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Production of polyhydroxyalkanoate (pha) by *pseudomonas aeruginosa* (ol405443) using agrowastes as carbon source

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ABSTRACT

The search for cost-effective substrates for the manufacturing of valuable products has led to the use of agrowastes as alternative sources of reducing sugar. Numerous bacteria build up polyhydroxyalkanoates (PHAs) as storage materials. This research aimed to produce PHA from *Pseudomonas aeruginosa* using agrowastes as carbon sources. The agrowastes (corncob, plantain peduncle and sugarcane bagasse) were treated with 1 % NaOH and analysed. The agrowastes were hydrolysed using cellulase produced by *Aspergillus niger* isolated from agrowastes dumpsite. The agrowaste hydrolysate was used in place of glucose for PHA production in a submerged fermentation. Nile blue A test and Sudan black test showed positive results for the isolate with a bright orange fluorescence on irradiation with UV light and was identified as *Pseudomonas aeruginosa* (accession number 0L405443). Sugarcane bagasse gave the highest potential for PHA production with PHA values of 5.86 mg/mL, followed by corn cob (5.29 mg/mL) and the least was obtained in plantain peduncle with a yield of 3.58 mg/mL. The findings using response surface methodology (RSM) for optimization show that all the four factors (carbon source, pH, temperature and incubation time) were statistically significant ($P < 0.05$) for PHA production. The optimum PHA production was attained under culture conditions of 24 h, 38 °C, pH 6.5, and 3 % carbon source. The PHA produced from 10 L of MSM was quantified to be 10.57 g under these conditions. The study revealed that *Pseudomonas aeruginosa* 0L405443 is a local bacterial strain utilized for the production of PHA using affordable, sustainable and easily available agrowastes hydrolysate as substrate.

Introduction

Polyhydroxyalkanoates (PHAs) are a group of polyesters synthesized by a range of bacteria including archaea (Koller et al., 2017). Different types of Gram-negative and Gram-positive bacteria produce polyhydroxyalkanoates. Over 300 distinct bacteria, including *Azotobacter* sp., *Pseudomonas* sp., *Bacillus* sp., and *Methylobacterium* sp., are known to produce and store PHAs intracellularly. Under nutrient-limited conditions with an abundance of carbon, these bacteria have the metabolic capacity to synthesize PHA particles and collect them as carbon and energy sources in their cytoplasm in the form of crumbs. The production of PHA is catalysed by an enzyme, PHA synthase, during the synthesis (Shreya and Anil, 2021).

Accumulation of wastes on land and in water bodies have significantly affected the healthy living of most developed and developing countries. Untreated industrial waste has generated many inorganic and organic pollutants. Agrowastes are associated with post-harvest operations and are abundant in the Nigeria environment, constituting a great menace to public health and safety (Oyewole et al., 2023). Therefore, the removal of organic and inorganic contaminants from the environment is of utmost importance. Bioremediation is the most preferable means as it poses no threat to human, plant and aquatic life (Qi et al.,

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2021). Polyhydroxyalkanoate (PHA) was created as a synthetic plastic replacement due to the surge in environmental pollution brought on by non-biodegradable polymers made of petrochemicals. Polyhydroxyalkanoates (PHAs) are entirely biodegraded to CO_2 and water at the end of their lifespan as the byproducts of mineralization (Ahmed et al., 2021). They are produced with environmentally friendly, renewable resources (Ahmed et al., 2021). Polyhydroxyalkanoates (PHAs) made from bacterial cells resemble polypropylene and other conventional polymers in their properties (Blaithín et al., 2020). They are therefore a fantastic substitute for thermoplastics made of petrochemicals. The advantages of polyhydroxyalkanoates over chemically produced polymers are their biodegradability, biocompatibility, cost-effectiveness, and non-toxic thermoplastics characteristics (Liu et al., 2019).

Polyhydroxyalkanoates (PHAs) have been engaged in production of garbage bags, building materials, packaging films, toys, and household and cosmetic products. The PHA value was also increased by their structural variety, which makes it easy for them to be converted into biofuels and undergo methyl esterification (Choonut et al., 2017). As a result, they may find use in a variety of industries, including packaging, feed additives, textile production, the creation of bio-implants, the delivery of drugs, the production of biofuels, and bioplastic agricultural film (Sameh et al., 2023). The objective of this study was to produce PHA by *Pseudomonas aeruginosa* (OL405443) using agrowastes (corncob, sugar cane bagasse, and plantain peduncle) as a carbon source.

Materials and methods

Collection of samples

Waste water samples were collected from various locations in Minna Nigeria including a plastic company, 3 bakery sites, and a cafeteria in Minna and designated as IMU, B, AL, R, and DH respectively. Soil samples of each agrowaste (sugarcane bagasse, plantain peduncle and corncob) were collected from their dump sites aseptically by using a sterile spatula into sterile sample bottles. Agrowastes (sugarcane bagasse, plantain peduncle and corncob) were collected from the local market in Kasuwan Gwari Minna Nigeria. All samples were securely transported to the Microbiology laboratory of Federal University of Technology, Minna, Nigeria and kept in the refrigerator at 4 °C until they were ready for use.

Isolation of bacteria from a wastewater sample

A 10-fold serial dilution of the wastewater was done and plated on nutrient agar using the pour plate method. Colonies that differ in size, colour and shape were subcultured in freshly prepared nutrient agar plates by streaking and then pure isolates that were obtained were streaked on nutrient agar slants. (Joyline and Aruna, 2017).

Screening for PHA bacteria

The isolates were examined for PHA production capacity, the bacterial colonies were stained with a 0.02 % alcoholic solution of Sudan black B, and the plates were left alone for 30 min. After decanting the excess colour, the plates were gently cleaned with 96 % ethanol (Anish et al., 2013).

Using the Nile blue A assay technique, the isolates that were positive to Sudan Black B staining were further tested for PHA synthesis. The bacterial isolates were smeared on glass slides before being air-dried and heat-fixed. The smear was inundated with 1 % Nile blue A and heated for 10 min in a water bath at 55 °C. The stain was washed with water and an 8 % aqueous solution of acetic acid for 1 min.

Identification of the PHA-producing isolates

The isolate with the potential for PHA production was identified using morphological, cultural, and biochemical tests (catalase, oxidase, triple sugar iron agar, starch hydrolysis, and carbohydrate fermentation tests), and the isolate with the best potential was identified using molecular characteristics including DNA extraction, polymerase chain reaction, gel electrophoresis, and sequencing.

Bacterial DNA extraction

Single colonies grown on media were transferred to 1.5 mL of liquid medium and shaken for 48 h at 28 °C. The cultures were then centrifuged at 4600g for 5 min. The pellets were resuspended in 520 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After that, 3μLz of Proteinase K (20 mg/mL) and 15 μL of 20 % SDS were added to the mixture. After one hour of incubation at 37 °C, the mixture was vortexed with 80 μL of a 10 % CTAB solution in 0.7 M NaCl and 100 μL of 5 M NaCl. After 10 min of incubation at 65 °C, the suspension was placed on ice for 15 min. After adding an equal volume of chloroform: isoamyl alcohol (24:1), the mixture was held on ice for 5 min before being centrifuged at 7200g for 20 min. Following that, a fresh tube was filled with the aqueous phase and isopropanol (1:0.6), and DNA was precipitated at −20 °C for 16 h. The DNA was extracted by centrifugation at 13000g for 10 min, followed by cleaning with 500 μL of 70 % ethanol, allowing it to air dry for about three hours at room temperature (28 °C ± 2), and resolving it in 50 μL of TE buffer (Achife et al., 2023).

Polymerase chain reaction

A 10 mL of 5x GoTaq colourless reaction, 3 mL of 25 mM $MgCl_2$, 1 mL of 10 mM dNTPs mix, and 1 mL of 10 pmol each comprised the PCR sequencing preparation cocktail. 27F Using an 8 μL DNA template and sterile distilled water, primer sets of 5′- AGA GTT TGA TCM TGG CTC AG-3′ and − 1525R, 5′- AAGGAGGTGATCCAGCC-3′, and 0.3 units of Taq DNA polymerase (Promega, USA) were generated in a total volume of 42 μL. A 5-minute denaturation at 94 °C was followed by 30 cycles of 30 s at 94 °C, 60 s at 50 °C, and 1 min 30 s at 72 °C, with a final 10-minute termination at 72 °C (Achife et al., 2023).

