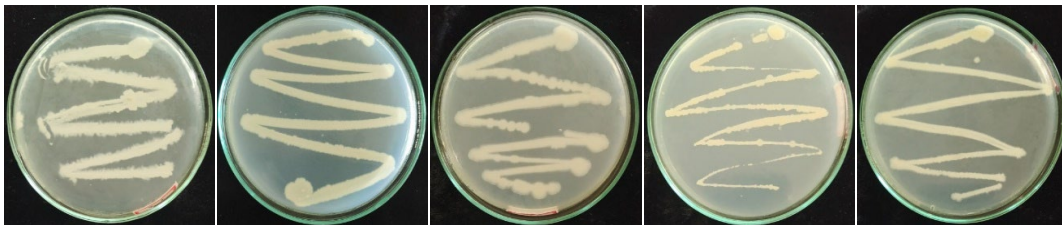


Figure 3. Morphological characterization of five potent PHB producing bacterial isolates

3.3.2 Biochemical characterization

Based on biochemical test results, the *Bacillus* sp. KUMBNGBT-64a isolate was positive for Vogues–proskauer, KOH, lecithinase, lipase, β -galactosidase, catalase and oxidase

and it was negative for urease, methyl red, indole, malonate, H₂S and catalase. Furthermore, it had the ability to utilize and hydrolyze different proteins such as gelatine and casein and also utilize carbohydrates such as starch and citrate. The *Bacillus* sp. KUMBNGBT-64a could ferment various sugars such as dextrose, sucrose, lactose and carbohydrates (starch and citrate), whereas it did not ferment hydrogen and carbon dioxide (Table 1) (Irsath et al., 2015).

Table 1. Morphological and biochemical characterization of *Bacillus* sp. KUMBNGBT-64a responsible for PHB production

Colony Morphology	Characteristics
Color	White
Shape	Circular
Size	2-3 mm
Texture	Sticky
Microscopic Characters	
Cell Shape	Rods
Cell length	2.8-3.2 µm
Cell width	1.3-1.5 µm
Motility	Non-Motile
Spore formation	Positive
Biochemical tests	
Catalase	-
Citrate utilization	+
H ₂ S production	-
Indole production	+
KOH	-
Lecithinase	-
Lipase	+
Malonate utilization	+
Methyl red	+
Nitrate reduction	+
Oxidase	-
Urease	+
Voges-Proskauer	-
β-Galactosidase	-
Hydrolysis	
Starch	-
Gelatine	-
Casein	-
Triple sugar iron test	
Carbohydrates	+
Dextrose	+
Gas	-
Hydrogen	-
Lactose	+
Sucrose	+

Note: ‘+’: Positive; ‘-’: Negative

3.3.3 Phylogeny analysis with 16S rRNA

The bacterium *Bacillus* sp. KUMBNGBT-64a was identified using molecular characterization of its 16S rRNA gene sequence, demonstrating maximum similarity with *Bacillus tequilensis*, with an amplicon size of 1484 base pairs and was deposited to GenBank NCBI with the assigned accession no. PV124813. The phylogeny of the isolate was constructed with reference to the type strains retrieved from GenBank NCBI using a bootstrap value of 500 and a maximum likelihood tree method is present in Figure 4 (Irsath et al., 2015).

The maximum likelihood (ML) tree construction is a crucial tool in phylogenetic analysis due to its statistical rigor, flexibility, and ability to handle complex models. ML uses a probabilistic framework, considering factors like nucleotide substitution rates, transition biases, and codon position-specific rates, providing a more accurate and statistically valid estimation of evolutionary relationships. It can accommodate a wide range of evolutionary models, allowing for better-fitting models and incorporating rate heterogeneity. ML also offers accuracy by optimizing tree topology, handling homoplasy and evaluating tree reliability through bootstrap support and model comparison. It can handle large data sets with high computational power, estimating evolutionary parameters like substitution rates and tree branch lengths and handling incomplete data more effectively than simpler methods like Parsimony. ML is an invaluable tool for comprehensive evolutionary analysis, especially in modern phylogenetic studies.

