



Morphological And Thermal Properties Of Polyhydroxybutyrate (Phb) Produced By *Pseudomonas oleovorans* From Waste Cooking Oils In Restaurants In Gashua Town, Yobe State, Nigeria

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are important polymers synthesized from hydroxyalkanoates from cheap substrates including waste cooking oils. This research is aimed at evaluating the morphological and thermal properties of polyhydroxybutyrate (PHB) produced from different types of waste cooking oil as cheap carbon source (as samples) using *Pseudomonas oleovorans*; through small-scale batch fermentation in the laboratory. The fermentation experiments in shake flask were set up using 10g/l of the waste oil samples in tryptone soya broth. After 48 hours, *P. oleovorans* and *R. eutropha* were able to synthesize PHB polymer from both oil samples. The resulting polymer was extracted using N-hexane/chloroform mixture, purified and freeze-dried. Physical properties like morphology of PHB produced showed dispersed homogenous aggregation of granular particles of varying sizes from 0.5 μm to 2 μm as revealed by scanning electron microscope (SEM). X-ray diffraction analysis showed crystalline nature of the PHB through display of characteristic patterns there are narrow, sharp and broad peaks that appear similar in the samples examined. Comparison of the results with literature findings indicates presence of a semi crystalline polymer as the Thermogravimetric analysis showed the thermal stability of the PHB samples produced by *P.oleovorans* from the oils as the PHB samples starts to degrade at 255°C and 248°C resulting in complete degradation at 340°C and 300°C respectively.

Keywords: Polyhydroxyalkanoates, polyhydroxybutyrate, *Pseudomonas oleovorans*

1.0 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are group of biodegradable polymers of hydroxyalkanoates (HAs) produced by various microorganisms through the accumulation of energy storage materials to overcome stress (Quiang *et al.*, 2018). The PHAs are synthesized through polymerization of hydroxyacyl-CoA thioesters in a reaction catalysed by PHA synthases (Khatami *et al.*, 2021).

The use of waste materials obtained from agriculture and industries as starting materials for the biosynthesis of PHA is an important strategy for the cost-effective production of the polymer and provides an alternative means to overcome waste disposal problems. Different convertible substrates

obtainable from variety of sources that includes waste cooking oil and plants oils provides triacylglycerols or fatty acids and glycerol which can serve as direct carbon source after hydrolysis of the constituent triacylglycerol backbone (Koller *et al.*, 2017). Food industry wastes including oils mainly disposed as wastes in the environment can serve as carbon sources for the microbial synthesis of PHAs, an important bioplastic material (Obruca *et al.*, 2017). Approximately 90 different genera of microbes comprising of aerobes, anaerobes, photosynthetic bacteria, *archaeobacteria* as well as lower eucaryotes show capabilities of accumulating and catabolizing these polyesters with polyhydroxybutyrate (PHB), PHA and their derivatives been the most widely produced among the microbial bioplastics. Other recombinant organisms including yeast and transgenic plants also possess this attribute (Masood *et al.*, 2021).

Synthetic polymers derived from petrochemicals are not biodegradable and their indiscriminate disposal results in increased environmental pollution problems notwithstanding their diverse domestic applications (Muneer *et al.*, 2021). The resistance of these synthetic plastics to degradation coupled with their disposal has attracted attention to develop alternative biodegradable polymers that are compatible with the ecosystem (Amaro *et al.*, 2019). This is the reason why a biodegradable alternative produced through the use of a cost-effective carbon source will bring about mitigation of waste disposal problem associated with environmental pollution problems in Gashua becomes a necessity.

The use of waste materials obtained from agriculture and industries as starting materials for the biosynthesis of PHA is an important strategy for the cost-effective production of the polymer and provides an alternative means to overcome waste disposal problems. Different convertible substrates obtainable from variety of sources that includes waste cooking oil and plants oils provides triacylglycerols or fatty acids and glycerol which can serve as direct carbon source after hydrolysis of the constituent triacylglycerol backbone (Koller *et al.*, 2017). Food industry wastes including oils mainly disposed as wastes in the environment can serve as carbon sources for the microbial synthesis of PHAs, an important bioplastic material (Raza *et al.*, 2018).

