UMYU Scientifica, Vol. 3 NO. 4, December 2024, Pp 355 – 368
https://doi.org/10.56919/usci.2434.030
ISSN: 2955 – 1145 (print); 2955 – 1153 (online)
A periodical of the Faculty of Natural and Applied Sciences, UMYU, Katsina

ORIGINAL RESEARCH ARTICLE

Optimization of Polyhydroxybutyrate Production by Bacillus Species Isolated from Dump Site Soil Using Multiple Linear Regression Analysis

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ABSTRACT

Petrochemical-based plastics cause considerable environmental degradation due to their non-biodegradable properties; however, biodegradable plastics, such as polyhydroxybutyrates (PHBs), provide a sustainable relief to the environment. This study used sugarcane bagasse as a carbon source to synthesize PHB by Bacillus species isolated from dumping site soil. *Bacillus velezensis*, which displays a high affinity to generate PHB based on the intensity of coloration upon radiation, was selected from 53 isolates. At 30°C, pH 7.5, and 48 hours of incubation, under the ideal conditions resulted in attaining the maximum PHB yield of 50.23% (w/w). Similarly, a predicted PHB yield of 66.05% (w/w) was estimated by a multiple linear regression (MLR) model utilizing ordinary least squares (OLS) regression, which identified temperature, pH, substrate concentration, and incubation time as critical variables. Scanning electron microscopy displayed intracellular PHB granules, and Fourier-transform infrared (FT-IR) analysis indicated the existence of unique PHB functional groups (C–H, CH_2, C=O, and C–O). Tests on biodegradability indicated that soil microorganisms can break down the produced PHB, highlighting the environmental benefits of this research. This study also demonstrates that sugarcane bagasse, an agricultural byproduct, is a cost-effective raw material for PHB synthesis. Additionally, the OLS model provided useful insights for optimizing yield, indicating promise for industrial applications. Future studies should examine other optimization statistical techniques like Response Surface Methodology (RSM) to boost PHB production in complex biological systems. These techniques could also address scalability, economic feasibility, and environmental benefits through cooperation with agro-waste companies and pilot-scale manufacturing activities.

ARTICLE HISTORY

Received September 29, 2024
Accepted December 10, 2024
Published December 20, 2024

KEYWORDS

Biodegradable plastics, polyhydroxybutyrate (PHB), *Bacillus velezensis,* sugarcane bagasse, multiple linear regression (MLR)

INTRODUCTION

One of the factors making the status of environmental deterioration worse is the growing use of plastic. The plastic material that is finding its way into our food chain is releasing microplastics and other toxic substances into the environment. Since then, efforts to create a sustainable, eco-friendly environment with a low carbon footprint have focused on biobased plastics research (Coppola *et al.*, 2021). Petroleum-derived conventional plastic polymers are indispensable to every aspect of human existence. But because these refractory polymers don't break down easily, disposing of them is a problem. Because of this, they stay in the ecosystem for a very long time and cause significant ecological harm by upsetting the natural order (George N *et al.*, 2021). Petroleum-based polymers should not be disposed of carelessly on land because burning in the open air can release toxic chemicals into the environment that endanger human and animal health as well as destroy biological systems and cycles (Coppola *et al.*, 2021).

Identifying an alternative to petroleum-based polymers is crucial to mitigate the environmental consequences associated with current polymers due to overconsumption and improper disposal. Consequently, the pursuit of novel biodegradable polymers is garnering heightened interest (Sunday *et al.*, 2023).

One answer to these problems is the identification of biodegradable polymers. Certain bacteria produce biodegradable polymers that can be decomposed by their enzymes (Mostafa *et al.*, 2020). Biodegradable plastics offer a feasible solution for environmental waste management, as they can replace conventional polymers in situations where recycling or incineration is unfeasible or prohibitively costly (Mostafa *et al.*, 2020).

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How to cite: Adamu, K. S., & Salisu, I. (2024). Optimization of Polyhydroxybutyrate Production by Bacillus Species Isolated from Dump Site Soil Using Multiple Linear Regression Analysis. *UMYU Scientifica*, 3(4), 355 – 368. https://doi.org/10.56919/usci.2434.030

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polymers that can be produced from renewable resources such as plant oils, sugars, and agricultural waste. A variety of bacteria can produce PHAs. Polyhydroxybutyrate, or PHB, belongs to the PHA family and is a subclass of biopolymer (Samrot *et al.*, 2021). It is one of the PHAs most frequently investigated and used commercially, and it is likely to find applications in the packaging, biodegradable plastics, pharmaceutical, and agricultural sectors (Markl., 2021). It is a desirable substitute for traditional petrochemical-based plastics since it is a biodegradable and compostable polymer (Padovani *et al.*, 2016).

It is known that certain bacteria, such as *Bacillus megaterium, Ralstonia eutropha*, and *Alcaligenes latus,* collect polyhydroxyalkanoates (PHAs) as a reserve food source. The most common biodegradable polymer and a viable substitute for manufactured non-biodegradable plastics is polyhydroxybutyrate, or PHB for short. It belongs to the biodegradable polymer family PHA. These polymers come together as an internal membrane-enclosed inclusion that can make up as much as 90% of the dry weight of the cell when it is under nutritional stress. These polymers function as materials that store energy. It can be molded, spun into monofilaments, turned into films, and used to create heteropolymers with other synthetic polymers. Furthermore, because it is immunologically compatible and biodegradable, it has a wide range of applications in packaging, medicine, and agriculture (Sunday *et al.*, 2023).

Bioplastics can be produced from proteins (casein, gluten), plant-based polysaccharides (starch, cellulose, chitosan/chitin), and other carbon sources. Similar to other proteins, wheat gluten can be transformed into beneficial bioplastics (Wang *et al.*, 2015). By allowing bacteria to absorb sugar, sugarcane can be used to make bioplastics (Pohare *et al.*, 2017). Last but not least, oil provides a valuable carbon source for the production of bioplastic. It has previously been demonstrated that crude palm kernel oil, jatropha oil, crude palm oil, palm olein, corn oil, coconut oil, cottonseed oil (Magar *et al.*, 2015), soybean oil (Park *et al.*, 2011), and crude palm kernel oil are all suitable substrates for the synthesis of biopolymers (Wong *et al.*, 2012).

The main concern in the microbial generation of PHB compared to petrochemical-generated polymers is high production costs, despite the fact that PHB has the advantage of being biodegradable. Among the strategies that must be used for successful green synthesis of polymers are screening for effective bacterial strains, using alternative, inexpensive sources of carbon and nitrogen, optimizing growth settings, and recovery protocols (Souvik *et al.*, 2024).

The impact of pH on the microbial production of PHB has been the subject of numerous investigations. According to Gasser *et al.* (2014), the metabolic pathways involved in the creation of PHB are heavily regulated by pH, with the right pH conditions encouraging the proliferation of bacteria and the accumulation of polymers. They found that maintaining a pH between 7.5 and 8.0 throughout fermentation resulted in a significant rise in PHB yields. In a similar vein, Bharathi *et al.* (2016) found that pH is crucial for both the activity of microbial enzymes involved in PHB production and the solubility of nutrients.

Another important factor that affects the microbial synthesis of PHB is temperature. According to research by Lee *et al.* (2022), PHB yields were reduced by temperatures above 35°C because these temperatures denatured important enzymes involved in the polymerization process. Conversely, it was shown that PHB production was enhanced by moderate temperatures (about 30°C) without compromising bacterial growth. These findings suggest that enhancing PHB synthesis requires careful temperature control.

Examining a number of environmental factors, including pH, temperature, substrate concentration, and incubation duration, is necessary to comprehend the optimal conditions for PHB synthesis because these factors might have a significant impact on bacterial metabolism and polymer growth. In order to maximize production, traditional experimental designs frequently change one factor at a time (OFAT). However, this method is laborious and less effective than statistical models like multiple linear regression (MLR), which can assess the effects of multiple variables at once (Kumar *et al.*, 2020).