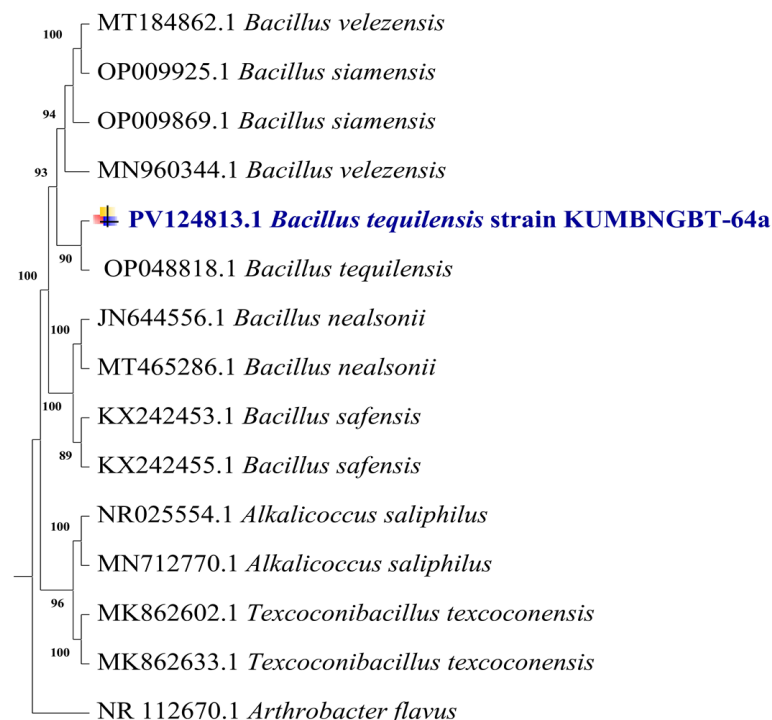


Figure 4. Phylogeny of analysis of *Bacillus tequilensis* KUMBNGBT-64a using maximum likelihood method. A 500 boot strap value was used for the analysis.

3.4 Utilization of different factors for optimization of PHB production by *Bacillus tequilensis*

3.4.1 Optimization of different media and incubation period

Bacillus tequilensis strain KUMBNGBT-64a was grown on five broth media, with nutrient broth showing the highest yield, i.e. 7.683 ± 0.11 g/L at 72 h compared to other broths (Figure 5a).

3.4.2 Effect of temperature, pH, carbon source, nitrogen source and carbon-nitrogen ratio (C/N ratio) on PHB production by *B. Tequilensis* KUMBNGBT-64a

To optimize the PHB production by *B. tequilensis* strain KUMBNGBT-64a, 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 affected the PHB production due to the presence of low polymerase activity (Figure 5b). *Bacillus tequilensis* strain KUMBNGBT-64a showed maximum PHB production at pH 7 compared to other pH ranges (Figure 5c). According to the results, acidic or basic pH was not suitable for PHB production. The impact of different carbon sources (glucose, fructose, sucrose, maltose and lactose) on PHB production was analyzed (Figure 5d). Among all the carbon sources utilized, the media supplemented with glucose showed maximum PHB yield by *B. tequilensis* strain KUMBNGBT-64a. The effect of different nitrogen sources (yeast extract, peptone, urea, sodium nitrate and ammonium chloride) on PHB production is revealed in Figure 5e. Among the the nitrogen sources tested, the maximum PHB production was observed in ammonium chloride by *B. tequilensis* strain KUMBNGBT-64a. For the media was supplemented with 2% carbon source and nitrogen sources tested, 4:1 is the best suitable carbon and nitrogen ratio for maximum PHB production (Figure 5f).