This study evaluates the morphological and thermal properties of polyhydroxybutyrate (PHB) produced from different types of waste cooking oil as cheap carbon source by employing *Pseudomonas oleovorans*; a bacterium through small-scale batch fermentation in the laboratory by evaluating the morphology of PHB synthesized relative to the oil samples, the crystalline nature of PHB produced from the oil samples and the thermal stabilities of the PHB synthesized from the oils.

2.0 MATERIALS AND METHODS

2.1 Collection of samples and bacteria

Two waste cooking oil samples (supplied by food restaurants in Gashua, Bade L.G.A, Yobe state) coded PS and TS are utilised individually as carbon source for PHA production. The oil samples differ in their fatty acids (FA) compositions with varying range of monounsaturated, polyunsaturated and saturated fatty acids evaluated by determining the iodine number of the oil samples.

Pseudomonas oleovorans and *Ralstonia eutropha* were used for the production of PHB from industrial waste cooking oils. These microorganisms were collected from the culture collection of microbiology laboratory, University of Maiduguri, Borno state, as streaked plates. The microorganisms were refrigerated at 4°C and cultures were maintained by streaking on TSA plates, once after every two weeks.

2.1 Growth media

The growth media used in this experiment are tryptone soya broth (TSB) and Tryptone soya agar (TSA). Tryptone soya broth (Lab M, UK) consist of water, 17g/l trptone, 3g/l soy peptone, 5g/l NaCl, 2.5g/l K₂HPO₄ and 2.5 g/l D-glucose. This was prepared by dissolving 30g/l of the powder in 1L of distilled water and stirring with a magnetic stirrer (VELP scientifica). 250ml of the broth was transferred into 500ml conical flasks and autoclaved at 121°C before use.

TSA (Lab M, UK) contains distilled water, 15g/l tryptone, 5g/l soy peptone, 5g/l NaCl and 12g/l Agar No.2. The agar was prepared by dissolving 37g/l TSA powder in 1L of distilled water (equivalent to 7.4g in 200ml of distilled water in separate bottles). This was autoclaved at 121°C and poured aseptically into Petri dishes and allowed to cool so that it becomes solid.

Ringer solution (Lab M, UK) was used for serial dilution (1/4 ringer) by dissolving one tablet in 500ml distilled water and dispensed into test tubes before autoclaving at 121°C. The solution aids in keeping the cells alive before they are plated on agar.

2.3 The fermentation process

Batch fermentation in conical flasks was carried out by preparing 250ml of TSB into each flask (of which 50ml of it was initially sonicated with 2.5ml oil to form a homogenate). The initial oil concentration in the mixture was approximately 10g/l. The oil- broth mixtures in the flasks was inoculated with 100µl of the starter culture. The shake flasks were incubated in a rotatory incubator at 150rpm, 30°C for 48 hours. However, the fermentation was stopped at intervals to enable sample collection at time (0, 3 and 6, 24, 27, 30 and 48 hours).

2.4 Determination of total cell dry weight and solvent extraction of polymer

Since the PHAs are accumulated within the cell inside inclusions; a procedure for the recovery or isolation and purification of the PHAs was used. This involves the use of hot chloroform to extract the polymer in an extraction device called the Soxhlet extractor (Thermo scientific) in a fume cupboard for three hours. Approximately 200ml of chloroform HPLC grade (Fischer Scientific) was used.

The total cell dry weight or biomass was determined by dispensing known quantity of the fermentation medium into 50ml centrifuge tubes after the fermentation is stopped after 48 hours. These tubes were centrifuged (HERMLE Z 300K) for 10 minutes at 3500rpm at 4°C and the resulting supernatant was discarded leaving the bacterial pellets. The pellets were washed by adding distilled water to the centrifuge tubes and centrifuged again at 3500rpm for 10 minutes at 4°C to get rid of any residual oil. The supernatant was discarded again leaving behind the bacterial biomass. This was kept in a -20°C freezer for 24 hours.