MLR has developed into a potent tool for evaluating the relationship between a dependent variable (like PHB generation) and independent factors (like pH and temperature). By using MLR, researchers may estimate the results of changing these components in a data-driven manner in addition to quantifying the impact of each ingredient on PHB yields (Zaman *et al.*, 2021). By identifying the key factors influencing microbial synthesis, this modeling technique has been successfully used in numerous studies to maximize PHB output.

This study explores the potential of *Bacillus sp.* isolated from dumping site soil to produce polyhydroxybutyrate (PHB) using sugarcane bagasse as a carbon source. Multiple linear regression (MLR) was applied to analyze the effects of pH and temperature on PHB yields. By optimizing these conditions, the research aims to enhance PHB production and provide insights for scaling up the process for industrial applications. Most literature research utilized only experimental techniques to optimize the synthesis of PHBs. But, in this work, both experimental and statistical approaches (multiple linear regression) were used to compare the effectiveness of the two methodologies.

MATERIALS AND METHODS

Sample Collection Isolation of Bacterial Cultures

The sample site was in Kumbotso and Gwale local government of Kano state, Nigeria. According to the 2016 National Population Census, Kumbotso Local Government has a total population of 295,979 and a land area of 158 km². It is located at 11◦53`17``N latitude and 8◦30`10``E longitude. According to the 2016 National Population Census, Gwale Local Government has a total population of 362,059 and a land area of 18 km². It is located at latitude 11°58' N and longitude 8°30' E. One hundred soil samples were aseptically taken from the topsoil of several disposal sites in the Kano State local governments of Kumbotso (fifty soil samples) and Gwale (fifty soil samples).

To extract only endospore-forming bacteria, 10 grams (10 g) of each sample were dissolved in 90 milliliters of sterile distilled water and heated to 80 degrees Celsius for 15 minutes. The samples were serially diluted, and then 100µl of the diluted samples were spread-plated onto nutritional agar plates (Christina *et al*., 2018). The plates were then incubated for 24 hours at 35°C (Singh *et al*., 2011).

Screening of PHB producers

Pure cultures of morphologically different colonies were obtained by cultivation on agar plates devoid of nitrogen for 48 hours. The lipophilic stain Sudan Black B was used to detect PHB formation (Sunitha and Ujwala, 2013). The stain was prepared by dissolving 0.3 g of powdered in 100 ml of 70% ethanol to create the stain. Smears of colonies were heat-fixed on clean, oil-free glass slides for microscopic examinations and then stained with a 0.3% Sudan Black B solution. The slides were immersed in Xylene and counterstained with safranin (5% w/v in sterile distilled water) after being left undisturbed for fifteen minutes. Under a microscope, blue-black cells were recognized as PHB-positive strains (Christina *et al*., 2018).

Nile blue was used to further screen isolates that tested positive for Sudan black B. Colonies containing PHB-accumulating strains were dyed with 0.0005g of Nile blue on carbon-rich nutrition agar media. When exposed to ultraviolet light, A displayed pink fluorescence. According to El-Hamshary *et al.* (2018), isolates exhibiting pink fluorescence were deemed PHB-positive. Agar slants containing 2% glycerol for preservation were used to preserve PHB-positive bacteria on two vials: working and stock vials.

Phenotypic Identification

Biochemical tests of isolated PHB producers were conducted to identify and characterize bacterial isolates using several methods. The Gram staining technique (Gram, 1884) was employed to differentiate between gram-positive and gram-negative bacteria, while the catalase test (Graham and Parker, 1964) assessed the presence of the catalase enzyme, indicating the organism's ability to neutralize hydrogen peroxide. The citrate test (Noel et al., 2011) evaluated the organism's capacity to utilize citrate as its sole carbon source. Additionally, the indole test (Sagar, 2019) determined the ability to produce indole from tryptophan, and the starch hydrolysis test (Tillie, 2014) assessed the breakdown of starch into simpler sugars, further aiding in the identification of the bacterial species.

Molecular identification of PHB-producing isolate

Molecular identification was carried out using 16S rDNA sequence analysis of the isolate that produced PHB. The extraction was performed using the Dongsheng Biotech Quick Bacterial Genomic DNA Extraction Kit, as previously described (El-Kadi, 2014).

A CSL-JADNA ready-to-use PCR reaction combination was used for the PCR, according to Ajuzie and Atuanya (2014). Inqaba Biotech performed nucleotide sequencing on the amplified products. The NCBI website's BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare nucleotide sequences with accessible 16S rDNA sequences in the GenBank database.

Production of PHB by Selected Isolates Using Mineral Salt Medium

The chosen isolates produced PHB using mineral salts medium (MSM), which has the following composition (g/L): urea (1.0), yeast extract (0.16), KH_2PO4 (1.52), Na_2HPO_4 (4.0), $MgSO_4.7H_2O$ (0.52), $CaCl_2$ (0.02), glucose (40), and trace element solution 0.1 ml. (g/L) of $ZnSO_4{\cdot}7H_2O$ (0.13), $FeSO_4{\cdot}7H_2O$ (0.02), $(NH_4)_6MO_7O_{24}.4H_2O$ (0.06), and H_3BO_3 (0.06) were present in the trace element solution. Before inoculation, the glucose and trace element solutions were autoclaved independently and reconstituted. For 48 hours, the production medium (100 ml) was incubated at 37°C at 120 rpm after being inoculated with 2 ml of standardized inoculum (Getachew and Woldesenbet, 2016).

Extraction, Purification and Quantification of the PHB Produced

The biomass powder was treated with equal volumes of chloroform and 3.6% sodium hypochlorite, heated at 80°C for one hour, and then centrifuged at 3000 rpm for 20 minutes, resulting in three phases. The upper phase contained the hypochlorite solution, the middle phase had undisturbed cells (cells that were not disrupted during the treatment), and the bottom phase contained chloroform and PHB granule suspension (PHB extracted from cells in the form of granules suspended in chloroform). The chloroform phase was collected, and PHB was precipitated using methanol and chloroform (9:1), filtered, dried at 60°C, and weighed to calculate the percentage of

PHB in the dry cell weight (DCW) (Zakaria et al., 2010). Sugarcane bagasse was pretreated with 5% sulfuric acid, autoclaved at 121°C for 30 minutes, filtered, and neutralized with sodium hydroxide (Ramadas et al., 2009). Reducing sugars in the pretreated bagasse were measured using DNSA reagent and glucose standards, as described by Darshen et al. (2014). The optimization of PHB production was conducted in shake flask cultures with varying substrate concentrations, incubation times, pH, and temperatures, using sugarcane bagasse as a carbon source, following the methods described by (Getachew and Woldesenbet, 2016).

Characterization of Polymer

HT-35000 SEM (Hitachi Ltd., Japan) was used to record SEM pictures of thin sections and perform a scanning electron microscopy analysis of PHB-producing cells at 80 kV. Five milliliters of chloroform were used to dissolve one milligram (1 mg) of PHB that had been extracted. After adding KBr to produce a pellet, Spectrum 65 FT-IR was used to record spectra in the 4000–400 cm−1 range (Hong *et al*., 1999; Shreema, 2014).

PHB Degradation Study

The granule agar suspension method of the solidified medium was used to investigate the biodegradability of the polymer. A mineral salt media containing 15% agar was created as a nitrogen-deficient medium. A portion of the MSM medium was put onto a sterile petri dish and left to harden after sterilization. After melting, another part of the material was chilled to between 40 and 500 °C. The medium (granule-agar suspension) was mixed with enough PHB granule suspension to create a thin layer, which was then poured over the solidified medium's surface. After that, soil samples that had been serially diluted were spread out throughout the medium and left to incubate for three to five days (Michael *et al*., 2012).