Integrity determination

Purification of amplified product

The amplified fragments were ethanol-filtered after gel integrity to remove the PCR chemicals. In a new, sterile 1.5 μL Eppendorf tube, each 40 μL PCR amplified product received 7.6 μL of Na acetate 3 M and 240 μL of 95 % ethanol. The mixture was completely mixed using vortexing, and the tubes were then refrigerated at −20 °C for at least 30 min. The pellet was cleaned with 150 μL of 70 % ethanol and mixed before being centrifuged for 15 min at 7500 g and 4 °C. After a 10-minute centrifugation at 13,000 g and 4 °C, the supernatant was recovered. The supernatant tube was emptied and inverted onto a paper towel, dried in the fume hood for 10 to 15 min at room temperature (28 °C ± 2), before resolving in 2 L of sterile distilled water and sequencing at −20 °C. The purified fragment was tested for an hour at 110 V on a 1.5 % Agarose gel to authenticate its existence and quantify it using a Thermo Scientific nanodrop model 2000 (Achife et al., 2023).

Sequencing of PCR products

According to the manufacturer's instructions, the amplified fragments were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit on an Applied Biosystems Genetic Analyzer 3130xl sequencer. MEGA 6 and Bio-Edit tools were used for all genomic research (Achife et al., 2023).

Determination of gene specific for PHA production in Pseudomonas aeruginosa

Molecular investigations of the species-specific PHA-C1 gene genomic areas responsible for the partial production of poly-hydroxyalkanoic acid in the bacterium isolates were carried out using straightforward PCR on the recovered DNA and genomic-specific primers. The following ingredients were employed in the reaction cocktail for each PCR primer set: 5X PCR SYBR green buffer (2.5 μL), $MgCl_2$, 10 pM DNTP, and 10 pM of each forward CCGCAGATCAA-CAAGTACTACA and reverse primer Two liters of the template were added to forward CATCCTCTACTGGAACAACGAC (0.25 μL), 8000 U of Taq DNA polymerase (0.06 μL), and sterile distilled water was brought up to 10.5 μL before the mixture was added. PCR profile was present. A five-minute denaturing period at 94 °C is followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C, with a ten-minute final period at 72 °C. During PCR in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA), the appropriate profile for each primer pair was utilized (Máčalová, et al.,2023).

Accession and culture deposition number

The NCBI Gene Bank received an isolate whose gene sequences showed the best potential for producing PHA, along with the appropriate accession number.

The pretreatment and hydrolysis of agrowatse

The pre-treatment of the agrowastes was carried out according to Makut and Ekeleme (2018) which involves rinsing of the substrates with clean running water, sun-drying for 5 days and milling into powder. The powdered substrates were weighed using the weighing balance of 20 g each and soaked with 200 mL of prepared NaOH solution (1:10) for 10 min. The substrates were microwaved at 2450Mhz for 5 min to delignify the substrates. The microwaved substrates were washed with clean water till a neutral pH was attained using a pH meter (The untreated and pretreated agrowastes are presented in Fig. 1).

Cellulase was produced following the method of Anchal et al. (2016) using *Aspergillus niger*. The *A. niger* was grown in a cellulase-producing basal medium containing, yeast extract, 2.0; $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; and $FeCl_3$, 0.001 mg/l. A 1 % concentration of carboxymethyl cellulose (CMC) was added, and the culture was allowed to develop for 7 days at 25 °C. The culture medium was filtered to obtain the filtrate using Whatman No. 1 filter paper and the culture filtrate was centrifuged using a speed cold centrifuge, and the supernatant served as the crude enzyme solution (Oyeleke et al., 2012a,b). The culture broth was taken every 24 h during growth to evaluate cell density by measuring absorbance at 540 nm. The cellulase assay was performed using the method described by Omonije et al. (2022). A 1 mL of 1 % CMC in 0.1 M citrate buffer (pH 5.5) was combined with 1 mL of crude enzyme in a test tube. The reaction was halted after 30 min of incubation at 50 °C by adding 1.5 mL of 3,5-dinitro salicylic acid (DNSA) reagent. The tubes were heated for 15 min in a boiling water bath at 100 °C before being allowed to cool to room temperature (28 °C ± 2). At 540 nm, the absorbance was measured. Enzyme activity was evaluated in millimoles of glucose released per second per millilitre of enzyme solution (culture filtrate).

The agrowastes were hydrolyzed by Makut and Ekeleme (2018) by weighing 20 g of each extract and dispensing in well-labelled 250 mL conical flasks, 20 mL of cellulase was added to each substrate and tightly corked. The conical flasks were inserted in a water bath at 60 °C for 48 h with frequent shaking to ensure uniform distribution of the enzyme.



Fig. 1. Agrowastes; A1 = Plantain peduncle, A2 = Plantain peduncle after treatment, B1 = Sugar cane Bagasse, B2 = Sugar cane Bagasse treatment C1 = Corn cob, C2 = Corn cob after treatment.

Upon incubation for 48 h, the hydrolysis was halted by raising the temperature of the water bath to 100 °C to completely inhibit the enzyme activity. The hydrolyzed substrates were sieved using a muslin cloth and further filtered using Whatman filter paper and hydrolysate was obtained (Makut and Ekeleme, 2018).

Mineral salts medium (MSM) medium composition for screening of the hydrolysate (g/L) were: Urea (1.0), Na_2HPO_4 (4.0), $MgSO_4{\cdot}7H_2O$ (0.52), yeast extract (0.16), KH_2PO_4 (1.52), $CaCl_2$ (0.02) and 0.1 mL trace element solution; H_3BO_3 (0.06), $ZnSO_4{\cdot}7H_2O$ (0.13), $(NH_4)6MO_7O_{24}{\cdot}4H_2O$ (0.06) and $FeSO_4{\cdot}7H_2O$ (0.02) were used for the production of PHA by the selected isolate in five different conical flasks containing the four different hydrolysates (sugarcane bagasse, corn cob, plantain peduncle) and the positive control contain glucose (Getachew and Woldesenbet, 2016). The conditions (pH = 7, temperature = 37 °C and 24 h incubation time) for the screening medium were all the same except for the carbon source that was different but the same quantity (10 mL) was added. Before inoculation, Separately, the substrate and trace element solutions were autoclaved and reconstituted. Each conical flask (production media) was filled with one millilitre of a 24-hour-old culture and incubated at 37 °C for 72 h.

The reducing sugar concentration of hydrolysate with the highest potential to produce PHA was determined by dispensing 1 mL of the hydrolysate into a test tube and 1 mL of 3,5-Dinotrosalicyclic (DNS) indicator was added, a blank was created, which served as a guide (i.e., the indicator will not be added to it) and heated at 50——100 °C for 5 min as colour change was observed (i.e., from yellow to red). The sample was diluted by adding 9 mL of distilled water, thereafter the absorbance of the sample was measured gravimetrically using a spectrophotometer at 520 nm (Model UV-1800, Shimadzu UV spectrophotometer Japan) (Al-Dhabi et al., 2020).

Analysis of agrowaste for PHA production

Before hydrolysis with cellulase enzyme, the agrowaste was analyzed using scanning electron microscopy (SEM), x-ray diffraction (XRD), and Fourier-transformed infrared spectroscopy (FTIR) (Anuj, et al., 2014).

SEM and EDX analysis of agrowaste

The agrowaste sample was mounted on stubs with adhesive carbon and coated in 20 nm Carbon with a QUORUM Q150R ES mini sputter coater before being examined with a Phenom PRO-X SEM equipped with an Oxford XMax 50 Silicon Drift Energy Dispersive X-ray detector at 15KV in high vacuum (Anuj et al., 2014).

