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Production and Characterization of Polyhydroxyalkanoate (PHA) Using Mango Seed Kernel as an Alternative to Glucose

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Authors' contributions

This work was carried out in collaboration between all authors. Author KONN designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors KONN, KOS and AYK managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To explore the possibility of using Mango (*Mangifera indica*) seed kernel as an alternative to glucose in the production of biodegradable plastic (polyhydroxyalkanoate) using soil isolated *Bacillus megaterium*.
Study Design: Experimental design.
Place and Duration of Study: This research was carried out at Biotechnology and Genetic Engineering Advanced Laboratory, Sheda Science and Technology Complex and Biochemistry Department, Federal University of Technology Minna, Nigeria, between the periods of June 2014 to October 2015.
Methodology: Using fed-batch fermentation technology, polyhydroxyalkanoate (PHA) pellets were

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produced, extracted and purified via solvent extraction. Polyhydroxyalkanoate produced was quantified using the crotonic acid assay at 235 nm by UV-VIS spectrophotometry. Fourier transform infrared spectrometry (FTIR) was used to characterize the PHA.

Results: Mango seed kernel produced a significantly greater ($p<0.05$) dry cell weight (DCW) and polyhydroxyalkanoate (PHA) (10.60±0.90 g/L; 64.22±0.55%) compared to glucose (10.19±0.01 g/L; 54.40±5.60%). Optimum conditions for maximum production of polyhydroxyalkanoate (PHA) were pH 7.5; temperature 35℃; substrate concentration of 2 g/100 ml; *Bacilli* inoculums volume of 2 ml and an incubation period of 72 hours. Fourier transform infrared spectrometry (FTIR) of extracted PHA revealed C-O, C=O, C-H, O-H functional groups at respective wave bands of 1125.5, 1644.37, 2938.65 and 3399.65 cm^{-1} for extracted mango seed kernel PHA; and 1086.92, 1646, 2937 and 3403.51 cm^{-1} for extracted glucose PHA.

Conclusion: Mango (*Mangifera indica*) seed kernel produced higher dry cell weight and polyhydroxyalkanoate than glucose hence, could be a substitute to glucose in PHA production.

Keywords: Polyhydroxyalkanoate; Bacillus megaterium; Dry cell weight; crotonic acid.

1. INTRODUCTION

Risk factors associated with non-biodegradable conventional plastics, such as release of dioxin (a toxin resulting from incineration of petro-chemical derived plastics) into the atmosphere, leaching of pollutants from landfills into ground water, build-up of plastics in the marine environment, food web contamination from potentially harmful chemicals like bisphenol-A and phthalates which are blended with plastics during their manufacture as well as, exhaustion of fossil fuels have paved the way for biodegradable alternatives such as; polyhydroxyalkanoate (PHA).

Polyhydroxyalkanoates are bioplastics in the family of biodegradable polyesters synthesized by a wide range of microorganisms such as *Cupriavidus necator*, *Alcaligenes latus, Bacillus megaterium*, *Pseudomonas putida* and recombinant *Escherichia coli* [1]. Polyhydroxyalkanoates (PHAs) are insoluble in water, and accumulate as intracellular food and energy reserves (granules) in response to nutrient limitation or imbalance in the environment [2]. These granules help prevent starvation when essential nutrients (such as nitrogen, phosphorous and oxygen) are limited and carbon source is excessive. Certain cyanobacteria as well as some halophiles are also known to accumulate PHAs not only under unsuitable environmental conditions but also under suitable conditions [3].

About 150 different PHA monomers have been identified and this number is on the increase with the introduction of new types through the chemical or physical modification of the existing ones, or through the creation of genetically modified organisms (GMOs) [4]. Polyhydroxyalkanoate (PHA) is classified based on the total number of carbon atoms within the monomer, and this classification reflects their different physical properties such that short chain lengths (scl-PHAs) are highly crystalline solid/semi-solid and medium chain lengths (mcl-PHAs) are latex-like elastomers [5]. The large degree of variation in the functional group, number of unsaturated bonds and chain length allows PHAs to be tailored towards a large number of applications [4].