Multiple Linear Regression Analysis

In this study, a multiple linear regression (MLR) analysis was employed to model the relationship between polyhydroxybutyrate (PHB) production and several independent experimental variables: substrate concentration, pH, temperature, and incubation time. In order to measure the contribution of each independent variable to PHB production and ascertain how modifications to these factors impact the yield of PHB in a controlled experimental setup, this statistical technique was selected. Following well-established machine learning best practices, we used the entire dataset for both training and prediction in order to overcome the problem of a small dataset (<100 samples) (Ng, 2018; Bengio *et al*., 2016). The regression model is of the following form:

$$\text{PHB Production} = \beta 0 + \beta 1 \times \text{Substrate Concentration} + \beta 2 \times \text{pH} + \beta 3 \times \text{Temperature} + \beta 4 \times \text{Incubation Time} + \epsilon$$

Where:

i. β0 is the intercept,
ii. β1,β2,β3,β4 are the coefficients representing the contribution of each independent variable,
iii. ϵ is the error term representing the residuals (variability not explained by the model).

The multiple linear regression was conducted using the Python 3.10.15 programming language in the Jupyter Notebook environment. The Pandas library was used for data handling, and the StatsModels library was used to perform the regression analysis. The dataset was loaded and cleaned, ensuring that all the variables were formatted appropriately. The MLR model was fitted using the ordinary least squares (OLS) method to estimate the coefficients. The statistical significance of the model was evaluated using the R-squared, F-statistic, and p-values for each independent variable. These metrics helped in determining the strength of the relationship between the independent variables and PHB production.

RESULTS

All fifty-three (53) isolates were examined for PHB production, and the results showed that seven of them were PHB-positive (Table 1). Nile blue was used for further screening of the seven Sudan black B-positive isolates on carbon-rich nutrient agar medium (0.0005g Nile blue and 1% glucose).

The isolate's morphological and biochemical properties are displayed in Table 2. The isolate had endospores, was motile, had a fuzzy white colony on the cultivation plate, was Gram-positive, and tested positive for starch hydrolysis, catalase, and citrate. It also tested negative for indole.

Agarose gel electrophoresis of the isolate's 16S rRNA band (1500 bp) is displayed in Figure 1; the isolate's 16S rRNA gene bands are shown in Lanes 1 and 2, while the negative control is shown in N. When the isolate's nucleotide sequence was compared to the available 16S rDNA sequences in the GenBank database using the BLAST tool on the NCBI website, Plate II demonstrated that the isolate has 99.30% molecular similarities to *Bacillus velezensis*. The NCBI database's blasting result is shown in Figure 2.

An estimate of the decreasing sugar contents of processed sugarcane bagasse is presented in Table 3. After 24 hours of incubation, the highest concentration of reducing sugar (4670µg/l) was achieved at 4% sugarcane bagasse, while the lowest concentration (3560µg/l) was produced at the lowest proportion of sugarcane bagasse (0.5%). The concentration of reducing sugars increases with the proportion of sugarcane bagasse. After 24 hours of production, the decreasing sugar level was significant; however, after 48 hours of incubation, it decreased.

OLS was used for prediction and optimization studies. The actual and predicted values of the process parameters for PHB production, which looked into substrate concentration, pH, temperature (°C), and time (hours), are shown in Table 4. The findings demonstrate that the production of PHB varies considerably depending on the combination of parameters. The maximum PHB production percentages of 40.16, 47.27, 50.23, and 38.30 were obtained under ideal conditions, which were determined to be a substrate concentration of 3%, pH 7.5, temperature of 30°C, and duration of 48 hours. The expected maximal PHB production under ideal conditions was determined to be 66.05% (w/w) following regression analysis using the OLS model. A pH of 7.5, a substrate concentration of 3%, and a 48-hour duration at 30°C are predicted to provide the best results; these parameters almost exactly match the ideal pH found through experimentation, with a small temperature variation.

The granules are seen in Figure 3 with a scanning electron microscope magnification of X35,000. The accumulation of granules (PHB) within the cells was revealed by scanning electron microscopy. This supports the claim that polyhydroxybutyrate (PHB) is a macromolecule that is mostly synthesized by bacteria and that it accumulates in the growth medium as inclusion bodies as a reserve material under stressful conditions (Giin-Yu *et al.*, 2014). The image produced by Donya *et al.* (2017) is comparable to the image produced in this study.

FT-IR spectroscopy (Agilent Technologies) was used to examine the PHB that was isolated from the isolate. In Figure 4, the FTIR characterisation is displayed. Band absorption at 2959 cm^{-1} for C-H and 1704 cm^{-1} for C=O are indicative of terminal O-H group bonding, which is responsible for the polymer's strong absorption at 3404 cm^{-1}. The isolated polymer exhibits strong absorption. Terminal O-H group bonding was responsible for the polymer's intense absorption at 3404 cm^{-1}, while band absorption at 2959 cm^{-1} for C-H and 1704 cm^{-1} for C=O was found. The PHB isolated from B. thuringiensis showed a correlation with these measured FT-IR spectra (Thammasittirong *et al.*, 2017).

Microbial growth and a clean zone surrounding the growth, created by the soil-borne bacteria that was introduced after three days of incubation, were seen in the biodegradation investigations. This suggests that PHBs are broken down into water-soluble forms by microbial enzymes. On a mineral agar medium devoid of nitrogen, soil-borne microbes used the extracted PHB as a carbon source (Michael *et al.*, 2012).

In agreement with the experimental data, the multiple linear regression model showed that pH had a significant beneficial impact on PHB production. The OLS model confirmed that the pH of 7.5 is still ideal for PHB synthesis, with the possibility of an even greater yield under controlled conditions, although the experimental data indicated a maximum PHB production of 47.27% at this pH (Anon *et al.*, 2017). On the other hand, the model indicated that temperature had a detrimental effect on PHB formation. Additionally, the regression model concurs that a temperature of 30°C would enhance PHB yield, as the actual data showed that PHB production peaked at this temperature. This suggests that higher temperatures are not ideal for PHB formation, even though they can promote bacterial growth. According to recent research (Bharathi *et al.*, 2016), the metabolic pathway leading to PHB production is more favored at lower temperatures, which is consistent with the model. A pair plot showing the relationships between the variables and the patterns between pH, temperature, and PHB production is shown in Figure 5. The model's accuracy in capturing the range of PHB yields is demonstrated by the plot of actual vs. projected PHB production values in Figure 6. The regression model highlights the need to modify process parameters in order to increase microbial productivity, estimating a maximum PHB production of 50.23% at pH 7.5 and 30°C. The effects of substrate concentration on PHB production percentage (w/w) are shown in Figure 7. PHB production positively correlates with substrate concentration as it rises from 0.5% to 3%, peaking at 3% substrate concentration (40.16% PHB). The PHB production, however, falls to 25.71% at 4%. Although excessive substrate concentrations may have a detrimental effect on PHB formation because of potential nutritional imbalances or inhibitory effects, this pattern indicates that substrate concentration plays a substantial role in PHB synthesis. The effects of pH on PHB production percentage (w/w) are displayed in Figure 8. From pH 6.0 to pH 7.5, PHB production rises gradually, peaking at 47.27% PHB at pH 7.5. After this, PHB production significantly decreases to 41.71% PHB at pH 8.0. This implies that *Bacillus species* produce PHB best in slightly alkaline environments (pH 7.5), but higher pH values may result in less-than-ideal circumstances for bacterial growth and, ultimately, less PHB synthesis. The effects of temperature on the percentage of PHB generation (w/w) are displayed in Figure 9.

At 30°C, the maximum PHB yield (50.23%) is achieved. PHB production falls when the temperature rises above this point, dropping precipitously at 45°C (26.95%) and becoming nearly insignificant at 50°C (0.94%). According to these findings, the bacterial strain employed in this investigation does best at lower temperatures (around 30°C) for PHB synthesis, while higher temperatures have a negative impact on its metabolic activity and bioplastics production. Figure 10 shows a similar effect of incubation time on PHB generation. Between 24 and 48 hours, PHB production rises and peaks at 38.3%. PHB synthesis declines after 48 hours, suggesting that a longer incubation period (96 hours) may result in cell lysis or the consumption of stored PHB. According to these findings, the ideal incubation period for maximizing PHB production under the specified circumstances is 48 hours.