X-ray diffraction analysis of agrowaste

The sample was finely ground and homogenized, and the average bulk composition was determined. After that, the powdered sample was produced with the sample preparation block and crushed in the flat sample holder to generate a flat, smooth surface that was afterwards put on the sample stage in the XRD cabinet. The reflection-transmission spinner stage with Theta-Theta settings was used to evaluate the material. The two-theta starting position was 4 degrees, and each step at 8.67 s was 0.026261. The tube current was 40 mA and the tension was 45VA. A Gonio Scan was used in conjunction with a Programmable Divergent Slit and a 5 mm Width Mask.

The intensity of diffracted X-rays is continuously recorded while the sample and detector rotate through their respective orientations (Anuj et al., 2014).

FTIR analysis of agrowaste

The FTIR is an intriguing tool for evaluating structural differences in samples caused by treatments with chemicals. The structural changes from untreated agrowastes to pre-treated agrowastes were analysed by FTIR spectroscopy with the help of a Cary 630, FTIR Agilent Technologies (Cary 630 ZnSe, Part no - G8043 64002, Model no - MY19322004, Country of Manufacture; Malaysia) spectrophotometer. The samples were oven-dried at 105 °C for 4–5 h before being combined with KBr in a 1:200 (w/w) ratio and pressed under vacuum to create pellets. The materials' FTIR spectra were acquired in the transmittance mode in the 4000–500 cm-1 range (Anuj et al., 2014).

Experimental design for optimization of PHA production conditions

Optimization of process parameters for PHA production using bacteria isolates with agrowaste as substrate was carried out using several parameters (i.e., carbon source, incubation time, temperature and pH) as demonstrated by Andler et al. (2021) with little modifications to assess the most suitable condition for its production and obtain high yield. Every parameter was optimized for required variants. The experimental set-up of batch cultures was carried out in a 250 mL Erlenmeyer flask with 100 mL production (fermentation) media. Optimization of pH was performed with the required number of flasks containing 100 mL of fermentation media in each flask (adjusted to the respective run values) was incubated with an agitation rate of 200 rpm for each flask separately. Readings were recorded at the respective temperature at the end of each run time and PHA was quantified using a spectrophotometer at 240 nm. The PHA production was optimized using Design Expert Software Version 11 as shown in Appendix A. A total of 29 fermentation experiments (runs) with different input values were created and carried out (Adeoye et al., 2015).

Quantifications of PHA by spectrophotometry using standard graph

Dissolution of Crotonic acid powder into sulfuric acid to make a standard solution of 0.1 μg Crotonic acid/ μl sulfuric acid. Sulfuric acid working standards of 0.125, 0.063, 0.032, 0.016, and 0.008 μg/3mL were produced. The 3 mL of sulfuric acid was added to make a blank. At 235 nm, the absorption was measured. A standard concentration vs. absorbance graph (Model UV-1800, Shimadzu UV spectrophotometer Japan) was created (Lingayya et al., 2015).

Production of PHA using submerged fermentation

The PHA was produced using the best values obtained from the optimized conditions (carbon source, pH, temperature, and incubation time). The production was done in 10 L of MSM. To obtain a standard inoculum, a loop of the culture was inoculated into 100 mL of nutrient broth packed in 250 mL conical flasks and incubated for 24 h at 35 °C.

Mineral salts medium (MSM) [composition (g/10*L*): Urea (10), Yeast extract (1.6), KH_2PO_4 (15.2), Na_2HPO_4 (40), $MgSO_4{\cdot}7H_2O$ (5.2), $CaCl_2$ (0.2) and 0.1 mL trace element solution was used for the production of PHA by the selected isolates. The trace element solution was containing (g/L): $ZnSO_4{\cdot}7H_2O$ (1.3), $FeSO_4{\cdot}7H_2O$ (0.2), $(NH_4)6MO_7O_{24}{\cdot}4H_2O$ (0.6) and H_3BO_3 (0.6) (Getachew and Woldesenbet, 2016). Before inoculation, the substrate (3 % sugarcane bagasse) and trace element solution were autoclaved separately and reconstituted. The culture was formed by subculturing the isolates twice in nutrient broth. The culture was then cultivated for 24 h at 38 °C with 1 % W/V of a 24-hour-old culture put into 10,000 mL production media (Martău et al., 2021).

Extraction and purification PHA

After incubating the MSM for 24 h at 37 °C, the culture broth was centrifuged at 10000 rpm for 10 min, the supernatant was discarded, and the pellet was the crude PHA. To lyse the cells, the pellet was treated with 10 mL sodium hypochlorite at 500 °C for 1 h. The suspension was centrifuged for 10 min at 3000 rpm after incubation. Following the removal of the top and intermediate phases, 5 mL of ethanol was added to precipitate the granules. Before determining the weight of PHA, the precipitate was allowed to dry for 2 h at 60 °C. The extracted PHA's dry weight was calculated to be g/L (Veenagayathri and Nabila, 2020). PHA extract (50 mg) was mixed in 10 mL of 3 % (v/v) H_2SO_4 and heated for 10 min at 100 °C (Veenagayathri and Nabila, 2020).

Results and discussion

Characteristics of bacteria isolates

The isolate was also positive for the Nile blue test and showed bright orange fluorescence of the PHA isolates on irradiation with UV light (Supplemented Data, SD 1) The biochemical characteristics of the isolate are presented in SD 2.

Molecular identity of Pseudomonas aeruginosa and gene-specific for PHA production

The *Pseudomonas aeruginosa* had a size of 1500 bp (Fig. 2a), an accession number of OL405443 and a specific gene deposited in the NCBI database (Table 1). Fig. 2b shows an agarose gel with positive amplification of the phaC2 gene of bacterial DNA using gene specific primers. The presence of 530 bp in extracted DNA indicates positive amplification.

Attempts were undertaken in this work to isolate and identify PHA-generating bacteria from wastewater obtained from various sources. Bacteriological investigation of the strain was accomplished using a variety of cultural and biochemical assays. According to this study, around 20 % of the bacteria isolated from waste water are PHA accumulators. This is because at least two out of every ten isolated bacteria tested positive for the Sudan B black dye, displaying blue-black coloration when the dye was applied. Further confirmation tests were performed on the two isolates using the Nile Blue A assay method, and both isolates were positive, displaying an orange colour when seen under a fluorescent microscope. Gram staining indicated that the isolates were Gram positive and Gram negative. Spiekermann et al. (2018) employed this technology to select PHA bacteria from waste water from the pulp, paper, and cardboard industries. Kitamura and Doi (2014) initially established this viable colony approach on agar plates by cultivating bacteria in an E2 medium with 2 % (w/v) glucose before staining with Nile blue A. After Nile blue, the colonies that accumulate PHA When exposed to UV light, staining revealed brilliant orange fluorescence, and the intensity of the fluorescence grew as the bacterial cells' PHA concentration rose. *Pseudomonas* sp. and *Bacillus* sp. were identified as IMU and DH, respectively. Other scholars have looked into the PHA potentials of these genera as well. Singh et al. (2009) investigated *Bacillus subtilis* as a potential producer of polyhydroxyalkanoate.

A local strain of *Pseudomonas aeruginosa* capable of producing PHA was discovered in this study. The sequence of the 16S rRNA gene for the isolate *Pseudomonas aeruginosa* revealed 99.60 % similarity to other *Pseudomonas aeruginosa* in the database. *Pseudomonas* species bacteria use two metabolic routes to provide precursors for PHA production. Each process starts with a different substrate to generate 3-hydroxyacyl (3HA) precursors, which are subsequently used to make PHAs. *Pseudomonas* use the β-oxidation process to break down aliphatic carbon sources like fatty acids. This route begins with either acyl-CoA or acetyl-CoA as an intermediate and terminates with monomer polymerization using PHA synthases. An NADPH-dependent acetoacetyl-CoA dehydrogenase (PhaB) is responsible for acetoacetyl-CoA reduction. Finally, P (3HB) is polymerised by the (R)-3-hydroxybutyrate) -CoA polymerase PhaC into P(3HB). *Pseudomonas* sp. uses the Entner-Doudoroff pathway to catalyze the conversion of glucose to pyruvic acid. In this scenario, PhaC, the polymerase gene responsible for polymerization into P(3HB), was encoded as the PhaC1 gene in *Pseudomonas aeruginosa*. The organism's capacity to generate a specific type of PHA might be attributed



Fig. 2. (A)-Agarose gel electrophoresis image of *Pseudomonas aeruginosa.* (B) -Agarose gel electrophoresis image of *Pseudomonas aeruginosa* showing positive amplification.

mostly to the substrate specificity of the PHA synthases. The new *Pseudomonas aeruginosa* OL405443 was deposited in the Genbank under the accession number JN969046. Many recent investigations have chosen *Pseudomonas* species as PHA producers. Sylvaine et al. (2012) investigated the production of medium-chain-length polyhydroxyalkanoates in engineered Pseudomonas putida KT2440 by sequential feeding of xylose and octanoic acid.