The most extensively studied PHA, polyhydroxybutyrate (PHB) with chemical formula of [–O–CH–(CH3)–CH2–CO–]n was first described in 1925 at the Pasteur Institute in Paris by Maurice Lemoigne; a French Microbiologist. Since then, it has attracted much commercial and research interest as a result of its biodegradability, biocompatibility, chemical-diversity, as well as its ability to be manufactured from renewable carbon resources [4]. These features give rise to diverse PHA properties that are beneficial for various applications ranging from biodegradable packing materials to medical applications. These properties of PHA have stimulated recent research interest in its production. Currently, the production costs of PHA are incomparable with that of petroleum-derived plastics due to high cost of raw material (glucose) used largely for its production [4]. This study exploits inexpensive production of PHA using Mango (*Mangifera indica*) seed kernel as substrate in a two-stage fermentation catalyzed by soil-isolated *Bacillus megaterium*.

2. MATERIALS AND METHODS

Mangos (*Mangifera indica*) were purchased from Gwagwalada-Abuja.

2.1 Sample Preparation

All seeds were manually separated from the fruit pulp, cleaned, washed in distilled water, air dried, and then shelled manually to remove the seed coat. The resulting kernels were crushed into pebbles (reduced particle sizes), detoxified by boiling in water bath at 100℃ for 30 min and allowed to cool overnight [6]. The supernatant was decanted and the residue washed several times (until the water was cleared).The detoxified kernels were oven dried at 50℃, milled in a blender (master chef, mc-9970B) then sieved using muslin cloth. The resulting flours were packed in a clean airtight polyethylene bag and kept at 4℃ until used [7].

2.2 Microorganism

Bacillus megaterium was isolated according to the method of Gulab et al. [8] with slight modifications. One gram of soil sampled from a depth of 10 cm within maize rhizosphere was suspended in 99 ml of distilled water, mixed vigorously and incubated in a water bath at 80℃ for 10 min. The suspension was then serially diluted in distilled water and dilutions from 10^{-1} to 10^{-6} were plated on nutrient agar prepared according to manufacturer's instructions and autoclaved at 121℃ for 15 min. The culture plates were then incubated at 37℃ for 24 h. Isolated colonies from nutrient agar culture were further sub-cultured according to the method of Abhilash et al. [9] on modified Sperber's medium, a selective media for *Bacillus megaterium*, containing 10 g/L glucose, 0.5 g/L yeast extract, 0.25 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2$., 15 g/L agar, pH 7.0 and sterilized in an autoclave at 121℃ for 15 min. The culture was then incubated at 37℃ for another 3 d. Isolated colonies were then preserved on nutrient agar slants at 4℃ for further identification and usage.

2.3 Identification of *Bacillus megaterium*

The isolated microorganism was identified on the basis of morphology (Sudan Black B Staining) and biochemical characteristics according to published methods [10,11].

2.3.1 Sudan black B staining

From the isolated colonies preserved on agar slant, 24 h growth culture was prepared and an aliquot was smeared on grease free slides using a sterile inoculating loop. The slides were allowed to dry, heat-fixed then stained for 10 min with Sudan Black solution prepared by dissolving 0.3 g of the powder in 100 ml of 70% ethanol [12]; [10]. Excess Sudan Black dye was drained from the heat-fixed slides, clarified with xylene, blotted dry, and counterstained with 0.5% aqueous Safranine solution for 5 s. The dye was rinsed with slow running tap water. The slides were subsequently air dried and viewed using X100 oil immersion lenses of Celestron digital microscope.

2.3.2 Amylase test

Starch agar medium containing 1% soluble starch, 0.3% beef extract, and 1.2% agar was prepared, sterilized and poured into Petri dishes to gel. The medium was inoculated with bacterial culture from nutrient agar slants by surface streaking and incubated at 37℃ for 48 h. The medium was then covered with iodine solution while rotating to distribute the iodine solution into a thin layer. Zone of hydrolysis around the colonies was used as a positive test for amylase activity.

