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Investigating the possibility of producing Polyhydroxyalkanoates (PHAs) Using Hydrolysates of Napier grass by Burkholderia Sacchari strain

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Abstract

Bacteria produces Polyhydroxyalkanoates (PHAs), which are macromolecules. They are inclusion bodies that build up as reserve resources while bacteria grow under various stress situations. PHAs have been chosen as alternatives for the development of biodegradable polymers due to their rapid degradability under natural environmental circumstances. This study aimed to produce reducing sugars from Napier grass for use as a carbon source in polyhydroxyalkanoate (PHA) synthesis. The effects of temperature, pH, and culture time on growth yields were adjusted using Response Surface Methodology using a Box-Behnken design. Burkholderia Sacchari strain was chosen from more than 300 bacterial strains capable of accumulating PHA strains based on its ability to consume both hexose and pentose sugars and has a capacity of accumulating high mass PHA granules inside its cells. At 200 rpm, optimal pH, fermentation temperature, and incubation time for the isolate to produce the highest PHA were 7, 35 °C, and 48 hours, respectively. When hydrolysates of Napier grass was employed as carbon sources and ammonium sulphate as nitrogen sources, the strain was able to collect 62.1% PHA from its total biomass. The extracted PHAs' FTIR spectra show strong peaks at wavenumbers that are unique to PHAs as C–H, CH2, O-H, C=O, and C–O groups. The extract's resemblance appropriateness for bioplastic production were validated by UV–Vis spectrophotometric analysis.

1. Introduction

With an ever-growing world population, climate change, globalization and industrialization; have intensified the uncontrollable and rapid use of non-renewable energy sources such as fossil fuels and minerals as planet earth is losing its natural resources [1]. While the growing environmental problem has many sources, the most common organizations directly or indirectly involved are synthetic polymers, in particular plastics. Petrochemical plastics such as polyethylene terephthalate (PET), polyvinylchloride (PVC), polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyamide (PA) have been commonly used as packaging materials. However, due to the high demand for their use for many purposes (low cost, durability and functional advantages), improper discarding, and environmental persistence, their use has been cut due to their low biodegradability, which causes significant environmental problems [2]. In view of rising energy demand and the decrease of fossil resources, the global economy is currently trying to replace recognized energy sources with greener, bio-based and renewable alternatives [3,4]. Environmental, health, and political concerns, as well as the problems, motivated the development of bioplastics such as Polyhydroxyalkanoates (PHAs); polyesters produced during the secondary metabolism of bacteria and archaea in the presence of excess carbon sources and insufficiency of nutrients inside the cell's cytoplasm, allowing for a lower environmental impact [5]. In the PHAs group, polyhydroxybutyrate (PHB) is gaining enormous consideration due to its extraordinary characteristics, such as a higher degree of crystallinity, tensile strength, and melting temperature (175 oC) with low permeability for H2O,O2, and CO2 [6].

The major challenge for industrial production of PHA is the reliable and scalable supply of carbon sources, as it makes up to 48-50% of PHA production costs [7,8]. Recently, great work has been devoted to lowering the cost of PHA production by employing strategies such as establishing efficient bacterial strains, optimizing fermentation and recovery processes, and so on. So, producing PHAs using cheap carbon source would reduce the production cost. According to Bugnicourt et al. biodegradable bio plastics are basically divided in to the groups according to their sources: biomass based (Proteins, Lipids, and Polysaccharide), microorganism based (PHA) and biotechnology based (Polyactides and Polylacticacid) [9]. In sustainable PHA production using non-edible, industrial and agricultural wastes, abundant and renewable carbon sources such as waste biomass are used. This will not only ensure agreement with zero waste policies but also enable upcycling of the generated wastes within bio refineries to high value products [10,11]. Cost optimization production process will be done by using waste materials (cheap carbon sources), recombinant strains techniques and use of mixed microbial culture (MMC) with open systems

that do not require sterile conditions, coupled to that of wastes or surplus based feeds [12,13].