Macroscopic examination of *Aspergillus niger* isolated from agrowaste dumpsite soil sample on czapek dox agar medium reveals that their growth is initially white but quickly turns yellow and black, producing conidial spore. On PDA, they begin as white colonies and progress to black colonies with umbonate elevation and an entire edge. The colonies' margins are pale yellow, with radial fractures. The microscopic investigation of *Aspergillus niger* shows smooth pigmented conidiophores and conidia. Conidia with a rough texture and a dark brown tint are produced by phialides. Fungi have been identified as degraders of cellulosic substrates and organic materials (Victoria et al., 2022). Because of their dominance in the soil and overall safety, filamentous fungi such as *Aspergillus niger* are commonly used. The level of hydrolysis in relation to different agrowastes may be due to biomass composition in each substrate, as hydrolysis effectiveness is strongly dependent on both the physical and chemical features of the feedstock (Victoria et al., 2022).

Concentration of hydrolysate produced from the agrowastes

Using the standard graph of PHA concentration (SD 3), sugarcane bagasse had the highest concentration of 5.92 mg/mL while corn cob was with the lowest concentration of 3.58 mg/mL and plantain Peduncle recorded a concentration of 5.29 mg/mL (SD 4). After the screening, it was observed that the sugarcane bagasse gave the highest PHA with a reducing sugar concentration of 21.45 mg/mL.

Sugarcane bagasse demonstrated the most exposed cell wall structure after treatment, allowing for better accessibility to hydrolytic enzymes via pores formed by thermal treatment with NaOH, facilitating the hydrolysis of lignocellulosic biomass. The porosities increased in general, indicating that a large number of strong particles (inorganic components or the most volatile components) were removed. Surprisingly, the majority of the pores originated in the cell wall's outer section. This is due to the bagasse's exterior being covered with wax layers, pectin, and other impurities such as hemicellulose and lignin.

Table 1
Sequencing and Accession number of *Pseudomonas aeruginosa* and gene Specific for PHA Production.

Sample ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Isolate IMU	*Pseudomonas aeruginosa*	2741	2741	99 %	0	99.60 %	1537	OL405443
Gene Specificity	*Pseudomonas aeruginosa* PhaC1 *gene*	972	972	99 %	0	99.44 %	539	JN969046.1

Additionally, these layers serve to safeguard the inner cellulose (Liu et al., 2019). The inner cellulose layers present is caused by the breakdown of hemicellulose and lignin, which led the fibers to swell and the linkages of hemicellulose and lignin to break down (Shuaishuai et al., 2021). According to a similar analysis from Getachew and Woldesenbet (2016), pre-treated sugarcane bagasse (56 %) was the best affordable carbon source, followed by corn cob (52 %). *Pseudomonas aeruginosa* was used by Paramjeet et al. (2012) to extract PHA from sugarcane bagasse.

Scanning electron micrograph of sugarcane bagasse

The analysis showed the morphological characteristics of the different agrowastes before and after treatment with NaOH at various objectives. The untreated sample represents the original state of the agrowastes, while the pre-treated sample was processed with NaOH to modify its structure and surface characteristics. The morphological characteristics of the untreated bagasse as well as pre-treated bagasse in the presence of NaOH, obtained by SEM are shown in Fig. 3. The untreated bagasse sample has a hard and compact morphology, but the NaOH-treated samples have a more chaotic morphology, with more fibre exposure and surface area. This pre-treatment is effective in removing lignin and hemicellulose, resulting in increased cellulose enrichment without the generation of excessive quantities of inhibitors.

Scanning electron micrograph of corncob sample

The SEM image of the untreated corn cob revealed a typical surface morphology characterized by a rough and irregular texture as shown in Fig. 3. The surface appeared heterogeneous, consisting of fibrous structures and agglomerated particles. The fibrous materials displayed varying lengths and thicknesses, interwoven in a complex network. The surface pores were relatively small and less pronounced, and the overall surface structure exhibited a natural composition without significant modifications compared to NaOH pre-treated samples. The NaOH-treated corn cob demonstrated distinct structural modifications. The surface morphology appeared smoother and more uniform, indicating the effectiveness of the NaOH treatment process in altering the surface characteristics and possible reduction in the lignin content of the treated corncob sample.

Scanning electron micrograph of plantain peduncle

The morphological characteristics of the steam-treated plantain peduncle in the presence of NaOH as well as of the untreated material, obtained by SEM is shown in Fig. 3. The SEM image of the untreated plantain peduncle revealed a distinctive surface structure characterized by an uneven and asymmetrical roughness. The treated plantain peduncle appeared gristly and unbundled. The gristly structures exhibited a more scattered arrangement, with individual fibres showing enhanced separation and reduced bundling. Suggesting the breakdown of some inter-fibre connections or loosened fibre structures. The pre-treatment process also induced an increase in surface porosity which provides a larger surface area for microbial attachment and enzymatic interactions.

Based on the micrograph results, it can be concluded that increasing the NaOH level resulted in an increased cellulose recovery. This could be justified by the fact that alkaline hydrolysis breaks the bonds between aryl-ether, C-C, aryl-aryl, and the ester groups between lignin and hemicelluloses. However, too much increase in NaOH will result in unrestrained hydrolysis of the functional groups present in cellulose, reducing the cellulose content of the product.

Energy dispersive X-ray (EDX) result of agrowastes

The morphological characteristics of the untreated and NAOH pre-treated corncob are shown in SD 5. The EDX analysis of the untreated corn cob revealed the elemental composition of the sample, with characteristic peaks corresponding to various elements. The major elements detected in the untreated corn cob included carbon (C), oxygen (O), potassium (K), chlorine (Cl), and molybdenum (Mo) at varying atomic and weight concentrations. The NaOH pre-treated corn cob exhibited potential changes in the elemental composition compared to the untreated sample. The EDX spectrum of the pre-treated sample also displayed peaks corresponding to carbon, oxygen, and potassium,

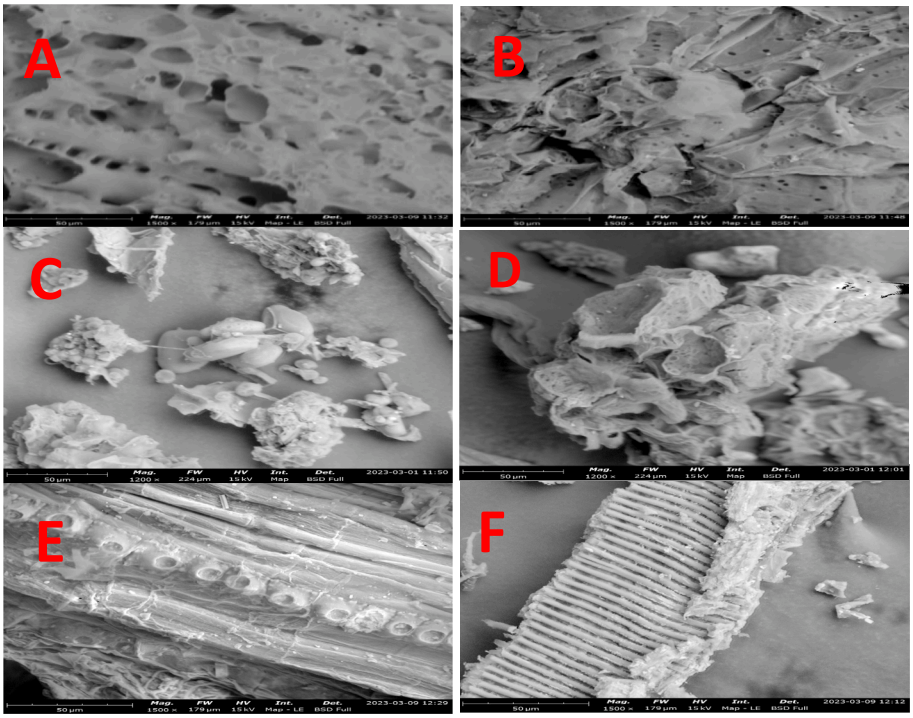

Fig. 3. SEM images of agrowastes. A = untreated bagasse, B = Treated bagasse, C = untreated corncob, D = Treated corncob, E = untreated plantain peduncle, F = Treated plantain peduncle.

indicating their continued presence. Additionally, silicon (Si), calcium (Ca), iron (Fe), and aluminium (Al) were detected in the pre-treated sample.