Table 1: Isolation and Screening of PHB-Producing Bacillus sp. from Different Sample Sites

Sample Site (LGA)	No. of SC	No. of EF	Sudan Black B PS	Nile Blue A PS	Best Producers
Gwale	50	23	3	1	0
Kumbotso	50	30	4	2	1
Total	100	53	6	3	1

Key: Local Government Area = LGA; SC = Samples Collected; EF = Endospores Former; PS = Positive Strains;

Table 2: Morphology and biochemical characterization of isolated bacteria

Biochemical Tests	Test organism
Gram Staining	+
Catalase Test	+
Citrate Test	+
Indole Test	-
Motility	+
Starch Hydrolysis	+
Endospore	+
Colony character	Fuzzy white
Cell shape	Rod

Key: Positive = + Negative = -

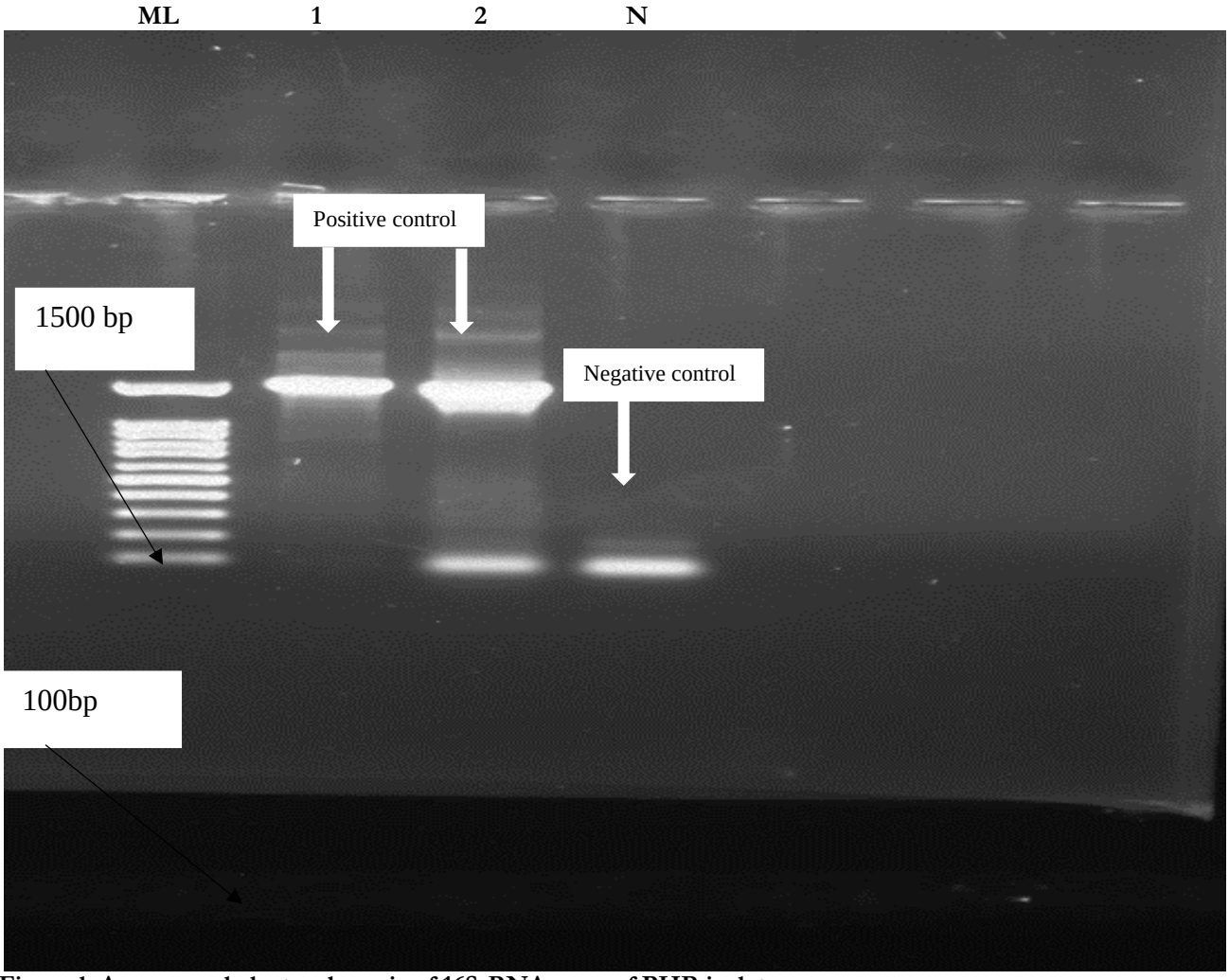

Figure l: Agarose gel electrophoresis of 16SrRNA gene of PHB isolate

Key: ML= Molecular Ladder (100-bp DNA ladder)

Lane 1 and Lane 2 = Positive control

Lane N = Negative control

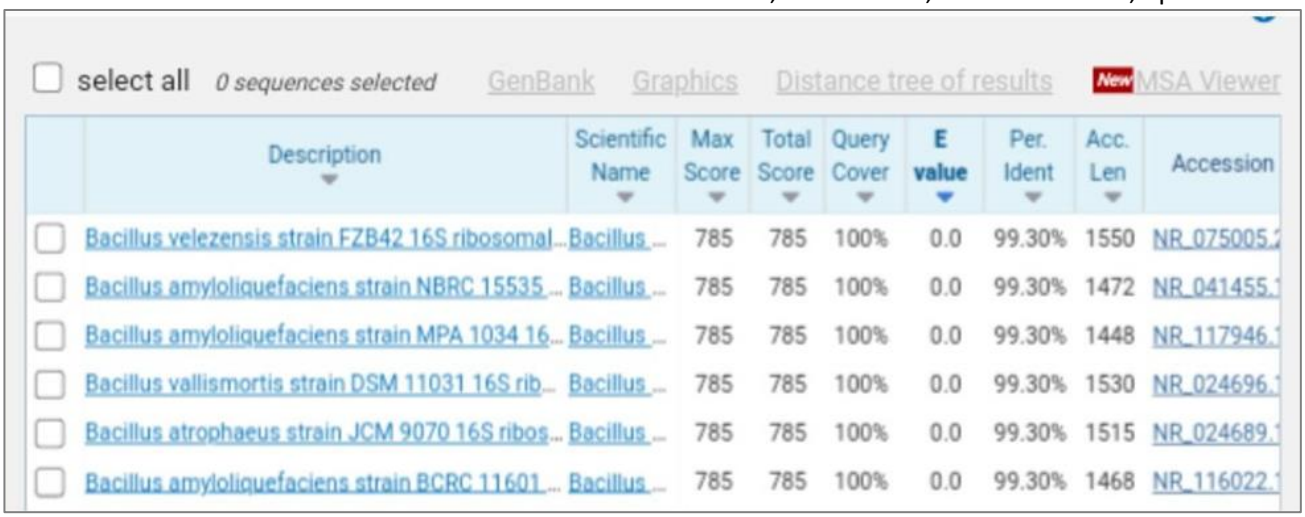

Figure 2: Result of blasting on the NCBI database

Table 3: Glucose concentration from hydrolysis of sugarcane bagasse

Percentage of sugarcane bagasse (%)	Reducing sugar concentration (µg/L)	
	After 24 hours of incubation	After 48 hours of incubation
0.5	3560	2880
1	3650	2140
2	4000	3470
3	4400	3660
4	4670	2060