Because of its high availability and low cost worldwide, lignocellulosic biomass, which mainly consists of cellulose, hemicellulose, and lignin as main component, has recently received increased attention as a promising sustainable and carbon-neutral feedstock for the development of biofuels and platform chemicals [3]. For instance, polysaccharides can be hydrolyzed by using different chemical, physical, enzymatic or combination of those methods from cellulose and hemicellulose, followed by biotransformation into different biofuels such as bioethanol, hydrogen, biodiesel, paper, lumber and bioplastics.

Napier grass is one type of lignocellulosic material that contains cellulose, hemicellulose and lignin as main alignment and has C4 metabolism [14,15]. It is a potential feedstock for production of biofuel like biogas [16], bioethanol [17] and bio butanol [18] as it has strong adaptableness, high growth rate, high biomass yield, drought resistance and wide availability properties. In order to extract monosaccharides from Napier grass, different pretreatment methods are being investigated by different researchers. Cellulose ethanol derived from Napier grass has been extensively studied by Yasuda et al by using Low-Moisture Anhydrous Ammonia (LMAA) Pretreatment combined with enzymatic hydrolysis of Napier grass to obtain a total sugar yield of 0.35 g- sugar per g-biomass, ethanol yield of 74.1% by using Escherichia coli [19]. Napier grass has been also converted to butanol by pretreatment, enzymatic hydrolysis and ABE fermentation [18].

In this study, Napier grass was chosen for carbon source and undergoes with NaOH pretreatment and enzyme hydrolysis to extract monosaccharides to produce PHA. To the best of the author's knowledge, this is the first study to explore the use of Napier grass as a feedstock to produce PHA.

2. Materials and methods

2.1. Materials

The raw material, mature stems of Napier grass (*Pennisetum purpureum*), was obtained from Menagesha Mushroom Farm in Ethiopia (Figure 1). The fresh biomass was milled first, then sun-dried for two weeks to reduce moisture content considerably. Once suitably cured, the material was manually cut into small segments of 1-2 cm length with scissors and hand cutters. The chopped grass was further refined by passing it through a mechanical cutting mill, which produced fine, consistent particle sizes. The material was categorized by running it through a standard 150 µm sieve to ensure homogeneity for future use.



Figure 1. Napier grass Processing

2.2. Methodology

Figure 2 depicts the overall experimental methodology, which consists of four sequential stages: (1) producing glucose from Napier grass, (2) inoculating, propagating, and fermenting using that glucose, (3) extracting and purifying the synthesized PHA, and (4) characterizing the final PHA product.

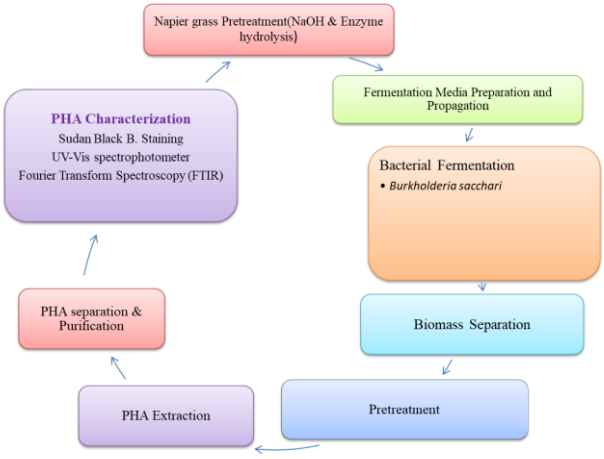


Figure 2. Major Steps in PHA Production Process

2.2.1. Alkaline Hydrolysis

In a sealed Erlenmeyer flask, 50 g of the Napier grass sample was submerged in 300 mL of 2 % (w/v) NaOH (a solid to liquid ratio of 1:6). The NaOH pretreatment bottles were autoclaved at 121 oC for 1 hour. After that, they were allowed to cool to ambient temperature [20]. Following the pretreatments, each pretreated solid was separated from the liquid fraction using a filter. The solid portions were rinsed in distilled water until their pH was neutral. Following filtering, the solid residue was dried for at least 48 hours in a hot-air oven at 80 ^{o}C before being kept in a sealed plastic bag at room temperature for later enzymatic hydrolysis. .