The morphological characteristics of the untreated and NaOH pre-treated plantain peduncle are shown in SD 5. The EDX analysis of the untreated plantain peduncle showed the elemental composition of the sample, with characteristic peaks corresponding to various elements. The major elements detected in the untreated plantain peduncle included carbon (C), oxygen (O), potassium (K), americium (Am), calcium (Ca), tellurium (Te) and plutonium (Pu) at varying atomic and weight concentration.

The EDX spectrum of the pre-treated sample also displayed peaks corresponding to carbon (C) and oxygen (O), and potassium (K), indicating their continued presence. Additionally, Iron (Fe), silicon (Si), chlorine (Cl), strontium (Sr) and bromine (Br) were detected, while elements like americium (Am), calcium (Ca), tellurium (Te) and plutonium (Pu) were not detected in the pre-treated sample due to the action of NaOH.

The EDX morphological characteristics of the untreated and NAOH pre-treated sugarcane bagasse are shown in SD 5. The EDX analysis of the untreated bagasse revealed the elemental composition of the sample, with characteristic peaks corresponding to various elements. The elements detected in the untreated bagasse sample included carbon (C), oxygen (O), silicon (Si), aluminium (Al), phosphorus (P), and indium (In) at varying atomic and weight concentrations. The NaOH pre-treated bagasse exhibited potential changes in the elemental composition compared to the untreated sample. The EDX spectrum of the pre-treated sample also displayed peaks corresponding to carbon and oxygen indicating their regenerated presence. Additionally, Iron (Fe) and Potassium (K) were detected, while elements like silicon (Si), phosphorus (P), indium (In), and aluminium (Al) were not detected in the pre-treated sample due to the action of NaOH.

The morphological structure of corncob was uniformly short fibrous and coarse in general. In contrast, the plantain peduncle image revealed masses of loose spongy lignocellulosic structures. The picture of sugarcane bagasse revealed a lengthy, homogeneous fibrous lignocellulosic structure. This is consistent with the findings of Santhebennur and Jogttappa (2012), who observed that SEM images of groundnut shells revealed masses of loose spongy lignocellulosic structures.

The agrowastes (sugarcane bagasse, plantain trunk and corn cob) showed varying concentration of PHA yield with sugarcane bagasse having highest yield of 5.86 mg/mL, corncob had yield of 5.29 mg/mL while plantain peduncle had the lowest yield of 3.58 mg/mL. This could be due to the high sugar contained in the sugarcane hydrolysate, even though the hydrolysates were not in complete lyophilized states. Unlike the hydrolysate, the glucose is easily converted into PHA by the 3(P)HB synthase to PHB during PHA synthesis by the bacteria. The poor yield recorded from plantain peduncle hydrolysate could be attributed to the percentage of ash content and the concentration of other non-sugar substances of the agrowaste.

X-ray diffraction (XRD) result of agrowastes

The XRD analysis presents the diffractograms of untreated corncob (A) and pre-treated corncob with NaOH (B) as shown in Fig. 4. The minerals present in untreated corncob include Refikite (74.3 %), davyne (4.0 %), chaoite (7.4 %), mellite (4.31 %), cristobalite (6.4 %) and chlorite (3.6 %) while those present in s corncob pre-treated with NaOH include Refikite (72 %), davyne (2.5 %), chaoite (9 %), mellite (6.3 %), cristobalite (7 %) and chlorite (3 %). The diffractograms of untreated plantain peduncle and pre-treated plantain peduncle with NaOH are also presented in Fig. 4. The minerals present in untreated plantain peduncle include Refikite (72 %), davyne (2.5 %), chaoite (9 %), mellite (6.3 %), cristobalite (7 %) and chlorite (3 %) while those present in s plantain peduncle pre-treated with NaOH include Refikite (72 %), davyne (4.7

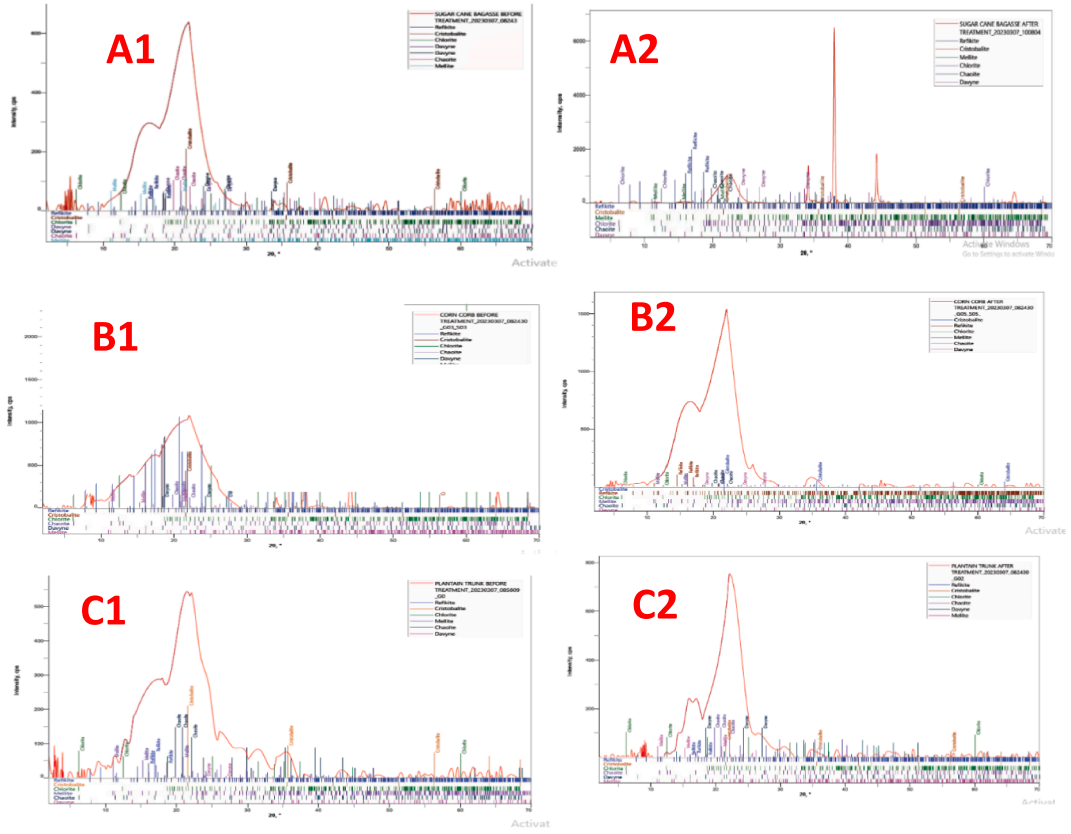

Fig. 4. XRD diffractograms of agrowastes; A1 = untreated bagasse, A2 = treated bagasse; B1 = untreated corncob, B2 = treated corncob; C1 = untreated plantain peduncle, C2 = treated plantain peduncle.

%), chaoite (7.2 %), mellite (3.4 %), cristobalite (7.5 %) and chlorite (5 %).