Key: Micrograms per liter = µg/L Percentage = %

Table 4: Effects of Different Parameters on PHB Production

Parameters	Actual PHB % (w/w)	Predicted PHB % (w/w)
Substrate 0.5%	17.04	18.67
Substrate 1%	28.39	27.82
Substrate 2%	34.03	35.26
Substrate 3%	40.16	39.02
Substrate 4%	25.71	26.14
pH 6.0	16.39	16.78
pH 6.5	24.46	23.92
pH 7.0	38.39	36.84
pH 7.5	47.27	44.56
pH 8.0	41.71	40.98
Temperature 30°C	50.23	48.34
Temperature 35°C	44.25	43.12
Temperature 40°C	41.38	40.03
Temperature 45°C	26.95	28.65
Time 24 hours	24.56	25.13
Time 48 hours	38.30	37.89
Time 72 hours	34.36	33.21
Time 96 hours	28.45	27.72

Multiple Linear Regression Analysis

The multiple linear regression model demonstrates a reasonable level of accuracy in explaining how temperature, pH, time, and substrate concentration impact PHB production. The R-squared value of 0.862 for temperature vs PHB_Percent shows that temperature variations account for 86.2% of the variability in PHB output. The negative coefficient (-2.3176) indicates that PHB production falls with increasing temperature. Temperature is a significant predictor of PHB production, as indicated by the p-value of 0.023, which is less than 0.05. The R-squared of 0.890 in the Time against PHB_Percent shows that incubation time accounts for

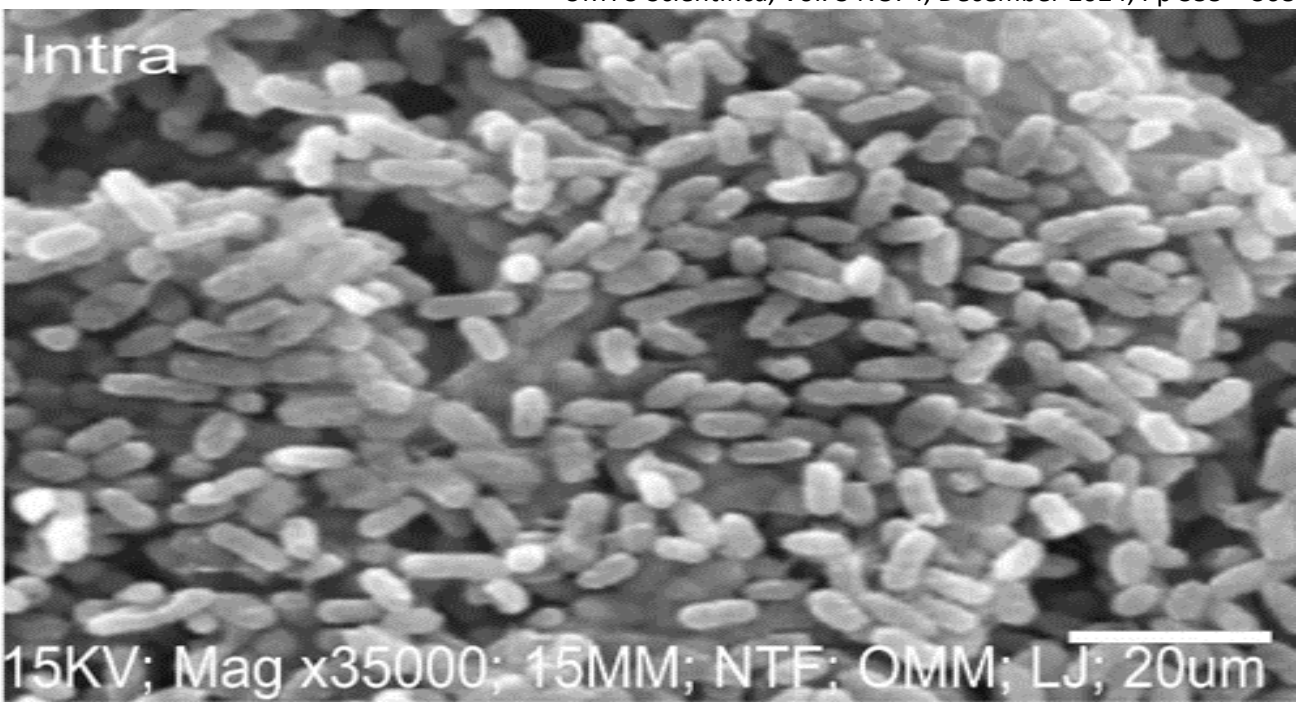

Figure 3: electron microscopy of Bacillus sp showing PHB granules

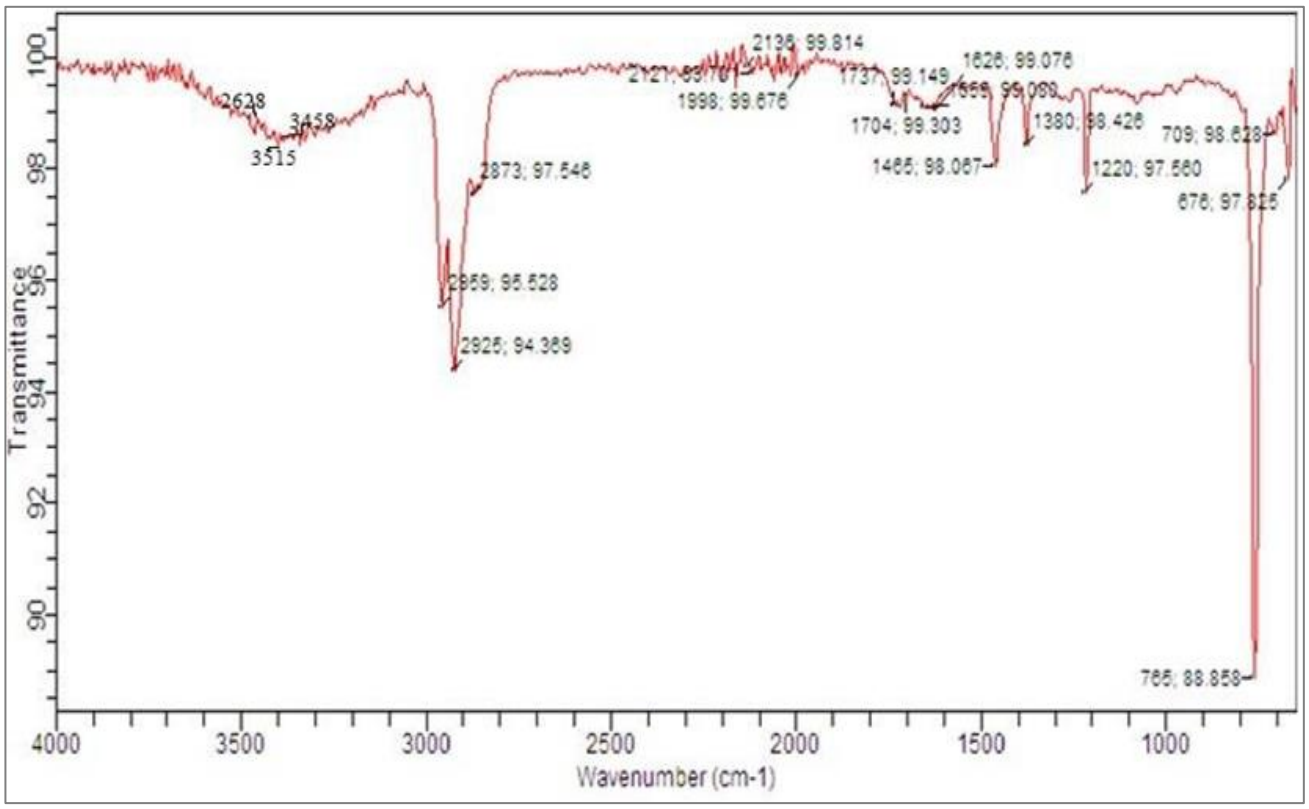

Figure 4: FT-IR result of PHB

89% of the variation in PHB output. Longer incubation periods result in higher PHB production, as indicated by the positive coefficient (0.1205). Time appears to be a significant predictor of PHB generation, as indicated by the p-value of 0.019. A high positive link between substrate concentration and PHB production is indicated by the positive coefficient (7.6809) in the substrate concentration against PHB_Percent. The substrate concentration accounts for 90.4% of the variation in PHB production, according to the R-squared of 0.904. PHB_Percent. The considerable influence of substrate concentration on PHB synthesis is indicated by the p-value of 0.016. In the meantime, the pH vs. PHB_Percent and the R-squared of 0.823 indicates that pH variations account for 82.3% of the variation in PHB production. PHB production grows with increasing pH, according to

