Production and Characterization Of Polyhydroxyalkanoates From Lactic Acid Bacteria Isolated From Dairy Wastewater, Fermented Cow Milk and 'Ogi'

ABSTRACT

Aims: This study aimed at screening, producing and characterizing polyhydroxyalkanoates (PHA) from lactic acid bacteria (LAB) isolated from dairy wastewater, fermented cow milk and "ogi".
Place and Duration of Study: Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan between August 2018 and February 2019.
Methodology: The initial screening of isolated LAB was carried out by Sudan Black staining method followed by secondary screening on liquid medium. Isolates that tested positive for Sudan Black stain and had the highest PHA yield from liquid medium were used for further analysis. The cell walls of selected isolates were lysed with sodium hypochlorite and PHA extracted using chloroform. Optimization of PHA production was carried out using different carbon and nitrogen sources, incubation temperature, pH and agitation speed. Extracted PHA was characterized by FTIR spectroscopy and the microstructure and surface morphology were observed using Scanning Electron Microscope.
Results: Seven (7) isolates tested positive for Sudan Black stain of which two isolates identified as *Lactobacillus plantarum* CW10 and *Lactobacillus casei* WWD3 had the highest PHA yield from liquid medium. From the optimization experiment, highest PHA production was observed in *Lactobacillus plantarum* CW10 (20.5%) and *Lactobacillus casei* WWD3 (19.7%) when glucose and ammonium sulphate was used as carbon and nitrogen sources respectively, and at pH 6, agitation speed of 200 rpm and incubation temperature of 35^0C and 40^0C. The spectra of extracted PHA as characterized by FTIR showed absorption peaks for the carbonyl, C-H, -OH, aliphatic $-CH_3$ and ester groups that are characteristics of PHA. The microstructure and surface morphology shows grains that are pseudo-spherical in shape with fairly regular distribution.
Conclusion: There is an appreciable production of PHA from the *Lactobacillus* strains and can be considered as part of the choice of organisms for PHA production in commercial quantity.

Keywords: PHA yield, Fermented products, *Lactobacillus* species, Microstructure, Plastics.

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are among the top group of biopolymers that have been intensively investigated and commercialized [1], and produced mainly by bacteria in the form of inclusion bodies and act as storage substances inside vegetative cells [2, 3]. Bacterial PHAs gained particular interest since they are completely biodegradable, non-toxic, biocompatible and also sources for commercially useful pool of chiral monomers [4]. Besides being biodegradable, PHAs are recyclable like the petrochemical thermoplastics. Since their

discovery, all these properties have made these microbial polyesters very attractive as a source of alternative biodegradable materials to conventional petrochemical-based plastics [4].

Depending on the carbon numbers in the monomeric constituents, PHAs can be classified as short-chain-length PHAs (SCL-PHA, C_3–C_5), which consists of 3–5 carbon monomers, and medium-chain-length PHAs (MCL-PHA, C_6–C_{14}), which consists of 6–14 carbon monomers in the 3-hydroxyalkanoate units. More than 150 different PHA monomers have been identified, which renders them the largest group of natural polyesters [5]. SCL-PHAs show thermoplastic material properties similar to polypropylene, while MCL-PHAs possess elastic material properties similar to rubber. Interestingly, some microorganisms synthesize PHAs having both SCL and MCL-monomers. Such SCL-MCL-PHAs exhibit material properties similar to low density polyethylene. The success in the biodegradable plastic strategy largely depends on the isolation of potent PHA producing bacteria and optimizing culture parameters for maximum PHA biosynthesis [5].

The last and the most promising scenario for plastic waste management is the production of biodegradable plastics. There are several bio-based polymers which are already on the market and have similar properties to petroleum-based plastics. They include polyhydroxyalkanoates (PHA), poly(lactic acid) (PLA), polyethylene (PE), poly(trimethylene terephthalate) (PTT), poly(butylene succinate) (PBS) and poly(p-phenylene) (PPP). Each of the polymers is biodegradable with certain mechanical and chemical properties allowing them to be used in a wide range of applications [6]. However, only hydroxyalkanoates can have rich structural variations along with being the only monomers made *in vivo* unlike the other monomers being polymerized by *in vitro* chemical synthesis [6, 7].