The diffractograms of untreated bagasse (A) and sugar cane bagasse treated with NaOH (B) obtained by XRD are shown in Fig. 4. The minerals present in untreated sugarcane bagasse include Refikite (50.5 %), davyne (13.1 %), chaoite (12.3 %), mellite (9.2 %), cristobalite (7.5 %) and chlorite (7.5 %) while those present in sugar cane bagasse treated with NaOH include Refikite (51 %), mellite (1 %), chlorite (1 %), cristobalite (16.2 %), chaoite (26.6 %) and davyne (4 %).

The high proportion of Refikite in both untreated and pre-treated samples is a result of the presence of perhydrophenanthrene (two axial methyl groups, one terminal carboxylic group and one terminal isopropyl group joined to the three fused rings in the same fashion as abietic acid. The chlorite (found as retrograde minerals in rock/sand particles) was significantly in very low proportion in the treated bagasse compared to the untreated bagasse due to the action of water during the treatment process since almost all the dust particles were washed off.

The crystallinity index of sugar cane bagasse was higher than that of corncob and plantain peduncle as revealed by XRD result of the treated agrowastes. This disagrees with Padmavati et al., 2012 who reported the increase in crystallinity index as a result of alkali treatment on corncob. It is worth noting that alkali treatment increase the digestibility of cellulose. The SEM and EDX results of the agrowaste also showed that the loose structure of sugarcane bagasse was able to undergo stable alkali treatment thereby enhancing the cellulose recovery. This could be the reason why the concentration of the sugarcane bagasse was higher compare to corncob and plantain peduncle when screened for the organism ability to utilize the agrowastes.

FTIR spectroscopy of agrowastes

The various frequencies from the infrared spectra of the untreated corncob, plantain peduncle and sugarcane bagasse and pre-treated corncob, plantain peduncle and sugarcane bagasse are represented in Fig. 5. Chemical groups affect the calorific value, binding characteristics, and ash content of agricultural waste. The spectral bands at 3280 cm^{-1} (O-H stretching intramolecular hydrogen bonds for), 2892 cm^{-1} (C-H stretching), 2370 cm^{-1} (C-O stretching vibration for the acetyl and ester linkages in lignin, hemicellulose, pectin, etc.). These bands are not seen in the FTIR spectra of bagasse after chemical treatment with NaOH. The 1364 cm^{-1} is due to the CH2 scissoring motion in bagasse, the 1319 cm^{-1} is due to C-H bending, 1028 cm^{-1} is due to the C-O-C pyranose ring stretching vibration, the 893 cm^{-1} is associated with the cellulosic -glycosidic links and the 1028 cm-1 is due to the C-O-C glycosidic ether band. In comparison to the spectral band's corncob and plantain peduncle, the spectral band 1423 cm^{-1} demonstrates considerable cellulose I content in sugarcane bagasse. The spectral band at 1371 cm^{-1} was very strong in treated bagasse, whereas the spectral band at 1319 cm^{-1} overlapped and diffused, and the other spectral band at 1032 cm^{-1} was faint and diffused. These peaks in the FTIR spectral can be used for analysis of the crystallinity of samples containing cellulose, amorphous cellulose, or a mixture of both components. The spectral ratio (1423/898 cm^{-1}) in pre-treated samples indicates the presence of cellulose fraction.

The Fourier transform infrared (FTIR) spectra of the agrowastes indicated characteristics of a carboxyl group with a band of 3213 cm-1. In addition, the agrowastes can be said to include uronate due to the symmetrical stretching discovered in the carboxylate at a band of 2109 cm-1 (Manish et al., 2016). Some sugar groups were also suspected due to the presence of absorption bands of 849 and 928 cm-1. The functional

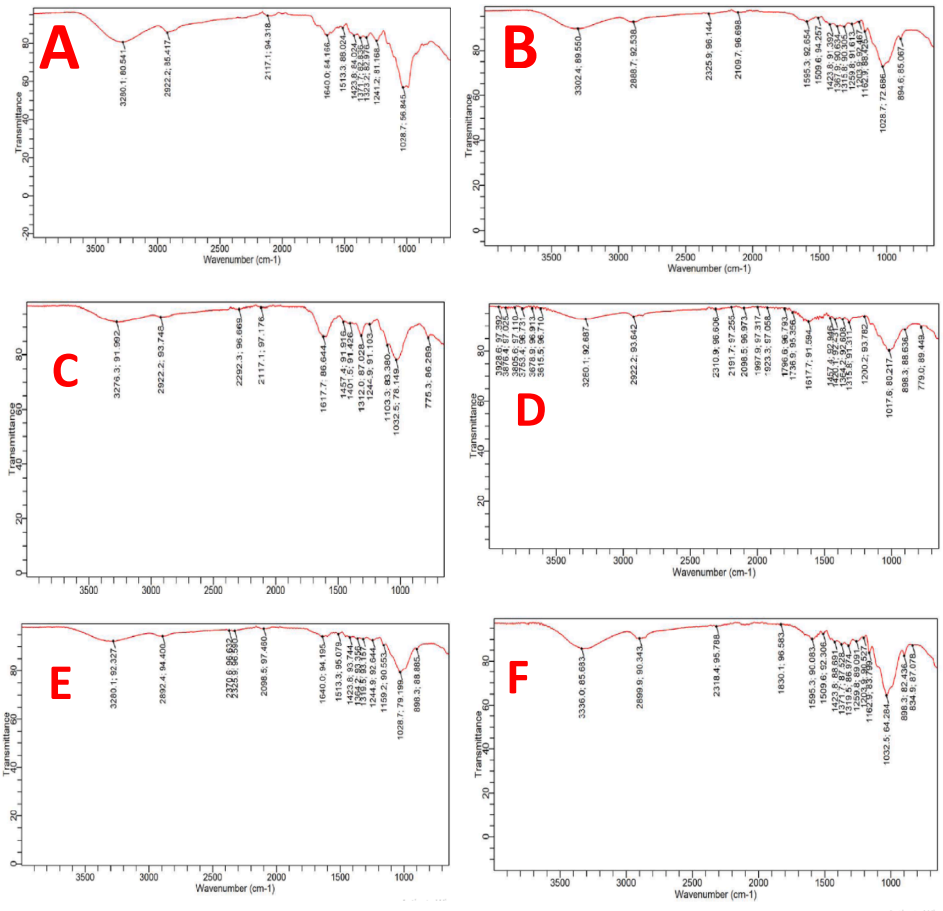

Fig. 5. FT-IR spectra of A-untreated corncob, B - pre-treated corncob, plantain peduncle. C-untreated, D - pre-treated, A-untreated bagasse, B - pre-treated bagasse.

groups reported in this paper played a substantial role in the incredible fermentation activity observed. Manish et al. (2016) found amino, hydroxyl, and carboxyl groups in biopolymers produced by a *Bacillus toyonensis* AEMREG6 strain. This is consistent with the findings of Santhebennur and Jogttappa (2012), who observed that SEM images of corncob revealed masses of loose spongy lignocellulosic structures.

Full factorial design for optimization and response of PHA yield

Table 2 reveals that 25 desirability solutions were generated for the effects of carbon source, temperature, pH, and incubation period on PHA yield form the 29 runs carried out concurrently (SD 6). The most desirable optimum condition gave a the highest PHA yield (76.363 mg/mL) with 3 % carbon source, 24 h incubation time, pH of 6.5 and temperature of 38 °C, While the least desirable optimum condition with PHA yield of 66.708 mg/mL was obtained with values of 3 % carbon source, incubation time of 13 h, pH of 6.4 and temperature of 38 °C.

Solutions are reported by desirability – most to least, based on how well the specified goals are met. The closer all goals are met, the higher the overall desirability will be. Desirability = 1.000, indicates that all goals are perfectly satisfied. D = 0 when one or more responses fall outside acceptable limits.

Linear model for optimization

The linear model was chosen because it contained the highest-order polynomial with significant additional terms and was not aliased. The F-value of the model is 253.89, indicating that it is significant (SD 7). Only 0.01 % of the time may an F-value this large be caused by noise. Because of the probability value (p-value) of the various models under consideration (0.0001), a Linear vs. Mean model was chosen.