2.2.2. Enzymatic Hydrolysis

Enzyme hydrolysis was done according to Kongkeitkajorn et al [21,22] methods. Multi component Cellulase (Novozymes, Denmark) was used to hydrolyze pretreated grasses. In a 500-mL laboratory container, 100 mL of 50 mM citrate buffer solution (pH 5.0) was mixed with the pretreated grass with loading of 15 %(W/V). After that, the enzyme was added at a loading volume of 2 ml/g substrate and combined, and the mixture was incubated in a water bath at 50 oC with continual mixing for 72 hours with shaking at 150 rpm. 0.5 g/L sodium azide was added to the process to avoid microbiological contamination. By boiling the contents for 5 minutes, the hydrolysis reaction was halted. After all samples are withdrawn, the hydrolysate was centrifuged for 15 minutes at 4 oC and 4000 rpm, and the supernatant was tested for total reducing sugar using the DNS method.

2.2.3. Inoculation

In this investigation, Burkholderia sacchari, which was recently reclassified as Paraburkholderia sacchari, brought from Switzerland, was utilized. To grow the organism, Luria Bertani broth (NaCl, 5 g/L; Tryptone, 10 g/L; and yeast extract, 5 g/L) was employed. In a 500 mL shake flask, 100 mL of media was inoculated with one overnight culture from an agar slant. The bacterial isolates were incubated for 24 hours at 30 oc and 150 rpm [23]. After 24 hours, the entire 50-mL culture was transferred aseptically to a fresh 250-mL LB broth culture (total 300 mL; enclosed in a 500-mL Erlenmeyer flask) and cultured as previously described.

2.2.4. Fermentation Media Preparation, Propagation and Fermentation

The following mineral salts media (MM) composition was modified to restrict nitrogen availability (in g/L): KH2PO4, 9.0; Na2HPO4 2H2O, 3.0; (NH4)2SO4, 2.0; MgSO4·7H2O0.2 g; CaCl2·2H2O, 0.02,; (NH4)5Fe(C6H4O7)2, 0.03 and trace elements solution (2 mL/L) [24]. The media was made in bulk and then distributed evenly across the test flasks in accordance with the protocols devised for maximum growth. This prior culture was applied as an inoculum to mineral salts media (10% v/v) [25]. Separate autoclaves were used for the MgSO47H2O, CaCl2, 2H2O, Hydrolysates and (NH4)5Fe(C6H4O7)2 solutions were added to the medium aseptically. Glucose rich hydrolysates were added 5(%W/V). pH was adjusted to the experimental run by adding NaOH (5M) or H2SO4 (5M) and 200 rpm in a shake incubator.

2.2.5. Measurement of Dry Biomass

Dry biomass is measured by taking samples (10 mL) from the culture media at set intervals and centrifuging them at 8000 rpm for 15 minutes. Remove supernatant and collect pellet at the bottom by rinsing with distilled water. The pellets were dried at 70°C, and weighed in an analytical scale to constant weight. The pellet samples are kept at room temperature in a dry place until PHA extraction is performed [26].