After freezing at -20°C, the pellets were lyophilized by freeze-drying using a freeze dryer (Edwards freeze dryer, Modulyo) for 72 Hours. The total cell dry weight in grams was estimated and recorded before solvent extraction and precipitation of the solubilized polymer (PHB) using cold n-hexane (Sigma Aldrich) in a 500ml beaker by slowly stirring using a magnetic stirrer. The precipitate is likely to be formed around the stirrer and settle as a visible precipitate at the bottom of the beaker.

The precipitated polymer was separated from the mixture of n-hexane-chloroform using a forceps to remove large clumps and purified by washing on a filter. The resulting polymer was dried in air and weighed using a weighing balance. The polymer extracted from the bacteria was used to calculate the percentage yield of the polymer relative to the amount of biomass in grams accumulated as shown below;

$$\% \text{ yield (PHB)} = \text{cell dry weight (g)} / \text{weight of polymer extracted} \times 100$$

2.5 Scanning electron microscopy

This involves the focussing of beams of electrons on the sample resulting in interaction between these electrons and the atoms contained in the sample leading to the generation of signals that give information on the surface morphology of the sample (Verlinden *et.al.* 2011). Scanning electron microscope (ZEISS EVO 50) using magnification of x 25, 000 at 2, 10 and 100µm. This technique will give an insight into the morphology of the polymer extracted from the various experiments.

2.6 Thermogravimetric analysis (TGA)

The thermal stability of the PHB samples extracted and the weight loss was determined using this technique (Rao, *et. al.*, 2019). A sample was placed in a pot (furnace) and heated between 20°C – 300°C in a thermogravimetric analyser (Perkin Elma, TGA 7) for 1hour. This is to determine the thermal stability of the polymer.

2.7 X-ray Diffraction

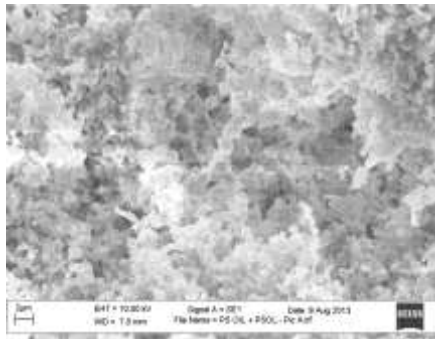
This technique was performed to examine the crystalline nature of the polymer samples extracted using X-ray diffraction (PANalytical EMPYREAM) device with Cu radiation (40kV, 40mA) at 2 theta position. The samples were grounded to powder after which a thin layer of a sample was prepared on a holder before been analyzed in order to establish the characteristic pattern in the polymer samples (Rao *et.al* 2019).

3.0 RESULTS AND DISCUSSION

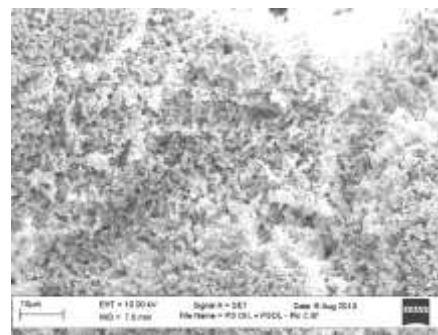
3.1 Scanning electron microscopy

In order to examine the morphology of the PHB samples extracted from the experiments; scanning electron microscopy was carried out at a magnification of $\times 2500$ at $2\mu\text{m}$, $10\mu\text{m}$ and $100\mu\text{m}$ in experiments involving *P.oleovorans* and *R.eutropha* with the oil samples. The images obtained are shown in the figure 1 and 2 below.