3.5 Development of PHB producing bacteria on different agro-industrial wastes

The limited commercialization of bio plastics is a result of their high production costs. Low-cost agro-industrial wastes served as the only carbon source needed to produce PHB, at lowered manufacturing costs. In our studies, the commercial products employed for the development of PHB-producing bacteria were replaced by a variety of low-cost substrates, including cotton cake, groundnut cake, castor cake, coconut cake, feed stock, sugarcane bagasse (SCB), areca nut husk, and rice bran. *Bacillus tequilensis* strain KUMBNGBT-64a grown on feed stock produced the highest PHB yield (1.086 ± 0.005 g/L) compared to other sources, as shown in Figure 6(a).

3.6 Quantitative assessment of PHB

In quantification method, the breakdown of PHB polymer into its monomers is due to its acidic nature present in the compound. These converted monomers were measured using a standard solution of crotonic acid and conc. H_2SO_4 . The λ -max of the PHB producing strain was at 270 nm when measuring by a Bio-spectrophotometer, and its maximal PHB content was directly correlated with the amount of UV light absorbed by crotonic acid as shown in Figure 6(b). The maximum amount of PHB present in the sample was directly associated with amount of UV radiation absorbed by crotonic acid (Reshma et al. 2017).

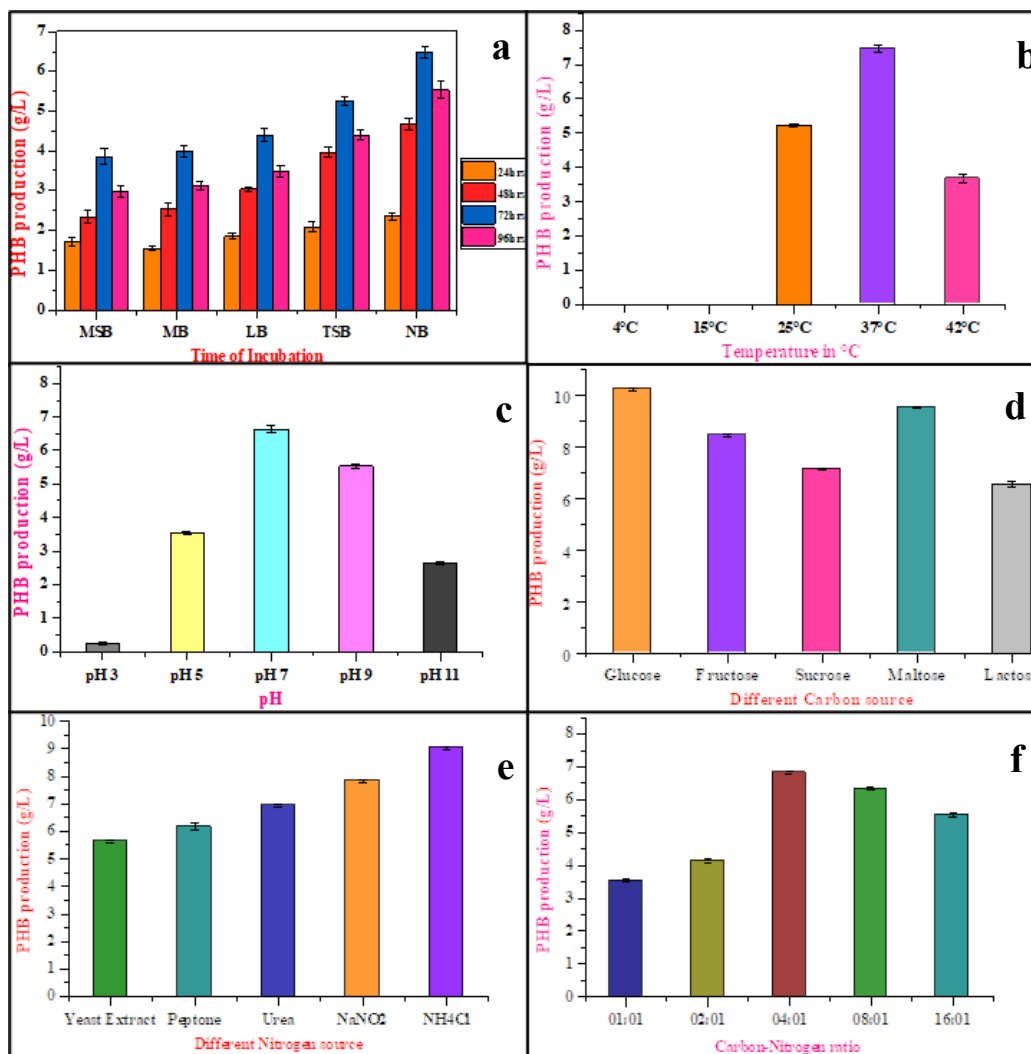


Figure 5. Influence of growth conditions required for the maximum PHB production using various physico-chemical parameters. a. Cultivation of PHB producing bacteria in different broth medium at different incubation period; b. effect of different temperature; c. Effect of different pH; d. Effect of different carbon sources; e. Effect of different nitrogen sources; f. Effect of different carbon to nitrogen ratio (C/N ratio). The statistical analysis was performed using one way ANOVA and p value ($p < 0.05$). Graphical representation with error bars were mean $\pm$ standard deviation with $n=3$.

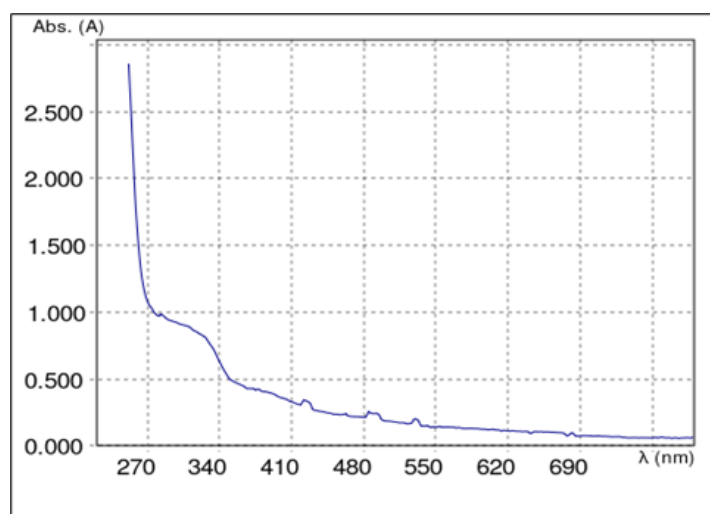
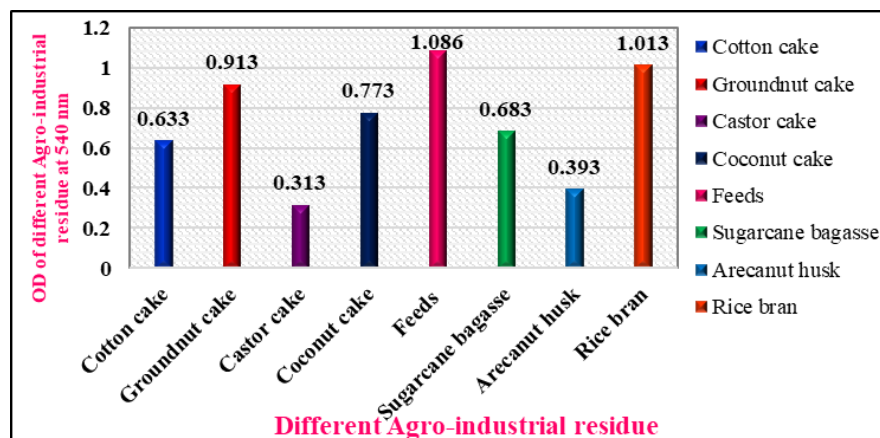


Figure 6. a. Production and quantification of PHB producing bacterial isolates using agro industrial residues; b. the amount of PHB present in the cells were quantified using UV-Visible spectrophotometric analysis.