2.2.6. PHA Extraction

Solvent extraction followed by non-solvent precipitation was employed to recover [27]. Following that, the lyophilized cells were placed in a conical flask with 100 mL sodium hypochlorite (5%) and 100 mL chloroform. For 12 hours, the mixture was stirred in a rotary shaker at 200 rpm and 30 °C. The suspension was placed in a separating funnel and allowed to stand for 30 minutes to allow the three phases to separate: an upper phase containing hypochlorite solution, a middle phase containing non-PHA material and a bottom phase containing PHA solubilized in chloroform. To precipitate the PHAs, the bottom phase was decanted into a beaker and nine parts methanol were added. Finally, the PHA-precipitate was dried at 30 °C by evaporation, yielding white flakes or powder [28].

2.2.7. Determination of Total Reducing Sugar using DNS Method

In the manual approach, 30 ml of modified DNS reagent was added to 10 ml of sample plus 10 ml of distilled water in a test tube, which was then heated in a boiling water bath for 15 minutes and chilled for at least 15 minutes. The absorbance was then measured at 540 nm. Several modifications in sample volume and wavelengths were investigated. Glucose standard curves were developed [29].

2.3. Characterization

2.3.1. Screening by Sudan Black B. Stain

After PHA production was established, ten milliliters of media were centrifuged at 6000 rpm for 20 minutes and Sudan staining was performed to confirm PHA production. A smear of 72 hour old strains was stained for 15 minutes with 0.3 percent (w/v) Sudan Black B. solution, then destaining with alcohol (50 percent v/v) and counterstaining with safranin solution (0.5 percent, w/v) [11].

2.3.2. UV–Vis Spectrophotometer Analysis of PHA

The extracted PHA was dissolved in chloroform and scanned in the range of 200–320 nm against a chloroform blanks a Perkin-Elmer Lambda 950 UV/VIS spectrometer, with the spectrum evaluated for a strong peak at 240 nm [30].

2.3.3. FTIR spectrophotometer analysis of PHA

PHA was validated using FTIR spectroscopy after 1 mg of sample and 10 mg of spectral pure anhydrous potassium bromide crystals were crushed thoroughly and formed into a pellet and relative intensity of transmitting light energy was evaluated against the wavelength of absorption 4000–400 cm^{-1} [10].

3. Results And Discussion

3.1. Benedicts' Test

The benedict test indicates that the color of the hydrolysate was changed to brick red after it was mixed with benedict solution and heated for 5 minutes and the color changes to brick red as it was shown in Figure 3, signifies the solution has more than 2% of reducible sugar [31]. Benedict's reagent comprises blue copper (II) sulfate (CuSO4)5H2O, which is reduced to red copper (I) oxide by aldehydes, resulting in carboxylic acids being formed. Copper oxide is water insoluble and thus precipitates. Depending on how many copper (II) ions are present, the color of the final solution can range from green to brick red [32,33]. Color change to bricks red indicates the

pretreated Napier grass using NaOH and Cellulase enzyme hydrolysis produces significant amount of reducible sugars that can be used as a feedstock for fermentation process.