2.3.3 Catalase test

Nutrient agar medium was prepared, sterilized and poured to gel in a slanting form. The agar slant was inoculated with bacterial culture from the agar slant stored at 4℃, then incubated at 37℃ for 24 h. Few drops of hydrogen peroxide (3%) were trickled down the slants. Gas bubbles indicating the presence of catalase activity was observed as a positive test.

2.3.4 Oxidase test

Colonies of the bacterial isolates preserved on nutrient agar slant were cultured at 37℃ for 24 h in nutrient broth containing: 15 g/L peptone, 3 g/L yeast extract, 6 g/L sodium chloride, 1 g/L D (+)- glucose at pH of 7.0, and sterilized at 121℃ for 15 min. Bactident Oxidase 1.13300 strips were used for the oxidase test after 24 h broth culture, allowed to air dry and the appearance of deep blue coloration on the strip indicated a positive test for oxidase activity.

2.3.5 Gelatinase test

Bacterial culture was spotted on sterile gelatin agar slants containing 1% gelatin, 1% peptone 1% beef extract, 0.5% NaCl, and 1.5% agar, then

incubated at 37℃ for 7 d. The tubes were then observed for liquefaction of gelatin as a positive test. To further confirm if liquefaction was due to gelatinase activity, the tubes were immersed in an ice bath for 30 min. Afterwards, the tubes were tilted to observe if gelatin had been hydrolyzed.

2.3.6 Urease test

Sterile urea broth was prepared by dissolving 0.01 g yeast extract, 0.91 g KH_2PO_4, 0.95 g K_2HPO_4, 2 g urea, and 1mg phenol red in 100 ml of distilled water with pH adjusted to 7.0. Bacterial isolates preserved on nutrient agar slants stored at 4^oC were inoculated into the urea broth and incubated at 37℃ for 48 h. Pink coloration was taken to indicate a positive test.

2.3.7 Indole test

Bacteria isolate preserved on agar slants were inoculated in peptone broth prepared by dissolving 1 g peptone and 0.5 g NaCl in 100 ml of distilled water. The pH was adjusted to 7 then sterilized at 121℃ for 15 min. The culture was incubated at 37℃ for 48 h. Kovac's reagent (5 drops) was added to the broth culture. After 5 min, a red layer at the top of the tube was observed as positive test and yellow coloration as negative test.

2.3.8 Methyl red test (MR Test)

Glucose phosphate broth (0.5% glucose, 0.5% K_2HPO_4 and 0.5% peptone) was adjusted to final pH of 7 and sterilized at 121℃ for 15 min. Bacterial isolates preserved on agar slants were inoculated into the broth, and incubated at 37℃ for 48 h. Methyl red indicator (3 drops) was added, development of red coloration indicated a positive test for mixed acid production from glucose phosphate broth fermentation.

2.4 Production of Polyhydroxyalkanoate (PHA)

Production of PHA was carried out in a two-stage fermentation process according to methods described in [1]; [13] and [14]. In the first production stage, primary inoculum was prepared by culturing *Bacillus megaterium* in 100 ml of sterilized nutrient broth, at 37℃ in an incubator shaker (New Brunswick Scientific, Innova 44) with a regulated speed of 120 revolutions per minute (rpm) for 24 h.

In the second stage, 2 ml of the 24 h culture was introduced in to 100 ml of two sterilized nutrient deficient media (pH 7.0) containing 0.02% $MgSO_4$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% yeast extract, and 0.25% peptone, with the carbon sources: 1% glucose and 1% mango seed kernel flour respectively and 0.1 ml of trace element solution (1.3 g/L $ZnSO_4.7H_2O$, 0.2 g/L $CaCl_2$, 0.2 g/L $FeSO_4$.7H2O, 0.6 g/L $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.6 g/L H_3BO_3). The cultures were then incubated at 37℃ in a shaker (New Brunswick Scientific, Innova 44) at 120 rpm for 48 h. All production studies were carried out in triplicate.