Analysis of variance (ANOVA) for linear model

The findings of the ANOVA for selected models is shown in SD 8. When the probability value (P value) is less than 0.05, the coefficient in this table is considered significant. P-values less than 0.0500 indicate that the model terms are significant in this case. In this scenario, the model terms A, B, C, and D are crucial. Values greater than 0.1000 indicate that the model terms are insignificant. Model reduction may improve your model if it contains a large number of insignificant model terms (other than those required to enable hierarchy). The Lack of Fit F-value of 5.78 suggests that there is a 5.03 % chance of a large Lack of Fit F-value occurring due to noise.

Effect of substrate concentration on PHA yield

The results revealed that there was a positive interaction between carbon source and PHA yield as shown in SD 9. The lowest PHA yield (55.213 mg/mL) in this interaction was obtained when the carbon source was low (1.5 %), while the highest PHA yield (78.667 mg/mL) for this interaction was obtained when a 3 % carbon source was used. This implies that increase in carbon source significantly affected the PHA yield of the optimization medium.

Effect of incubation time on PHA yield

The results revealed the effect of incubation time on PHA yield. SD 10 revealed that there was a continuous increase in PHA yield as the incubation time kept increasing. The lowest PHA yield (66.708 mg/mL) in this interaction was obtained when the incubation time was 13 h (low), while the optimum PHA yield (78.667 mg/mL) for this interaction was obtained when the incubation time was 24 h. This means the incubation time had a significant effect on the PHA yield.

Effect of temperature on PHA yield

There was a slow increase in PHA yield as the temperature increased (SD 11). The lowest PHA yield (75.539 mg/mL) in this interaction was obtained when the temperature was 36.7 °C (low), while the highest PHA yield (77.157 mg/mL) for this interaction was obtained when the temperature of was 38.3 °C. This means temperature also had a significant effect on PHA yield.

Effect of pH on PHA yield

The results revealed that there was a negative interaction between pH and PHA yield as shown in SD 12. The highest PHA yield (76.209 mg/mL) was obtained when the pH was 6.5, while at the least pH 6.1, the PHA yield obtained was 76.446 mg/mL. This implies that the slight change in pH from significantly affected the PHA yield.

Multi response optimization

The collected data was numerically optimized for minimum and maximum PHA yield. Desirability is an objective function with a value ranging from zero outside of the boundaries to one at the goal. The

Table 2
Desirability Responses for 25 solutions generated from analysed data for PHA optimization.

Number	Carbon source	Incubation Time	Temperature	pH	PHA	Desirability	
1	**3.000**	**24.000**	**38.353**	**6.500**	**76.363**	**1.000**	**Selected**
2	2.990	24.000	38.353	6.500	76.209	0.999	
3	3.000	24.000	38.353	6.410	77.157	0.993	
4	2.933	23.997	38.353	6.500	75.376	0.993	
5	3.000	23.857	38.353	6.558	75.723	0.992	
6	3.000	23.342	38.353	6.500	75.785	0.991	
7	2.905	24.000	38.343	6.500	74.966	0.990	
8	2.889	24.000	38.353	6.500	74.733	0.989	
9	3.000	23.962	38.322	6.320	77.900	0.985	
10	2.991	22.789	38.353	6.500	75.161	0.982	
11	3.000	24.000	38.353	6.238	78.667	0.981	
12	2.971	23.195	38.158	6.500	75.133	0.980	
13	2.799	24.000	38.353	6.500	73.406	0.979	
14	2.730	24.000	38.353	6.500	72.392	0.972	
15	3.000	22.457	38.353	6.370	76.152	0.969	
16	3.000	21.072	38.285	6.500	73.753	0.955	
17	2.999	23.995	36.771	6.500	75.539	0.949	
18	3.000	23.993	38.560	6.696	74.734	0.947	
19	2.693	24.000	37.608	6.500	71.470	0.945	
20	2.488	24.000	38.353	6.500	68.834	0.944	
21	2.226	24.000	38.353	6.500	64.976	0.912	
22	3.000	17.989	38.339	6.500	71.078	0.903	
23	3.000	20.238	38.682	6.134	76.446	0.868	
24	1.563	24.000	38.353	6.500	55.213	0.808	
25	3.000	13.003	38.353	6.500	66.708	0.790	

numerical optimization identifies a position where the desirability function is maximized. All goals are incorporated into one desirability function for many responses and circumstances. The Ramps display the attractiveness of each component and reaction, as well as the sum of the desirability. A highlighted point indicates both the exact value of the element and how effectively that aim was met. The desirability responses revealed that 76.363 mg/mL of PHA was the uptimum PHA yield during optimization and was best achieved with actual factors for 3 % carbon source, 24 h incubation time, pH of 6.5 and temperature of 38 °C according to the surface response factor coding using design expert v11 software. The desirability of 1.000 was obtained for actual factors as depicted in the Ramp function plot.

Validation of the optimization process

The following equation is in terms of actual factors (carbon amount, temperature, incubation period, and pH) and can be used to estimate the response (PHA concentration) for given levels of each factor. For each factor, the levels should be indicated in the original units. Because the coefficients are scaled to fit the units of each element, and the intercept is not at the center of the design space, this equation should not be used to evaluate the relative impact of each factor.

$$PHAc = +23.33692 + 17.22476CA + 2.19213\ t + 0.487175\ T - 5.13419pH - 0.028444\ CA * t + 0.005333\ CA *T - 0.361143CA * pH + 0.002769\ t * T\text{-}0.201587\ t * pH - 0.000262\ T * pH$$

where PHAc = PHA concentration, CA = Carbon Amount, T = Temperature, t = Incubation Time.

On the validation of the optimized process, it was observed that the predicted concentration of PHA was 76.363 mg/mL under the conditions of 3 % carbon source, 24 h incubation time, pH of 6.5 and temperature of 38° C, while the experimental value was 87.993 mg/mL under the same condition. The difference between the predicted and experimental PHA concentration was 11.630 which represented 15.23 % deviation. This is within acceptable deviation range.

The reducing sugar concentration of sugarcane bagasse was 21.45 mg/mL which is within the 20–25 mg/mL normal range for sugar cane bagasse. This is clear from the SEM used to assess microstructural changes in bagasse after treatments. These alterations are favorable for enhancing enzyme accessibility to cellulose, and hence enzyme hydrolytic performance was increased. As a result, the alkali treatment can improve the overall saccharification rate of enzymatic hydrolysis.

The results revealed that all the four parameters (carbon source, temperature, incubation time and pH) played significant role in achieving optimum PHA yield. Although, the carbon source, pH and incubation time showed the most significant ($P < 0.05$) effect in achieving PHA yield as compared to the temperature effect.

In this investigation, the pH range employed was 3.0–10.0. The PHA produced was 76.363 mg/mL at pH 6.5 which slightly increased to 76.446 at pH of 6.1. Although, *Pseudomonas* sp grows best in a neutral pH range of 6.0—7.0. The optimal pH for optimization using *Pseudomonas aeruginosa* was 6.5 in this investigation. The response surface methodology produced the best PHA desirability output (76.209 mg/mL) at this pH 6.5. This slightly disagree with report of Getachew and Woldesenbet (2016) that highest PHA produced by isolate AWW occurred within the pH range 7.0–7.5. Another reason for this could be that the initial pH of the MSM utilized in PHA synthesis was neutral (pH 6.5). The graph of response surface methodology on the effect of pH on PHA yield shows that increase in pH from neutral to basic pH decreases the PHA yield.

The concentration of the substrate was changed between 0.5, 1.75, and 3 %. The concentration of substrate is the most significant factor in organism development and nutrition. The optimum carbon source concentration for good PHA synthesis was discovered to be 3 %. The lowest PHA yield was found at carbon source concentration 1.5 %, indicating that the organism required a high substrate concentration to reach log phase. Varsha et al. (2019) obtained comparable results from Eichhornia crassipes utilizing glucose as the carbon source. With an inexpensive substrate like agricultural waste, PHA production will be both profitable and economically beneficial. The RSM graph of the effect of carbon source on PHA output revealed an inversely proportionate relationship between carbon source and PHA yield, implying that any change in carbon source affects PHA production. This demonstrates that carbon is the most significant component for microbial growth and nourishment, and for PHA formation, carbon used by the organisms is transformed into granules in the organism's cytoplasm via PHA synthesis.