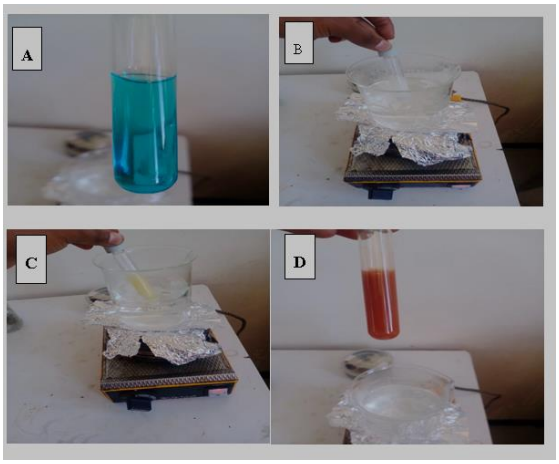


Figure 3: The Benedict Test of the Produced Reducible Sugar

3.2. Total Reducing Sugar Analysis of the Hydrolysates

The DNS approach was used to estimate sugar reduction [29]. To estimate the total reducing sugar after alkaline pretreatment and enzymatic hydrolysis, a standard curve was performed by the DNS technique. The sample absorbance at 540nm was computed from the calibrated data, and the total reducing sugar content was determined to be 28.74 g L-1. In this study, 3.2 g of sugar was recovered, which is less than a previous study that yielded 3.9 g of sugar from a 5 g sample load [18]. This indicates from Napier grass, high amount of reducible sugars were produced using NaOH and Cellulase enzyme, is a potential candidate for PHA production. Higher quantities of fermentable sugars in the hydrolysate are preferred for fermentation to generate higher PHA concentrations. The major cause of this disparity is the age during cultivation and kind of Napier grass used for this investigation.

3.3 Sudan Black B. Staining

Sudan Black B staining was used to detect PHA buildup within the bacterial isolate. This technique distinguishes PHA-producing cells by coloring the intracellular polymer granules with a distinctive blue-black color. The data, shown in Figure 4, show a considerable favorable response. A quantitative study of the stained slide revealed that revealed that approximately two-thirds of the total cell count exhibited positive staining. This high frequency of accumulation, consistent with [34]. Findings, demonstrates that PHA biosynthesis is resilient and efficient under the test conditions.

This approach takes advantage of the Sudan Black B dye's lipophilic properties, which have a high affinity for the hydrophobic surfaces of intracellular PHA granules. When applied to fixed cell cultures put on glass slides, the dye preferentially enters the cell wall and membrane and binds to the lipid inclusions. This interaction produces a strong visual difference under bright-field microscopy: PHA-accumulating cells have a pronounced blue-black coloration, whereas non-producer cells are unstained or have a weak, counterstained hue.

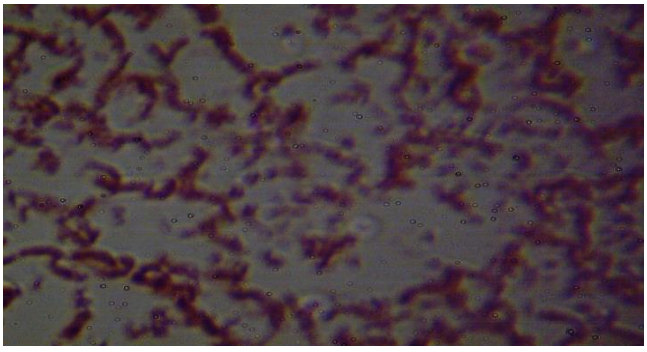


Figure 4: Microscopic Image of PHA Granules by Sudan Black B. Staining

3.4 UV-Vis Spectrophotometer Analysis of PHA

In this study, chloroform was chosen as the solvent blank because of its well-documented efficacy as a superior solvent for a wide range of polymeric compounds, including plastics and, more specifically, Polyhydroxyalkanoates (PHA) [35]. Its application is crucial in ultraviolet-visible (UV-Vis) spectrophotometry because it offers a baseline absorbance profile against which the analytes individual absorbance may be correctly measured. The presence and content of PHA in the extracted samples were next assessed by measuring their absorbance patterns across the UV spectrum, from 200 to 320 nm, as shown in Figure 5.

This quantitative analysis is based on the Beer-Lambert equation, which states that the absorbance of a solution is directly proportional to the concentration of the absorbing species inside it. As a result, a higher measured absorbance at a specific wavelength correlate directly with a larger concentration of PHA in the chloroform solution [36].

The obtained spectra indicated a prominent and sharp absorption peak at wavelength 240 nm. This spectral characteristic is recognized as a fingerprint for PHA polymers because the polymer's molecular links absorb light strongly at this energy level. The existence of this distinctive peak, rather than a flat or featureless baseline, provides strong confirmation of both PHA granule formation and effective extraction from the bacterial sample. This conclusion is consistent with recognized spectroscopic techniques for PHA identification, as demonstrated by Ranganathan et al. [37]. The sharpness of the peak indicates a relatively clean extract with little

contamination from other biological components that may absorb at different wavelengths [37].