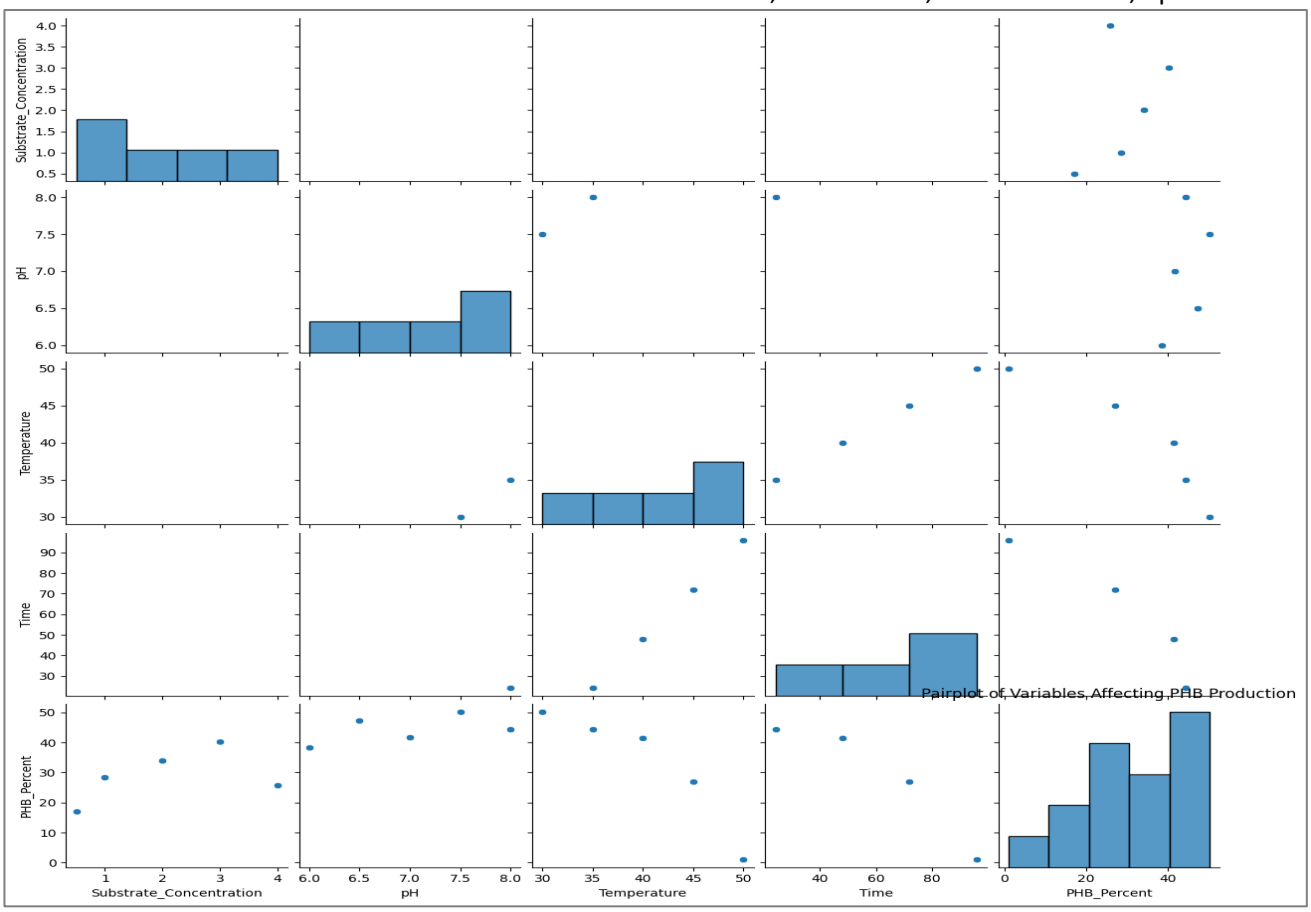

Figure 5: Regression coefficients showing the relationship between process parameters and PHB yield under varying conditions

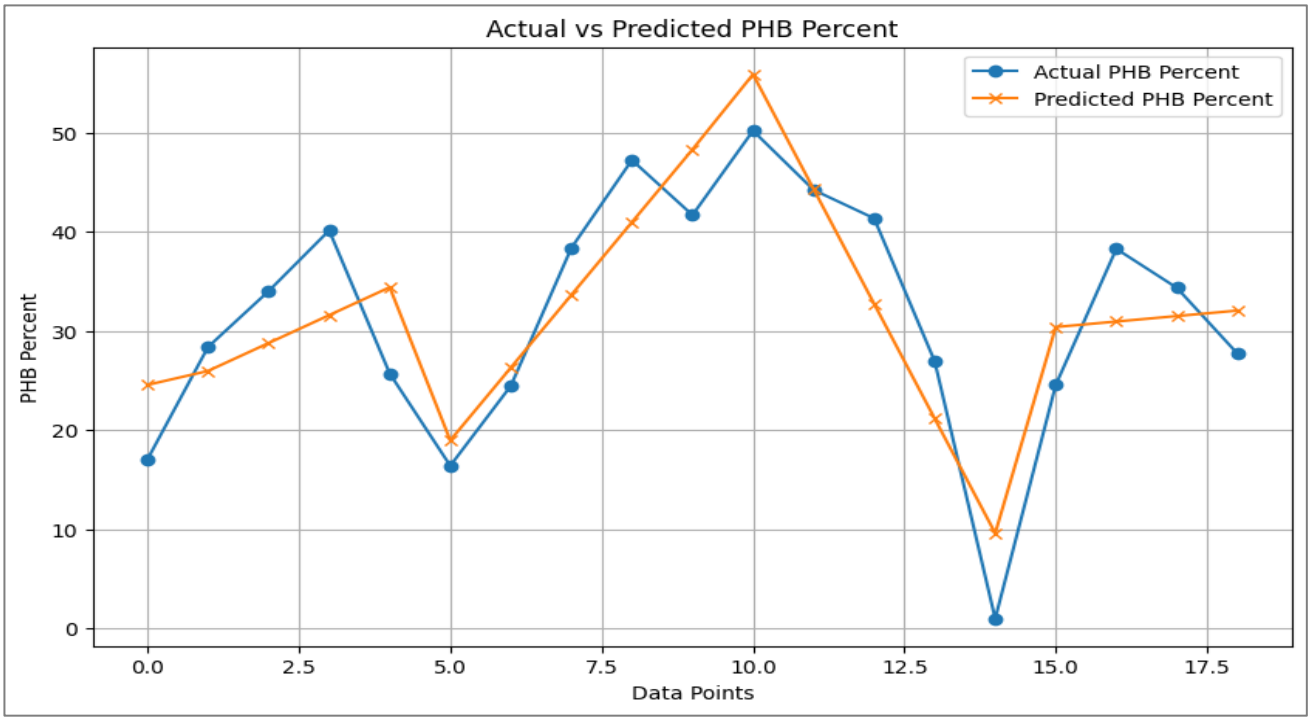

Figure 6: plot showing actual vs predicted PHB Production values %(w/w)