3.7 Discussion

In our current study, the isolated strain was gram positive, aerobic, endospore forming and motile bacteria, with specific colony characteristics (forming smooth, raised, opaque, sticky and yellow circular colony). While Ramachandran and Abdullah (2010) observed colonies of orange fluorescence grown on nutrient-rich media exposed to ultraviolet (UV) light, indicating the presence of PHB producers. As the amount of bacterial cells increased, so did the intensity of its fluorescence. Molecular characterization and identification of bacteria involved 16S rRNA gene sequencing, phylogenetic analysis and biochemical and phenotypic characterization. The 16S rRNA gene sequence is highly conserved across different species and provides a detailed information about the bacterium's genetic makeup. Phylogenetic tree was constructed by comparing the sequences or entire

genomes of the bacterium with those of known species. Biochemical tests and phenotypic characteristics were also used. Key molecular findings included 16S rRNA gene sequence identity, genome-based similarity, phenotypic features, and functional genomic features. These findings helped confirm the bacterium's classification (Gajdiss et al., 2020). Nutrient broth was found to be optimal for PHB production, yielding 7.683 g/L at 72 h. These results were correlated with the previous investigation of Sharma and Dhingra (2015). According to their study, *Pseudochrobactrum asscharolyticum* showed maximum production at time interval of 72 h. In our studies, the isolate strain, *B. tequilensis* strain KUMBNGBT-64a, had the highest output at 37°C, which was considered the optimal temperature for PHB synthesis. These results were compared with the earlier findings of Grothe et al. (1999). According to their findings, the largest PHB generation occurred at 33°C. At a pH of 7, the isolate produced PHB at its highest rate. At pH 7, residual glucose was at its lowest, indicating that the organism was efficiently utilizing carbon sources for development and PHB accumulation. These data suggest that basic and acidic pH are unfavorable for PHB synthesis, which was in agreement with the findings of Elsayed et al. (2013). Based on their results, pH 7-7.5 was suitable for PHB production. Glucose produced the highest amount of PHB after 72 h of incubation. These results were consistent with the study conducted by Khanna and Srivastava (2005), who discovered that the addition of fructose caused *Ralstonia eutropha* to produce the greatest amount of biomass. It was discovered that *B. tequilensis* utilized ammonium chloride as the optimal nitrogen source, resulting in the maximum synthesis of PHB. Analogous findings were reported by Borah et al. (2002), where *Bacillus mycoides* yielded the maximum amount of PHB when beef extract was added. It was discovered that a carbon-to-nitrogen ratio of 4:1 is ideal for PHB accumulation because PHB is a biopolymer produced by bacteria as a carbon storage compound under nutrient imbalance conditions. A 4:1 C-ratio balances carbon availability for PHB production, while a 16:1 C-ratio may lead to excess carbon, affecting PHB production. Nitrogen limitation affects the ratio, with higher C-ratios favoring growth and biomass production, resulting in lower PHB levels. PHB formation decreases at greater C-N concentrations. These findings were contrasted with past research by Sharma and Dhingra (2015), who produced PHB at an ideal 16:1 C:N ratio in their investigation. Ammonium chloride and glucose are essential nutrients for microbial growth and metabolic processes. Ammonium chloride provides nitrogen, a nutrient essential for protein synthesis, enzyme production, and cellular growth. It maintains a balanced nutrient environment, especially in waste residues with low nitrogen content. Glucose is a primary carbon source for microbial growth, providing energy for easy metabolism and supporting growth and reproduction. It also acts as an energy substrate, facilitating the production of metabolic by-products for further processes like secondary metabolites or biofuels.