2.5 Extraction and Quantification of PHA

Extraction and quantification was done according to the methods described in [13,8,15] with slight modifications. From each of the 48 h cultures, 50 ml aliquots were taken and centrifuged at 8000 rpm for 15 min in a Thermo Scientific Heraeus multifuge $3SR^+$ centrifuge. The supernatant was discarded and cell dry weight was estimated. Cell pellet was lysed by adding 10 ml of sodium hypochlorite solution then incubated at 50℃ for 1 h. The mixture was centrifuged at 10,000 rpm for 15 min and the supernatant discarded. The pellet was washed with 10 ml of distilled water, acetone and ethanol respectively.

The pellet was then dissolved in 10 ml of chloroform (BDH) and heated at 100℃ to evaporate to dryness. The dried PHA was converted to crotonic acid by adding 3 ml of concentrated H_2SO_4. The mixture was heated in a water bath at 100℃ for 10 min then left to cool. Absorbance was determined at 235 nm using a UV-VIS spectrophotometer (Biochrom-Libra S22). The concentration of extracted PHA (g/ml) was extrapolated from a standard curve for crotonic acid prepared according to the method described in [16] and PHA accumulation (%) was calculated using the formula:

PHA (%) = Conc. of extracted PHA (g/L) X 100/DCW (g/L).

2.6 Dry Cell Weight Determination

Following each production processes, after the initial centrifugation, the washed pellet was re-suspended in 1 ml distilled water, transferred to pre-weighed boat and dried to constant weight at 60℃. This weight was recorded as cell dry weight (DCW) in gram per litre (g/L) [14].

2.7 Optimization of Culture Conditions for PHA Production

Different factors (incubation temperature, substrate concentration, pH, cell volume and Incubation time) affecting PHA production by *Bacillus megaterium* were optimized by a modification of the methods in [1,8,14].

2.7.1 Optimization of incubation time

PHAs were collected, extracted and quantified at different time intervals of 0, 24, 48, 72, 96 and 168 hours.

2.7.2 Temperature optimization

PHA was produced at temperatures of 25℃, 30℃, 35℃ and 40℃ in an incubator shaker at 120 revolutions per minute (rpm). Samples were then collected after 48h for PHA extraction and quantification.

2.7.3 Optimization of pH

PHA production was carried out in media prepared using sodium phosphate buffer solution at the following pH values; 6.0, 6.5, 7.0, 7.5 and 8.0. Samples were collected after 48 h for PHA extraction and quantification.

2.7.4 Optimization of substrate concentration

PHA production was carried out using 0.5%, 1%, 2% and 4% carbon sources (glucose and mango seed kernel) respectively. Samples were collected after forty-eight hours for PHA extraction and quantification.

2.7.5 Optimization of cell volume

Inoculums volumes of 1, 2, 3, 4 and 5 ml of the twenty 24 h culture were used for PHA production. Samples were collected after 48 h for PHA extraction and quantification.

2.8 Characterization of Polyhydro-xyalkanoate (PHA)

To identify the various functional groups present in the extracted PHA, FTIR analysis was performed using a Shimadzu FTIR-8400S instrument. Each of the extracted PHA polymer fractions (2 mg) was dissolved in 500 µl of chloroform and layered on NaCl crystals. After evaporation of the chloroform, PHA polymer film was subjected to FTIR in a spectral range of 4500-500 cm-1 [14].

2.9 Statistical Analysis

All analyses were carried out in three replicates for all determinations and the results were expressed as mean ± SEM. Data were exported from Microsoft Excel and analyzed using IBM SPSS Ststistics. Version 20.0. Statistical differences between means were compared using Duncan Multiple Range Test and considered statistically significant at $p<0.05$.