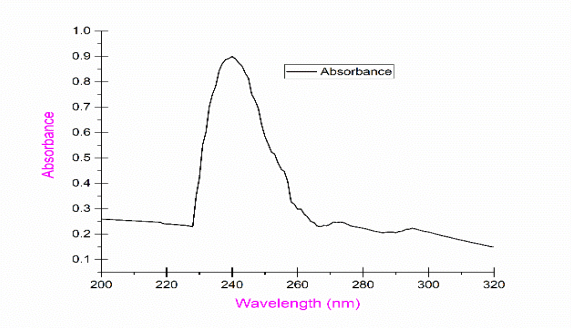


Figure.5: UV- Vis Spectroscopy of PHA

3.5. FTIR Spectrophotometer Analysis of PHA

Fourier Transform Infrared (FTIR) spectroscopy was used to analyze the functional groups of the polyhydroxyalkanoate (PHA) biopolymer derived from Burkholderia Sacchari. This analytical approach is based on the idea that when exposed to infrared radiation, chemical bonds inside a molecule vibrate at specific frequencies, resulting in a distinct absorbance spectrum that serves as a molecular "fingerprint" for the material. Figure 6 depicts the resulting spectrum for the retrieved PHA, and the key aspects are discussed in depth below [38].

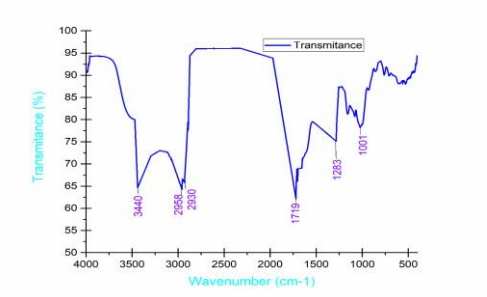


Figure 6: FTIR Spectra of the Extracted PHA

The spectral analysis of the PHA polymer revealed complex absorption patterns indicative of its intricate structure. Notably, a broad absorption band around 3440 cm^{-1} correlates with O-H stretching vibrations, signaling terminal hydroxyl groups that can form strong intermolecular hydrogen bonds. Additionally, distinct peaks in the high-frequency range of 2800 to 3000 cm^{-1} are attributed to aliphatic C-H stretching, with specific peaks at 2930 cm^{-1} and 2958 cm^{-1} corresponding to the asymmetric stretching of methylene (CH2) and methyl (CH3) groups in the PHA monomer units.

The 1700-1750 cm^{-1} spectral region confirms the sample's polyester composition. A prominent absorption band at 1719 cm^{-1} reveals the carbonyl (C=O) stretching vibration of an ester functional group, which is the characteristic of the PHA polymer backbone. The C-O stretching vibration has an additional absorption band at 1283 cm^{-1}, whereas the C-O-C stretching vibration has a band at 1184 cm^{-1}. The peaks C=O stretch (~1720 cm^{-1}), C-O stretch (~1280 cm^{-1}), and C-O-C stretch (~1180 cm^{-1}) confirm the ester functional group that connects the hydroxyalkanoate monomers in the PHA chain [39,40].

In conclusion, the FTIR spectral data support the effective separation of a polyester from Burkholderia sacchari. The combined pattern of absorption bands—from the aliphatic C-H stretches, the prominent ester carbonyl, and the related C-O and C-O-C vibrations—is compatible with the conventional infrared spectrum of Polyhydroxyalkanoates, thus verifying the chemical identity of the collected polymer [3,40].