The synthesis of PHA by diverse groups of bacteria depends mainly on the type of carbon sources used [8] and the downstream processing [9, 10].

The aim of this study is to produce and characterize PHA from lactic acid bacteria isolated from dairy waste water, fermented cow milk and “ogi”.

2. MATERIAL AND METHODS

2.1 Sample collection

Fresh cow milk sample was collected from Bodija abattoir, Ibadan while dairy waste water was collected from Fan milk Plc, Ibadan. The samples were collected in sterile screw cap tubes and were brought into the Postgraduate laboratory, Department of Microbiology, University of Ibadan for microbial analysis. The cow milk was left to ferment spontaneously at room temperature for three days before analysis was carried out on it while the dairy waste water was analyzed within three hours from the time of collection.

2.2 Isolation of Lactic acid bacteria

The pour plate method was used in isolating Lactic acid bacteria cultures from the samples on sterile MRS agar. The plates were incubated anaerobically at 35^0C for 48 hours. The microbial loads on the plates were recorded as colony forming unit per milliliter of each sample. Suspected lactic acid bacteria would present a cream or white shiny or dull small colony. Suspected lactic acid bacteria were selected and repeated subculturing on medium of isolation was carried out until pure colonies were obtained.

2.3 PHA SCREENING

2.3.1 Primary screening for PHA production on solid medium

This was done according to the modified method of Baptista [11]. Medium A2 containing 20 g/L of starch, 2 g/L of yeast extract, 1 g/L of peptone, 18 g/L of Agar, 1litre of distilled water was autoclaved at 121 ºC for 15 minutes. The medium was dispensed into sterile Petri plates and was allowed to solidify. The suspected pure isolates were streaked on the plates and incubated anaerobically for 24 hours at room temperature. After which 5mL of Sudan black B stain was used to flood the plates for 20 minutes. Then it was discarded gently.

Isolates that retained the dye (blue-black) contain PHA granules and were further subjected to the secondary screening.

2.3.2 Secondary screening for PHA production on liquid medium

To confirm the production of PHA, the isolates that retained the blue black colour of Sudan Black B stain were selected and grown in liquid medium. The PHA positive isolates were grown in 250 mL Erlenmeyer flasks containing 100 mL of the Medium A2 (without agar) and incubated at 30 ºC on a Shaker at 150 rpm for 72 hours. After which the bacterial cells were harvested and centrifuged. The biomass was analyzed for PHA content.

2.4 Extraction and Quantification of PHA

This was done according to the method of Amrita *et al.* [12]. The positive LAB culture was centrifuged at 4000 rpm for 20 minutes to obtain the cell pellet and dried to estimate the cell dry weight (CDW) in units of gramme per litre (g/L). Then, 5mL of sodium hypochlorite was added and centrifuged at 4000 rpm for 20 minutes. The pellet was washed sequentially with distilled water, acetone and ethanol, respectively. The pellet obtained after washing was suspended in 5mL of chloroform, and evaporated in a glass Petri dish at 60^0C for 1 hour. The weight of the residue obtained after evaporation of chloroform was recorded. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the cell dry weight.

2.5 Optimization of production conditions for PHA

2.5.1 Effect of different carbon substrates (monosaccharides, disaccharides and polysaccharides)

This was carried out according to the modified method of Baptista [11]. The selected isolates with the high PHA content were grown in 1litre liquid culture to determine better growth parameters. A compounded medium, medium E, which is specific for high yield of PHA was used and consists of the following; Ammonium phosphate ($(NH_4)_2HPO_4$) 3.3g/L, Dipotassium hydrogen phosphate (K_2HPO_4) 5.8g/L, Potassium dihydrogen phosphate (KH_2PO_4), 3.7g/L, Magnesium sulphate ($MgSO_4$), carbon substrate 20g/L. The carbon sources used includes glucose, galactose, fructose, lactose, sucrose, starch, raffinose and glycerol. Inocula were prepared in 1000mL of the medium using one isolated colony from agar plates and incubated for 120 hours at 30 ºC and 150 rpm.