3. RESULTS

Sudan black stained *Bacillus megaterium* appeared as gram positive rod shaped cells under oil immersion (Plate I) and tested positive for amylase, catalase, oxidase gelatinase and methyl red test but negative to indole and urease test (Table 1).

Table 1. Biochemical confirmation of *Bacillus megaterium*

Biochemical test	Inference
Amylase	+
Catalase	+
Oxidase	+
Gelatinase	+
Urease	–
Indole	–
Methyl red	+

Key: + = present, - = negative

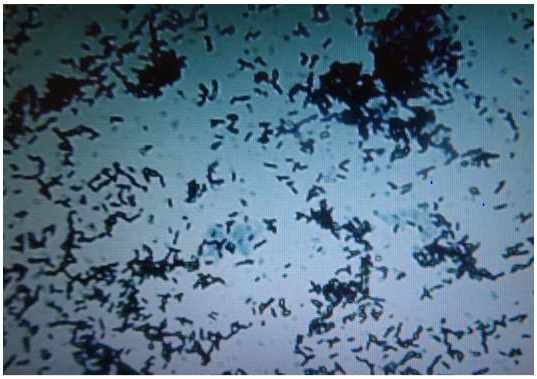

Plate 1. Sudan black stained *Bacillus megaterium* (1000 magnification)

Mango seed kernel fed cultures of B *megaterium* produced a significantly higher (*P<0.05*) DCW of 10.60 g/L with corresponding PHA accumulation

of 64.22% than did glucose fed cells with 10.19 g/L DCW and 54.40% PHA accumulation (Table 2).

Production pH ranged from 6.0 to 8.0 (Fig. 1). Product increased as the media pH changed from acidic conditions to neutral and then decreased as the media pH became alkaline. Highest product was obtained at pH 7.5. Although at this pH there was no significant difference ($P>0.05$) in DCW produced from the two substrates, but in the corresponding PHA accumulation, mango seed kernel fed cells (55.24%) had significantly higher ($P<0.05$) product than glucose fed cells (50.70%).

Incubation period ranged from 0 to 168 h (Fig. 2). Product accumulation increased as the incubation time increased up to 72 h after which a decrease was observed as production period increased. Highest production was recorded at 72 h and lowest production at 168 h. After 72 h, mango seed kernel fed cells (9.87 g/L; 64.85%) accumulated a significantly higher ($P<0.05$) DCW and PHA than glucose (8.34 g/L; 59.22%).

Table 2. Dry cell weight and Polyhydroxyalkanoate accumulation of substrates

Substrate	Dry cell Weight (g/L)	Polyhydroxyalkanoate (%)
Mango seed Kernel	10.60±0.90[a]	64.22±0.55[a]
Glucose (control)	10.19±0.01[b]	54.40±5.60[b]

Results in the same column with different superscripts are significantly different at (P<0.05)

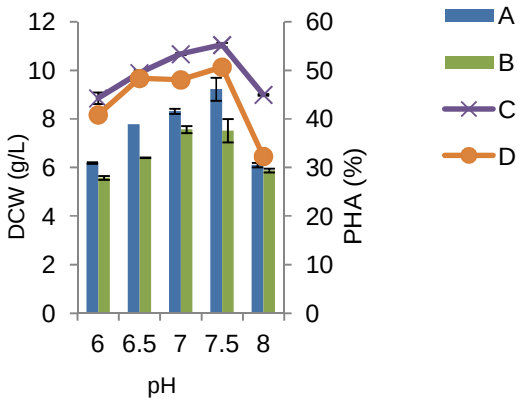

Fig. 1. Effect of pH on polyhydroxyalkanoate production

Key: DCW = dry cell weight, PHA= polyhydroxyalkanoate, A= Mango Seed Kernel, B= Glucose, C= mango seed kernel PHA accumulation (%) and D = glucose PHA accumulation (%)