3.6. Experimental Results on PHA Production

A Box–Behnken design (BBD) was employed to optimize the fermentation medium for achieving high yields of Polyhydroxyalkanoates (PHA). This statistical approach examined three parameters: incubation temperature (A), pH (B), and incubation time (C), with a total of 17 experimental runs conducted to analyze their effects on PHA yield. The results indicated that Trials 1 and 17 corresponded to the lowest and highest PHA yields of 3.15 g L-1 and 7.42 g L-1, respectively (Figure 7), which were attained through varying temperature (33 °C and 35 °C), maintaining pH at 7, and adjusting the fermentation time (24 and 48 hours). The study highlights that PHA accumulation in bacterial cells is primarily facilitated by limiting essential nutrients while providing an excess of carbon sources, as noted by Doi et al. [41].

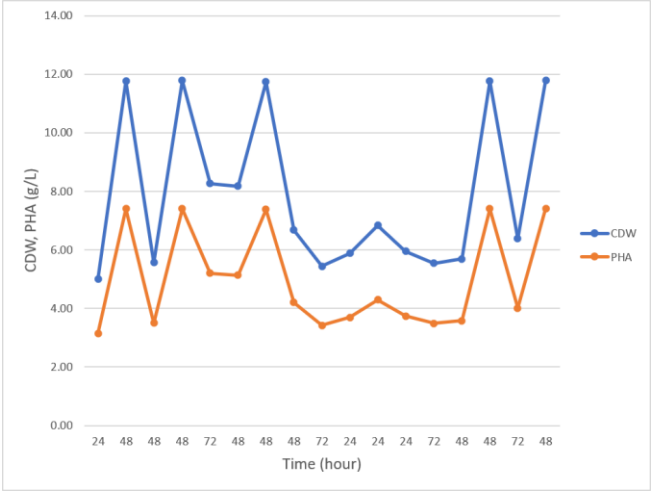


Figure 7: Burkholderia sacchari Growth and PHA Production

In a study of *Burkholderia sacchari*, growth and polyhydroxyalkanoate (PHA) production were examined under varying temperatures, pH levels, and incubation periods. The results, illustrated in Figure 4.6, indicate that

cell growth persisted until nitrogen depletion in the medium occurred, with PHA accumulation beginning at a cell dry weight (CDW) of approximately 5 g L^{-1} and peaking at around 12 g L^{-1}. The optimized medium facilitated the highest PHA production, reaching a maximum of 7.42 g L^{-1} at 48 hours, with a maximum PHA accumulation of 59% from CDW and a minimum

3.7. Interaction Effects on PHA yield

3.7.1. Interaction Effect Between Temperature and pH

When the temperature is increased from 34 to 37 ºC and the pH is increased from 6.5 to 7.5, the yield of PHA varies from 3.15 to 7.42 gL^{-1}. Figure 8 shows that when pH increasing from 6.5 to 7 and temperature from 33 to 35 oc maximum yield of 7.42 gL-1 of PHA is obtained and PHA yield is starts to decrease after pH 7 and temperature of 35 ºC. However, productivity couldn't exceed more than 7.42 gL^{-1} as high temperature slows down the metabolic activity (enzyme activity) of bacteria, reducing their ability to produce PHA [42] and at high and low pH because of its effect on the bioavailability of trace elements and the regulatory enzymes responsible for PHA synthesis [37]. Temperature is a critical factor in bioprocesses. Temperature changes can cause a variety of microbiological reactions. pH may have effect on the bioavailability of trace elements and the regulatory enzymes responsible for PHA synthesis, ketothiolase, acetoacetyl-CoA reductase, and PHA polymerase, pH had a strong influence on PHA accumulation [37]. As a result, the yield was influenced by both pH and temperature at the same time. This finding is comparable to that of de Andrade et al, who produced PHA from Burkholderia sacchari using Cheese whey as a carbon source and co substrate and obtained a higher product at a temperature of 35ºC and a pH of neutral [43] B. sacchari grown with glucose at 35 °C demonstrated increased productivity and polymer output.