P.oleovorans with PS sample

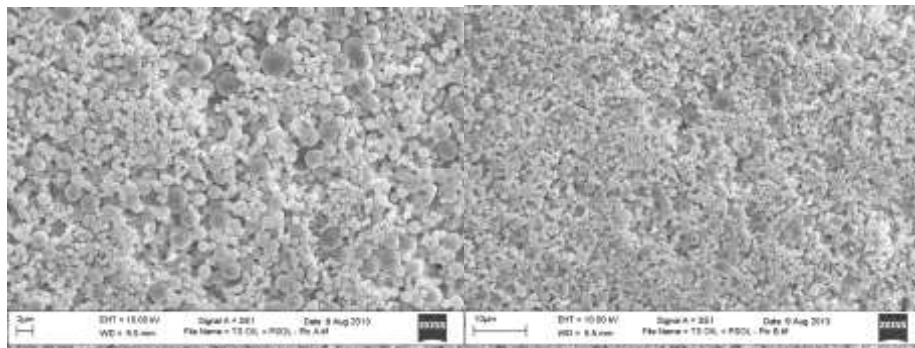


a. $2\mu\text{m}$



b. $10\mu\text{m}$

P.oleovorans with TS sample



c. $2\mu\text{m}$

d. $10\mu\text{m}$

Fig 1: Scanning electron microscopy of PHB from *P.oleovorans* with PS oil (a and b) and TS oil (c and d) at $\times 2500$ magnification and 10kV.

R. eutropha with PS sample

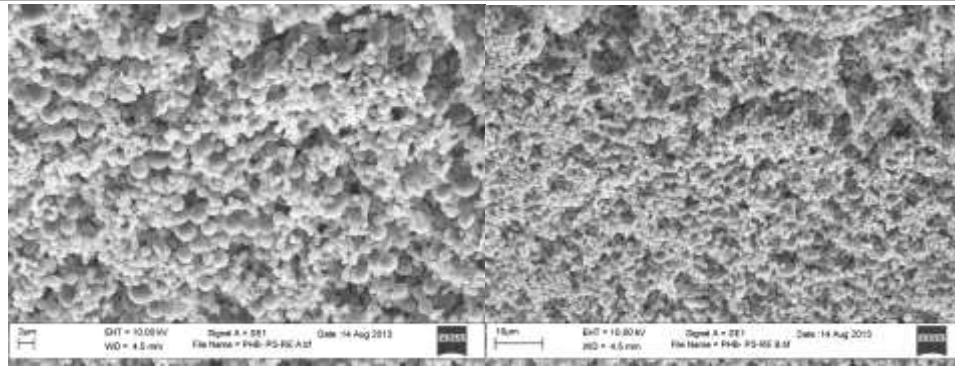
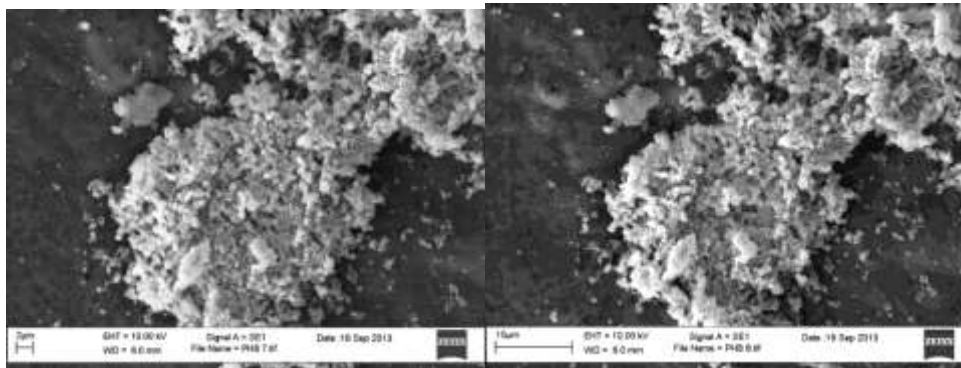
a. 2 μm b. 10 μm ***R.eutropha* with TS sample**c. 2 μm d. 10 μm

Fig. 2: Scanning electron microscopy of PHB from *R.eutropha* with PS oil (a,b) and TS oil (c,d) at x 2500 magnification and 10kV.