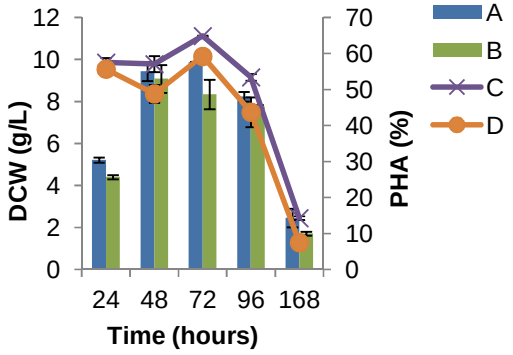

Fig. 2. Effect of incubation period on polyhydroxyalkanoate production

Key: DCW = dry cell weight, PHA= polyhydroxyalkanoate, A= Mango Seed Kernel, B= Glucose, C= mango seed kernel PHA accumulation (%) and D= glucose PHA accumulation (%)

Product accumulation increased as substrate concentration increased from 0.5 to 2.0 g/100 ml (Fig. 3). Highest product accumulation was observed at a substrate concentration of 2.0 g/100 ml, a point where mango seed kernel fed cells (10.60 g/L; 64.20%) accumulated significantly higher (P<0.05) product than glucose fed cells (9.92 g/L; 59.50%). As substrate concentration changed from 2.0 to 4.0 g/100 ml, PHA accumulation in mango seed kernel fed cells became relatively constant but dropped significantly from 59.50% to 54.40% in glucose fed cells.

Highest DCW (7.77 g/L) and PHA (66.09%) accumulations was obtained at 40℃ and 35℃ respectively (Fig. 4). The optimum temperature for PHA production was 35℃ and at this temperature there was no significant difference (P>0.05) between DCW accumulation in mango seed kernel (7.42 g/L) and glucose (7.92 g/L) fed cells but, mango seed kernel (66.09%) fed cells produced significantly more (P<0.05) PHA than glucose fed cells (43.90%).

Inoculum volume ranged from 1 to 5 ml and as this volume increased from 1 to 2 ml, DCW and PHA production increased but began a decline at 3 ml (Fig. 5).

Functional groups; C-O, C=O, C-H and O-H were observed at wave bands of 1125.50 cm^{-1}, 1644.37 cm^{-1}, 2938.65 cm^{-1} and 3399.65 cm^{-1} respectively in PHA extracted from mango seed kernel fed cells (Fig. 6). The same functional groups were observed for wave bands of 1086.92 cm^{-1}, 1646.30 cm^{-1}, 2937.68 cm-1 and 3403.51 cm^{-1} respectively in PHA extracted from glucose fed cells (Fig. 7).

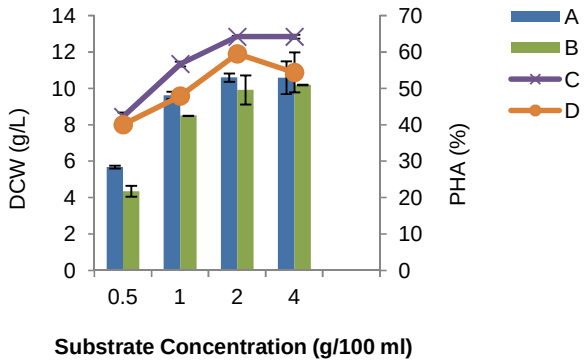

Fig. 3. Effect of substrate concentration on polyhydroxyalkanoate production

Key: DCW = dry cell weight, PHA= polyhydroxyalkanoate, A= Mango Seed Kernel, B= Glucose, C= mango seed kernel PHA accumulation (%) and D= glucose PHA accumulation (%)

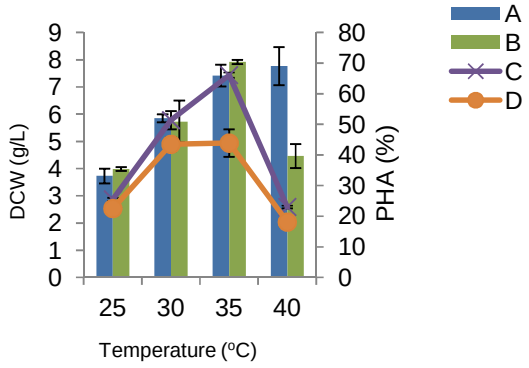

Fig. 4. Effect of temperature on polyhydroxyalkanoate production

Key: DCW = dry cell weight, PHA= polyhydroxyalkanoate, A= Mango Seed Kernel, B= Glucose, C= mango seed kernel PHA accumulation (%) and D= glucose PHA accumulation (%)