Eight distinct agro industrial by-products were chosen for PHB manufacturing in the current investigation. The feed stock (1.086 ± 0.005 g/L) exhibited the highest amount of PHB generation among the eight by-products. In comparison to feed stock, the PHB accumulation in cotton cake (0.633 ± 0.005 g/L), ground nut cake (0.913 ± 0.005 g/L), and coconut cake (0.773 ± 0.005 g/L) was relatively low. In comparison to feed stock, the PHB accumulation in castor cake (0.313 ± 0.005 g/L), rice bran (1.013 ± 0.01 g/L), sugarcane bagasse (0.683 ± 0.005 g/L) and arecanut husk (0.393 ± 0.005 g/L) was moderate. These results were consistent with the studies of Salgaonkar and Bragança (2017), which revealed that *Halogeometricum borinquense* strain E3's maximal PHB accumulation was 50.4 ± 0.1 and 45.7 ± 0.19 (%), with specific productivity (qp) of 3.0 and 2.7 (mg/g/h).

The PHB produced by the bacterial cells were quantified using spectrophotometric method and maximum PHB production at 270 nm was obtained which confirms the presence of PHB. The results were in comparison with earlier results of Reshma et al.

(2017). Polyhydroxybutyrate (PHB) is a biopolymer composed of 3-hydroxybutyric acid (3-HB) units. Under acidic conditions, PHB undergoes hydrolysis or depolymerization, breaking down into its monomeric unit, 3-hydroxybutyric acid. This process is typically carried out in the presence of concentrated sulfuric acid (H_2SO_4), which catalyzes and facilitates the breakdown. The acidic environment is crucial as the ester bonds in PHB are susceptible to cleavage, leading to the release of 3-HB as the main monomeric product. Crotonic acid is often used as a marker or derivative in assays to quantify PHB monomers. Sulfuric acid (H_2SO_4) is used in the hydrolysis process to ensure complete hydrolysis and enhance the yield of monomeric units for quantification.

Polyhydroxybutyrate (PHB) is a form of intracellular carbon storage that bacteria produce when carbon is available in excess, especially under nutrient imbalances. PHB serves as an energy reserve for bacteria when environmental conditions change or resources are scarce. Factors influencing PHB production include excess carbon availability, nutrient limitation, oxygen or stress conditions, environmental stressors, and genetic factors or mutations. Bacteria may accumulate PHB to avoid carbon toxicity or imbalance in metabolism and protect cellular structures from damage. They initiate PHB biosynthesis as a survival strategy, and possess specific genetic pathways or mutations that enhance PHB biosynthesis. In order to cut expenses without using pricey glucose supplements, the authors recommend more research on PHB generation in the future. They advise looking into alternate carbon sources such as co-product use, integrated bioprocesses, microbial strain genetic engineering, agricultural waste, and less expensive nutrient additions. Additionally, they recommend investigating microbial strains with higher PHB yields and more effective metabolic pathways in order to decrease the need for substrates and production expenses. These recommendations may include synthetic biology techniques as well as natural strain selection. The authors hope to increase PHB production without using pricey glucose supplements by investigating these possible avenues.

4. Conclusions

Bacillus tequilensis KUMBNGBT-64a, a bacterium isolated from trash yard soil, shows potential for polyhydroxybutyrate (PHB) production. The bacterium can utilize organic waste materials as carbon sources for PHB biosynthesis. The yield of PHB is influenced by factors like carbon source concentration, incubation time, and temperature. This suggests that optimizing these parameters could enhance production. The ability of *B. tequilensis* KUMBNGBT-64a to produce PHB in such an environment suggests its potential for use in bioremediation and sustainable plastic production. Given the growing concern for plastic waste and the need for biodegradable alternatives, *B. tequilensis* KUMBNGBT-64a could be a significant organism for biotechnological PHB production, offering an eco-friendly solution for plastic waste management and bio plastic synthesis.

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6. Author Contributions

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

7. Conflicts of Interest

The authors confirm that they have no known conflicts of interest or competing financial interests that could have influenced the work produced.

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