The SEM images from the PHB samples generated from the experiments shows that there is a slight difference in the surface morphologies of the PHB with respect to the oil samples as well as the different bacteria used in the experiment. In fig.1, it is evident that the PHB from *P.oleovorans* with the oil samples shows a homogenous aggregation of spherical granules with varying sizes. The particle size vary in the range of approximately 0.5 μm to 1 μm in PHB from PS oil with *P.oleovorans* compared to the particle sizes ranging from approximately 0.5 μm to 2 μm in PHB obtained from the same bacteria with TS oil sample with large and small particles distributed in the polymer. However, with *R.eutropha* (Fig. 2), the PHB morphology seems to be similar in shape but with uneven particle sizes approximately 0.5 μm to more than 2 μm with PS oil. The PHB morphology from the TS oil sample shows much smaller particles ranging from approximately less than 0.5 μm to 1 μm . Scanning electron microscopy of PHB samples from the experiments showed that there is a homogeneous aggregation of spherical particles in the polymer produced from the oil samples which appear porous. The surface morphology of the polymer shows spherical particles that vary in sizes.

3.2 Thermogravimetric analysis (TGA)

The thermal stability of the PHB samples extracted and the weight loss was determined using this technique (Rao, *et al.*, 2019). A sample was placed in a pot (furnace) and heated between 20°C – 300°C in a thermogravimetric analyser (Perkin Elma, TGA 7) for 1hour. This is to determine the thermal

stability of the polymer. The figures 3 below are showing the results obtained after 10mg of the samples were exposed to heat for one hour in the thermogravimetric analyzer.