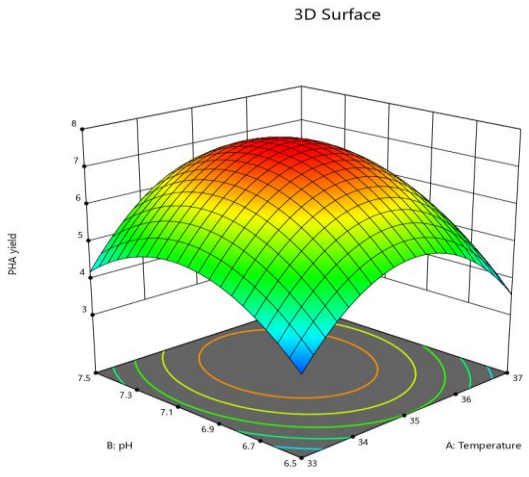


Figure 8: 3D plot of Interaction Effect Between Temperature and pH on the Yield of PHA

3.7.2. Interaction Effect Between Temperature and Fermentation Time

The interactions of temperature and fermentation period had little effect on PHA yield. This conclusion is derived from both quantitative analysis of variance (ANOVA) and visual representation of the response surface model. They both rise at the same time, with minimal interaction. The P-value for the interaction effect of incubation temperature with time in the ANOVA table is 0.5670, indicating that there is no significant interaction between these two independent variables on PHA yield. This suggests that adjusting the fermentation temperature has a consistent and independent effect on the final PHA concentration throughout the various fermentation time levels studied, and vice versa. The result depicts a circular contour map of the interaction impact of temperature and pH, indicating that the interaction between variables is not significant [44].

3.7.3. Interaction Effect Between pH and Fermentation Time

From the ANOVA Table, the p-value of pH and fermentation time was <0.0001, indicates both pH and fermentation time have a greater impact on PHA yield. The observed yield decreases at severe pH levels and temperatures above 35°C is entirely consistent with accepted microbiological principles. Numerous investigations on PHA-producing bacteria demonstrate that optimal polymer production is closely linked to optimal growth conditions. Stresses such as non-optimal pH or supra-optimal temperature cause cellular stress responses, impede important metabolic pathways, and can result in cell lysis, all of which are harmful to high PHA formation [45]. As a result, the discovered optimum at pH 7.05-7.1 and 48-50 hours is more than just a statistical result; it also reflects the microbial catalyst's fundamental biological requirements [46].

4. Conclusion

It may be concluded that B. sacchari grew better in cultivations using hydrolysates of Napier grass as the sole carbon source at 35 °C, and neutral pH than in other examined scenarios as the strain can consume both hexose and pentose sugars that can be extracted from lignocellulosic materials. In this study, alkaline hydrolysis of Napier grass was done first, followed by enzymatic hydrolysis and glucose rich hydrolysates were obtained. After evaluating the recovered hydrolysates, PHA, biocompatible bioplastic, was synthesized by fermentation and employing the Burkholderia Sacchari strain. This modeling yielded a second-order polynomial equation, indicating an optimal production temperature of 35.38 °C, a pH of 7.05, and a fermentation time of 48.18 hours. The statistical model has a high coefficient of determination (R^2) and substantial F-value, indicating its robustness. The improved settings resulted in roughly 7.43 g L^{-1} of PHA, validating the model's accuracy. The results show that B. sacchari is efficient at metabolizing mixed carbohydrates (such as glucose and xylose) from the hydrolysate, hence boosting PHA production and accumulation. This study emphasizes the possibility of combining agricultural waste usage with modern microbial processing and statistical methodologies to provide a sustainable pathway for bioplastic manufacture. To advance the

potential of polyhydroxyalkanoate (PHA) as a viable bioplastic, further investigations can be performed on optimizing Napier grass hydrolysates (via NaOH and cellulase), exploring recombinant strains and efficient fermentations with low-cost substrates, and conducting preliminary design and economic feasibility studies to establish scalable PHA manufacturing.

Declaration of competing interests

The authors declare that they have no competing interests.

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