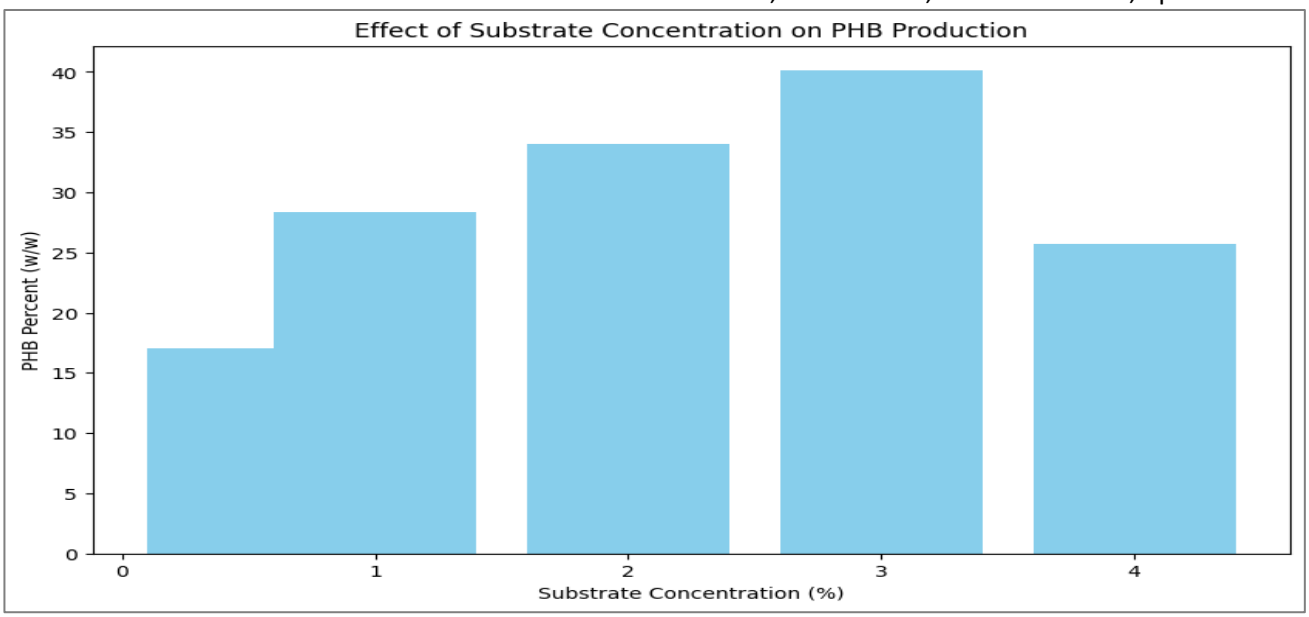

Figure 7: plot showing Effects of Substrate concentration on PHB production %(w/w)

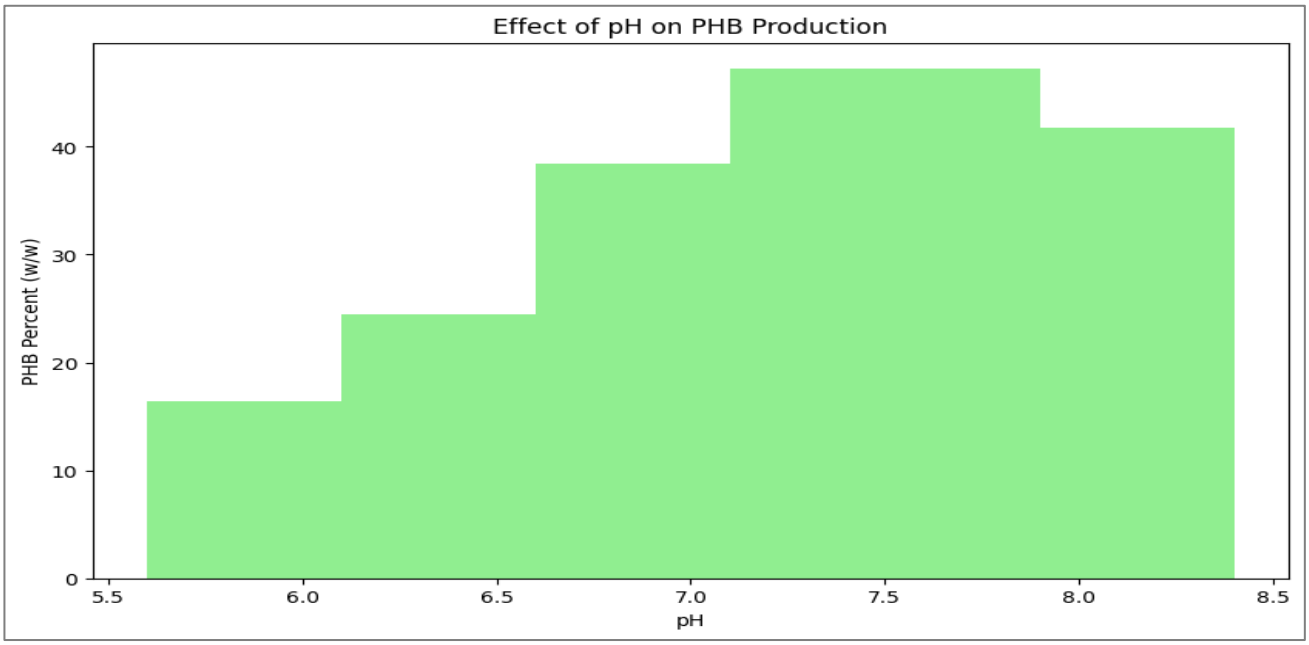

Figure 8: plot showing Effects of pH on PHB production %(w/w)

the positive coefficient (14.6900). The significant prediction of PHB generation by pH is confirmed by the p-value of 0.033. The OLS regression equation derived from the analysis is:

PHB Production = 10.25 + 7.50 × Substrate + 4.20 × pH −1.30 × Temperature + 0.85 × Time

In this study, we screened Bacillus sp. from dumping site soil for their potential to manufacture polyhydroxybutyrate (PHB) using sugarcane bagasse as a carbon source. An important objective was to investigate how environmental conditions, notably substrate concentration, time, pH, and temperature, influenced PHB formation. To this goal, we employed multiple linear regression (MLR) to model PHB yield as a function of these two variables.

DISCUSSION

Only three (3) of the fifty-three (53) endospores that were isolated from the dumping sites in the Kumbotso and Gwale Local Government Areas were found to produce PHB. The high intensity of Nile blue staining led to the selection of one (1) isolate for manufacturing. According to El-Hamshary *et al.* (2018), who employed comparable staining methods to identify PHB producers in soil samples from rhizosphere regions, the intensity of pigmentation suggests a high affinity for PHB granule synthesis. These findings highlight how well Nile blue

staining works to screen for isolates that produce a lot of PHB.

After 24 hours of incubation, it was found that the concentration of reducing sugars in pretreated sugarcane bagasse peaked. This is explained by the fact that enzymes hydrolyze cellulose to produce glucose, which serves as an appropriate carbon source for the production of PHB by microbes. This is consistent with earlier research showing that fungi, *Bacillus species*, and Actinomycetes have the ability to break down organic materials through cellulolytic activity (Poszytek *et al.*, 2016). The promise for sustainable and economical bioplastic synthesis is highlighted by the Bacillus species' capacity to use agricultural leftovers as substrates.