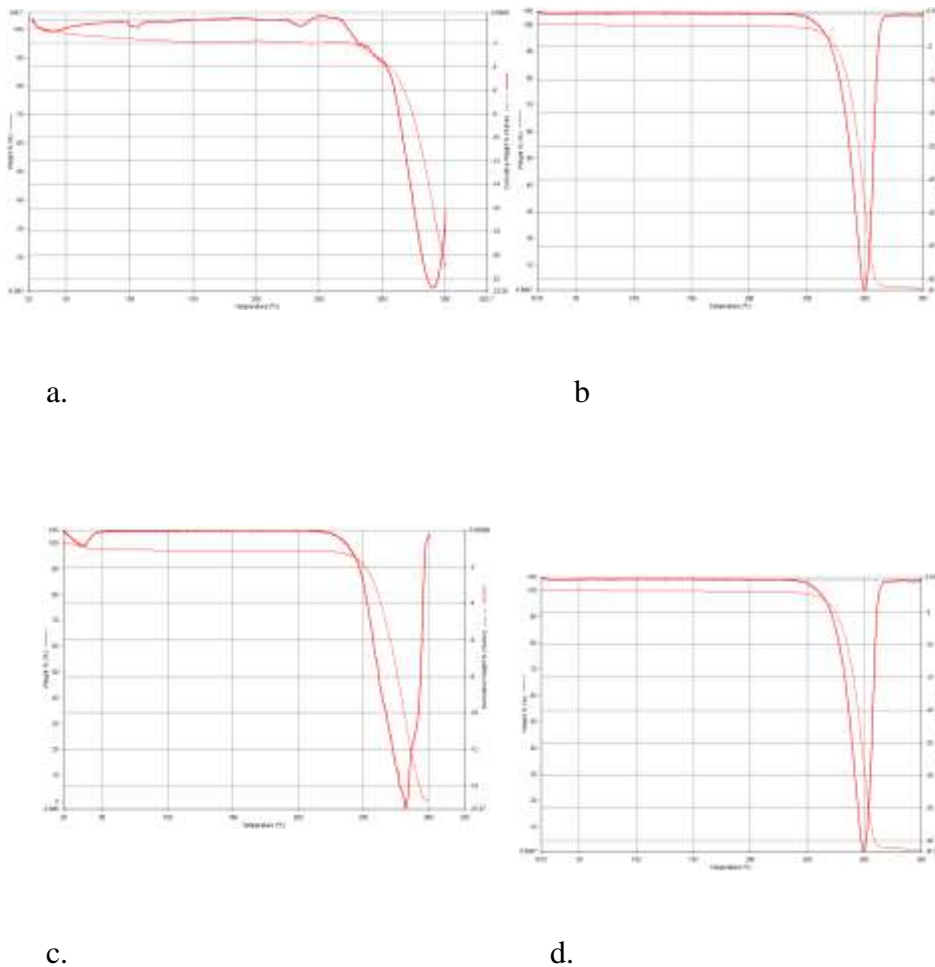


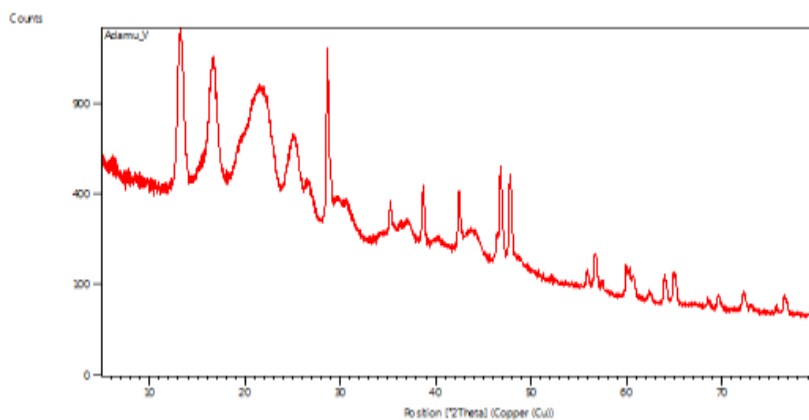
Fig 3: Thermo-gravimetric curve of PHB from *P.oleovorans* with (a) PS oil (b) TS oil and *R. eutropha* with (c) PS oil and (d) TS oil samples.

The thermographic curves (figure 3a and b) shows that the PHB samples from *P.oleovorans* with PS and TS oil samples starts to degrade at 255°C and 248°C resulting in complete degradation at 340°C and 300°C respectively. There was a slight loss of weight at about 100°C and 104°C for both samples as evident in the slight fall in the curves between 20°C to 50°C. The polymers were stable up to 255°C and 248°C before reaching maximum degradation at 347°C and 300°C respectively. With *R.eutropha* and the oil samples (figure 3c and d), PHB from PS oil was shown to start degrading at 229.50°C reaching maximum degradation at 280°C with a slight loss in weight between 20 to 50°C compared to PHB from TS oil sample which starts degrading at 239°C and degrades completely at approximately 300°C a slight loss in weight is observable between 19.34°C and 50°C. It was also established by Oliviera *et al.*, (2006) that a complete loss of weight in non-irradiated PHB was recorded at 293°C close to the temperatures for maximum degradation of PHB recorded from samples in this experiment. It was also observed that temperatures for optimal degradation of the samples are different in all the analysis carried out. This variation in degradation temperatures is likely to be caused by variation in the heating rates of the samples during the analysis since it was reported by Li *et al.*, (2001) that degradation temperature of PHB is affected by the rate of heating, and was shown to increase as the heating rate increases as well. It was also

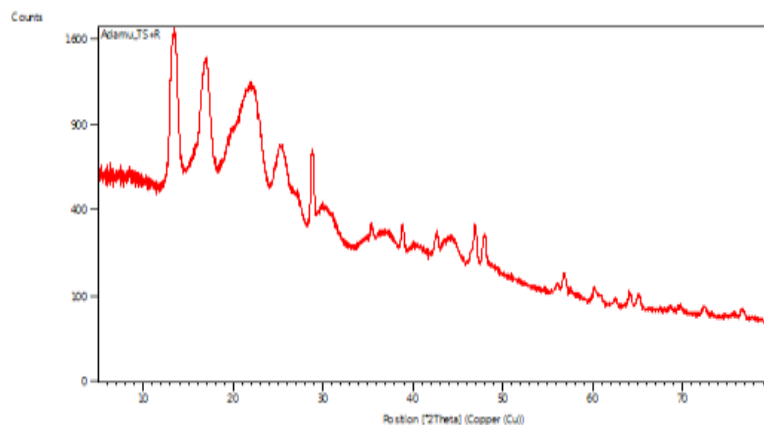
mentioned that, temperature changes or shifts in PHB thermal profiles using conventional methods can be caused by the differences in the source of the PHB and inefficiency in the technique utilised (Gonzalez *et al.*, 2005)