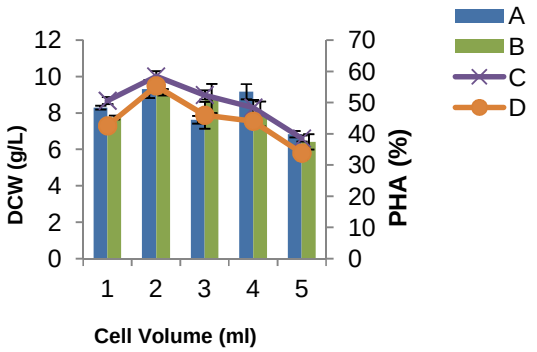

Fig. 5. Effect of cell volume on polyhydroxyalkanoate production

Key: DCW = dry cell weight, PHA= polyhydroxyalkanoate, A= Mango Seed Kernel, B= Glucose, C= mango seed kernel PHA accumulation (%) and D= glucose PHA accumulation (%)

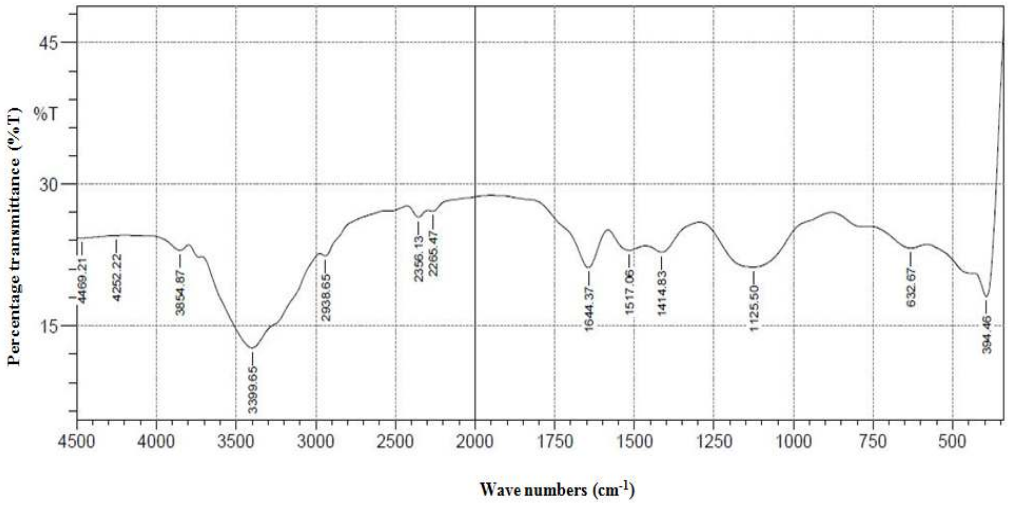

Fig. 6. FTIR curve of mango seed kernel-extracted PHA

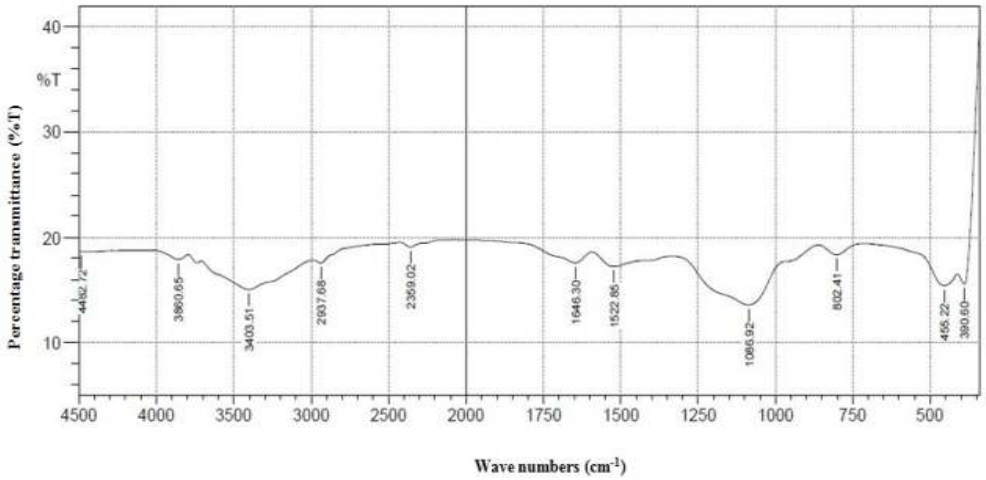

Fig. 7. FTIR curve of glucose-extracted PHA

4. DISCUSSION

Higher product accumulation was recorded for cells fed mango seed kernel flour (Table 2) which can be attributed to mango seed kernel flour having a higher carbohydrate content (74.75 to 76.14% [17] and [18]) than glucose. The amounts of PHA produced in this study are close to the amounts reported in [8] for raffinose (60.57%), lactose (59.52%) and sucrose (53.35%) fed *Bacillus* species.

Optimum production at pH 7.5 (Fig. 1) may be due to the fact that while acidic and alkaline media present adverse cultural conditions for bacteria survival, neutral media presents optimal conditions for the enzymes involved in the biosynthesis of PHA [19] and [20]. This pH optimum 7.5 is in accordance with the optimum pH of PHA production reported in [13,21].

Looking at the trend of DCW and PHA accumulation for the various incubation periods (Fig. 2), 24 h and 48 h could be said to be the log phase, 72 h; the stationary phase, 96 h and 168 h; the death phase of the microorganism. In the log phase, the isolate rapidly utilized the available carbon to enter the stationary phase and theoretically had a balance between new and dying cells. At this phase, PHA production is higher since this is the point in the growth curve in which metabolite production is at its peak [16]. The reduction in PHA accumulation after 72 h may be due to depletion of carbon and/or degradation of accumulated PHA by PHA depolymerase [16]. Sporulation and an increase in metabolites which also contribute to the negative effect on the PHA accumulation after the stationary phase [22] may also account for the low production recorded after 96 h.