Temperature has a crucial effect on PHA growth; temperatures were varied at 20, 50, and 80 °C. The best temperature for PHA accumulation using RSM was discovered to be 38 °C (based on the desirability response). Varsha et al. (2019) also found that 37 °C was the optimum temperature for PHA accumulation and highest cell density. Temperature changes can cause changes in PHA content because lower temperatures slow down the enzyme activity and metabolism of the organism, reducing their ability to create PHB. Essentially, PHA generation is achievable at temperatures where the organism thrives the maximum. *Pseudomonas aeruginosa* can survive at temperatures as high as 50–60 °C, which could explain why PHA synthesis did not fully cease when the temperature was elevated. The desirability response having 38 °C as the optimum temperature for PHA production using *Pseudomonas aeruginosa* could be because this organism thrive best at this temperature. Another cause for the lack of development at high temperatures could be the influence of heat on PHA-producing enzyme activity.

The incubation period was adjusted from 6 to 15 to 24 h during the optimization. The influence of incubation period on growth is significant in this study. The optimum incubation time for PHA growth and accumulation utilizing RSM was discovered after a 24-hour incubation period. The lowest level of PHA was recorded after a 13-hours incubation time, indicating that the organism requires a long incubation time to enter log phase. The observation is straightforward: increase in incubation time pushed for increase in microbial growth, resulting in increased PHA production. However, after the log phase of microbial development is completed, microbial growth begins to drop, resulting in a decrease in PHA production. Desouky et al. (2014) found that optimizing PHA production from *Bacillus thuringiensis* resulted in the greatest amount of growth and PHA accumulation after 72 h of incubation.

The desirable PHA yield was at its optimum when carbon source was 3 %, temperature 38 °C, 24 h incubation time and pH of 6.5. This implies that changes in these four factors (carbon source, pH, temperature and incubation time) influenced the experimental PHA yield, statistically making it significant ($P < 0.05$). This finding is consistent with Getachew and Woldesenbet (2016) that optimal PHA synthesis was achieved within pH range 6.0–7.5. After 48 h, Noha et al. (2013) showed maximal PHA accumulation and cell density at 37 °C. Temperature changes can cause changes in PHA content because severe temperature decelerates the metabolism (enzyme activity) of bacteria, reducing their ability to create PHB. This is consistent with the findings of Getachew and Woldesenbet (2016), who found that greater carbon supply in the medium, longer incubation period, 38 °C temperature, and 6.5 pH resulted in the highest PHA output. The slopes from the graphs shows that the carbon source, temperature, incubation time and pH is inversely proportional to the PHA yield. The production of PHA had low P-value (0.0500) which further support statistical significance.

Verification studies were carried out with numerically optimized levels of experimental variables in order to demonstrate the applicability of maximum response generation. The software suggested that the optimal circumstances for acceptable PHA and biomass synthesis were 3 % carbon supply concentration, 24 h time, and pH 6.5, and the predicted PHA output was 76.363 mg per 100 mL culture at desirability 1.000. The PHA yield obtained following the validation experiment under the predicted conditions was 76.879 mg per 100 mL culture. As a result, the

verification trials proved accuracy of the projected model.

Extraction and purification of PHA produced

The recovery and purification of intracellular PHA by sodium hypochlorite digestion is a widely used method, and it was chosen for this thesis study to extract PHA from *P. aeruginosa* growth. The PHA during extraction (centrifugation) appeared sticky but turned to powder form after purification with H_2SO_4. The purified PHA appear creamy in colour as shown in Fig. 6. The PHA produced from 10 L of MSM was quantified to be 10.57g after purification.

The extraction of intracellular PHA by sodium hypochlorite digestion is the most generally used approach, and it was chosen for this study to extract PHA from *P. aeruginosa*. The 10.57 g pf PHA obtained from 10*L* production medium is equivalent to 1.06 g/L. a yield of 2.30 g/L was obtained when Choonut et al. (2017) used higher concentration (20 g/L) of sugarcane molasses as carbon source, similar extract method was also used. Yu-Hong et al. (2011) also observed that PHB production in MM9 medium was 4.15 g/L when measured under the following conditions: Temperature of 30 °C, pH of 7 and agitation speed of 200 rpm. A higher quantity of PHA obtained could be as a result of the production medium with an increased carbon source since carbon source is a determinant in the quantity of PHA produced.

Conclusion

A local strain of *Pseudomonas aeruginosa* capable of accumulating PHA was molecularly confirm using the 16 s rDNA sequence analysis. PhaC, the polymerase gene responsible for polymerization into P(3HB), was encoded as the PhaC1 gene in the isolate. Statistically, it was observed that sugarcane bagasse had the highest PHA production potential as compared to the other agrowastes when screened for the capability to be exploited in PHA production. The hydrolysate extracted from the sugarcane bagasse was used as the carbon source for PHA production by the *Pseudomonas aeruginosa* (OL405443). Response surface methodology (RSM) for the optimization study showed that all the factors were statistically significant ($P < 0.05$) for PHA production. At 38 °C, 3 % carbon source, pH 6.5, and 24-hour incubation period, the bacterium was able to synthesize polyhydroxyalkanoate optimally. The interesting part of this is the fact that *Pseudomonas aeruginosa* is cheap to obtain and can survive in either aerobic or anaerobic conditions. The PHA produced from 10 L of MSM was quantified to be 10.57 g after purification and drying. *Pseudomonas aeruginosa* is a promising strain for the conversion of sugarcane bagasse into PHA polymer, as well as a viable choice for industrial PHA production. Prospects should focus on genetic modification of *Pseudomonas aeruginosa* for enhanced PHA production, industrial-scale production of the PHA, and application of the PHA in bioplastic production and in other industries.

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CRediT authorship contribution statement

Oluwafemi Adebayo Oyewole: Conceptualization, Supervision. **Shehu Usman Abdulmalik:** Investigation, Writing – original draft. **Asiya Onozasi Abubakar:** Investigation. **Konjerimam Ishaku Chimbekujwo:** Writing – review & editing. **Yemisi Dorcas Obafemi:** Writing – review & editing. **Benjamin Oyegbile:** Formal analysis, Validation. **Olabisi Peter Abioye:** Funding acquisition, Supervision. **Olalekan David Adeniyi:** Writing – review & editing. **Evans Chidi Egwim:** Methodology.

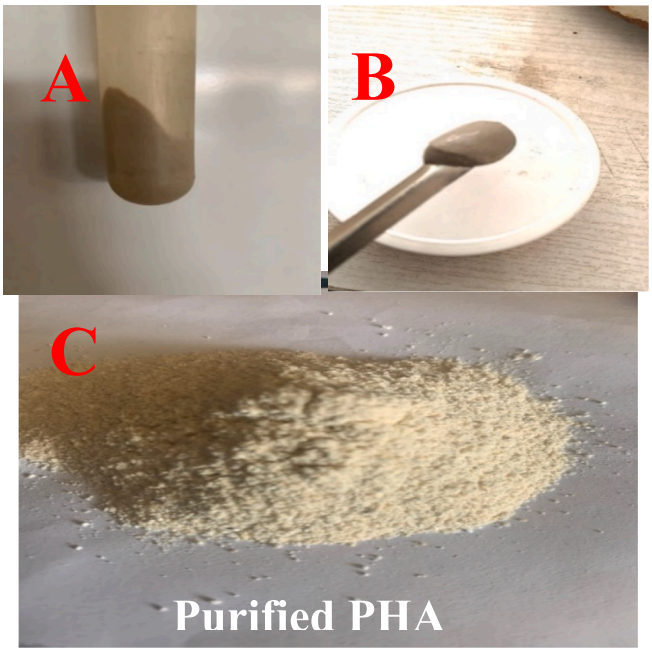

Fig. 6. Extracted and purified PHA; **A**- PHA after spinning (centrifugation), **B** – PHA extract, **C**-Purified PHA particles/granules.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Olabisi Peter Abioye reports financial support was provided by Tertiary Education Trust Fund.

Data availability

The data have been included in the submission

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clema.2024.100217.

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