3.3 X-Ray diffraction analysis

The crystalline nature of the polymer samples examined by X-ray diffraction using EMPYREAM device with Cu radiation (40kV, 40mA) at 2θ so that the differences in the patterns from the samples can be identified was evaluated. The results obtained are shown in the figures 4 and 5 below;



a.



b.

Fig. 4: X-ray diffraction patterns of PHB obtained from *R.eutropha* with (a) PS oil and (b) TS oil

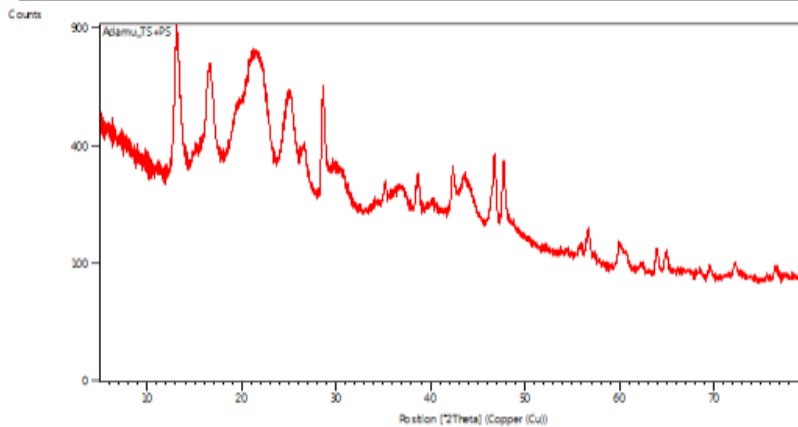


Fig 5: X-ray diffraction pattern of PHB obtained from *P.oleovorans* with PS oil

From the diffraction patterns from the polymer samples, it can be observed that all three samples gave similar patterns irrespective of the oil sample used or the bacteria. In all cases, there are narrow sharp peaks and broad peaks that appear similar in the samples examined. Comparison of the results with literature findings indicates presence of a semi crystalline polymer in the samples as shown in figure 6 below.

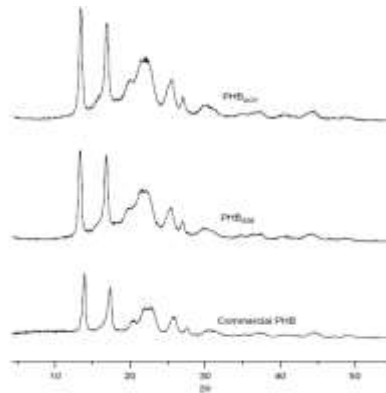


Fig. 6: X-ray diffraction patterns of PHB from different sources compared to commercial PHB (Oliviera *et al.*, 2007).

4.0 CONCLUSION

Results from the experiment revealed that *P. oleovorans* and *R. eutropha* are crystalline in nature since they exhibited similar trends in the patterns of the peaks consistent with results obtained as reported by Oliviera *et al.*, (2007) and Santos *et al.*, (2009) as displayed by X-ray diffraction analysis conducted on the PHB samples. TGA analysis has shown that the PHB samples produced have varying thermal stabilities since they start to degrade at different temperatures (T_0) which vary according to the carbon source and the bacteria. This varies from 255°C and 248°C with *P. oleovorans* with the oil samples compared to 229°C and 239°C with *R. eutropha*.

Thermal stability studies should be extended to differential scanning calorimetry (DSC) to improve on the analysis conducted so far in this study by acquiring more information on the melting and crystallization temperatures of the polymer.

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