Increase in PHA production as substrate concentration increased 1 to 2 g/100 ml may be due to increase in microbial growth in the presence of excess carbon and limited nitrogen supply, the stagnancy, then decline in production observed as substrate concentration increaseded from 2.0 to 4.0 g/100ml (Fig. 3) may be due to substrate inhibition [23].

A drastic fall in PHA but not DCW accumulation at 40℃ (Fig. 4) can be attributed to the thermophilic nature of *Bacillus megaterium*. Hence, elevated temperature favoured biomass accumulation but not that of PHA. A temperature optimum of 35℃ is in accordance with a 33℃ optimum incubation temperature reported in [21].

As the volume of inoculums was increased from 2 to 5 ml (Fig. 5) a rapid reduction in accumulation of PHA was observed, which was not obvious in DCW accumulation. This may be as a result of having excess isolates feeding on limited carbon substrate in the media for growth rather than for PHA production.

FTIR analysis of the extracted PHA revealed bands (Figs. 6 and 7) that are characteristics of PHA. These characteristic absorbance bands are similar to those reported in [24,25].

5. CONCLUSION

This study investigated the potential of *Bacillus megaterium* isolated from soil to utilize mango seed kernel as a carbon substrate in PHA production. The results indicated that substantial amounts of PHA can be produced. However, further study will be done using hydrolyzed mango seed kernel flour in addition to the crude flour in an effort increase the economic feasibility of using mango seed kernel as carbon source in fermentative production of PHA.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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