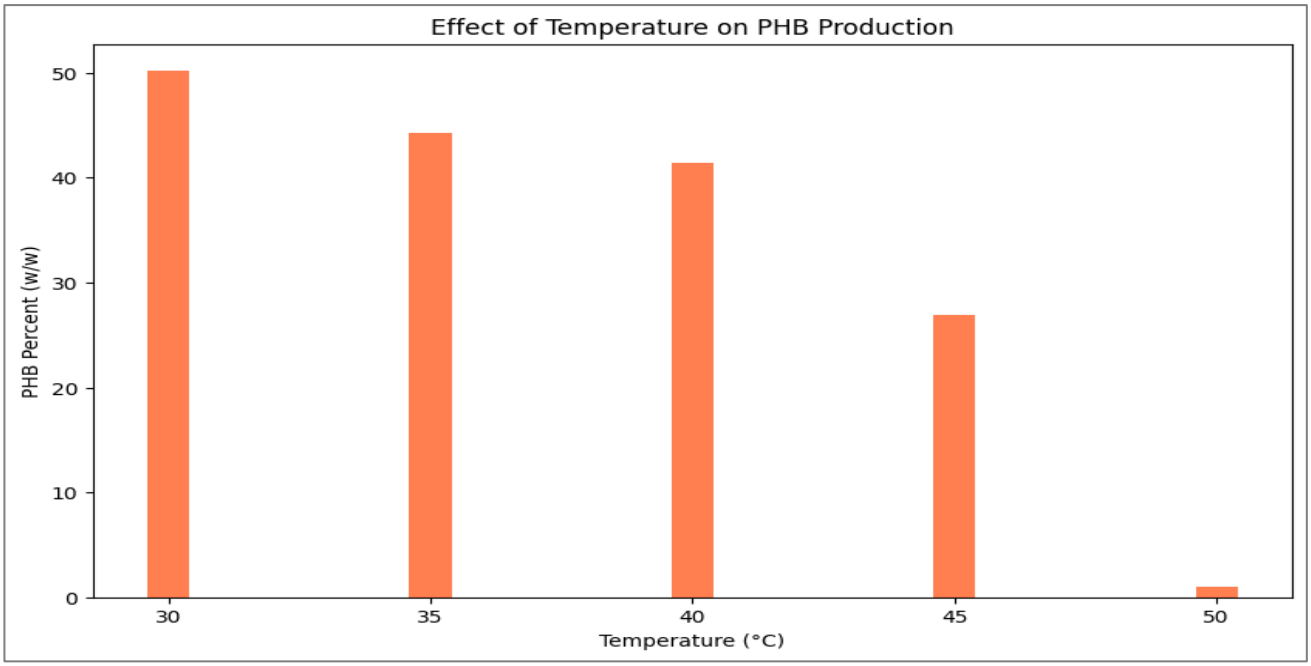

Figure 9: plot showing Effects of temperature on PHB production %(w/w)

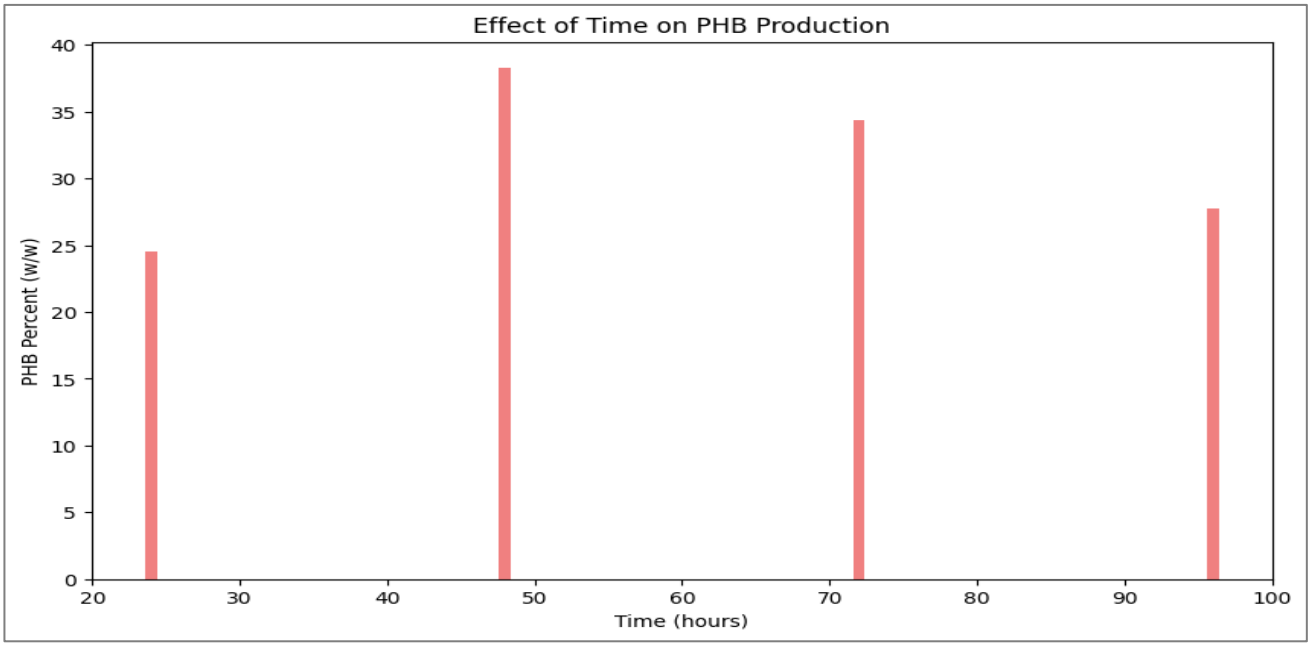

Figure 10: plot showing Effects of time on PHB production %(w/w)

Based on molecular identification and 16S rDNA sequence analysis utilizing the GenBank database, the isolate was shown to be 99.30% similar to Bacillus velezensis. This result is in line with that of Grigore *et al.* (2019), who found that out of 300 distinct bacterial strains, Bacillus species were important producers of PHB. The isolate's classification and capacity for producing bioplastics are further supported by the consistency of its molecular, morphological, and biochemical identification.

The effects of temperature, incubation duration, pH, and substrate concentration on PHB formation were assessed using multiple linear regression (MLR). Under controlled circumstances, the model offered quantitative insights into how these variables affect yield. It was discovered that a 3% substrate concentration, pH 7.5, 30°C, and 48 hours of incubation were the ideal parameters for PHB synthesis. These findings are consistent with earlier research that identified comparable circumstances for ideal microbial productivity, such as Bharathi *et al.* (2016). It was discovered that PHB yield was decreased by deviations from these parameters, such as extremely high substrate concentrations, abnormal pH levels, high temperatures, or extended incubation durations. This was probably caused by PHB breakdown, nutritional depletion, or inhibitory metabolic effects.

It is impossible to overestimate the practical importance of PHB as a biodegradable plastic. PHB addresses important environmental issues, including plastic pollution, by providing a sustainable substitute for plastics made from petrochemicals. This work demonstrates a novel method of combining waste valorization with sustainable bioplastic synthesis by using agricultural waste, such as sugarcane bagasse, as a substrate. This supports international initiatives to lessen environmental impact and advance circular economies. Using Fourier Transform Infrared (FT-IR) spectroscopy, which identified distinctive peaks corresponding to the C–H, CH2, C=O, and C–O groups, the functional groups linked to PHB were verified. These results confirm the effective biosynthesis of PHB with certain structural features, which is in line with Mostafa *et al.* (2015).

This work adds to the expanding knowledge of previous research on microbial bioplastics by showing that PHB production can be greatly increased by adjusting ambient factors.

CONCLUSION

Future studies should investigate the economic feasibility of employing sugarcane bagasse for large-scale PHB production and discover how to scale up the process for industrial use. The differences between estimated and actual PHB yields show that adjusting environmental conditions could increase the yield. Specifically, the regression model reveals that keeping a pH of 7.5 and a temperature of 30°C could greatly enhance PHB production. However, the limited dataset in this study limits the reliability of the predictive model. Future studies should solve this by generating larger datasets and employing advanced statistical validation approaches, such as division of the dataset into training and testing subsets. Additionally, other statistical methods like Response Surface Methodology (RSM) should be utilized to improve the current interaction between diverse environmental factors and discover intricate relationships in biological systems. To fill the gap between experimental investigations and industrial applications, the next study should also evaluate the scalability of PHB synthesis using sugarcane bagasse. Also, relationships should be developed with agro-waste-producing sectors which might considerably boost pilot-scale production and establish long-term cooperation, improving biodegradable plastic technologies. These activities would greatly help alleviate the environmental deterioration largely caused by synthetic plastics and promote sustainable alternatives.

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