2.5.2 Effect of nitrogen sources on PHA production

Using the best carbon source, the nitrogen sources were optimized. Two organic (urea and peptone) and two inorganic nitrogen (sodium nitrate and ammonium sulphate) sources were used.

2.5.3 Effect of pH on PHA production

For optimizing pH, the optimized medium E was prepared and adjusted by varying the pH from 5.0 to 9.0 before sterilization using NaOH/Phosphoric acid.

2.5.4 Effect of incubation temperature on PHA production

The inoculated culture in optimized medium E was incubated at different temperature (25ºC, 30ºC, 35ºC, and 40ºC) for temperature optimization.

2.5.5 Effect of agitation speed on PHA production

The effect of agitation speed on PHA production was carried out using the optimized medium E and the incubation speed was set at 100 rpm, 150 rpm and 200 rpm.

2.6 Characterization of PHA using Fourier-Transform Infrared spectrometer (FTIR)

This was done according to the method of Nwinyi and Owolabi [13]. FTIR analysis of the extracted product was carried out in a Perkin Elmer Spectrum1 FTIR instrument in the IR range of 4000 to 450 cm^{-1}. The samples of PHA from the isolates were mounted onto FT-IR; the various absorption bands depicting the presence of some functional groups were noted. Peak values obtained were used to interpret the presence of specific functional groups in the extract.

2.7 Determination of microstructure and surface morphology of produced PHA using Scanning Electron microscope

The PHA samples were mounted onto the sample holder that was covered with carbon tape. The sample holders were placed into the TESCAN VEGA 3 LM SEM coupled with a selected EDS microanalysis (Oxford system). Voltage of 20 KV was supplied and used for analyzing the PHA materials. For quality control and assurance, about 85% of the study area of PHA was scanned leaving the edges that may have extraneous particles. The microstructure and surface morphology of the samples were studied using Scanning electron microscope [13].

2.8 Phenotypic and Molecular Characterization of best PHA producing isolates

The best isolates were identified phenotypically using their morphological and biochemical characteristics as well as their sugar fermentation pattern. Genus level identification of the unknown bacterial strain was accomplished by using Bergey's Manual of Systematic Bacteriology [14]. Molecular characterization of the isolates involving DNA extraction, PCR amplification, gene sequencing and blasting was also carried out.

3. RESULTS AND DISCUSSION

The microbial load of lactic acid bacteria (LAB) isolated from the samples is shown in table 1 with highest lactic count recorded in fermented cow milk (5.1×10^5 CFU/mL) and least count of 4.4×10^3CFU/mL in fermented Ogi. A total of 20 catalase negative, gram positive rods and cocci were selected and subjected to primary screening for PHA production. Seven (7) out of the 20 isolates were observed to be PHA producers using presumptive (staining) screening method with varying degrees of intensity of the Sudan B Black stain with isolate CW10 and WWD3 showing the highest intensity (Table 2). This was in accordance with the report of Natarajan and others [15] who used Sudan black B stain in their research to confirm the presence of the polymer in *Lactobacillus* strains.

As Sudan B Black staining is not specific to PHA but could also bind to hydrophobic polymers such as lipids, and therefore to confirm the content of PHA, the seven (7) isolates were grown in liquid medium for further analysis of PHA content. LAB strains with the intense absorption signal were selected in order to determine their maximum PHA storage capacity. This is in line with the work of Aslim *et al.*[16] who obtained the highest PHA yield from LAB strains at 72 hours.

Table 3 shows the result of secondary screening of selected LAB to confirm the quantity of PHA each LAB isolate produced. From the table, highest PHA production was recorded in isolate CW10 (25.3%) followed by isolate WWD3 (16.1%) and least production recorded in isolate CW8 (4.6%). Highest PHA weight (0.41g) and least cell dry weight (1.54g) was observed for isolate OG8. The least PHA weight (0.10g) and higher cell dry weight (2.16g) were recorded for isolate CW8 while the highest cell dry weight (2.92g) was recorded in isolate OG9.

Table 1: Microbial load of Lactic acid bacteria isolated from the samples.

Samples	Microbial Load (CFU/mL)
Fermented cow milk	5.1×10^5
Dairy wastewater	7.3×10^4
Ogi	4.4×10^3

Table 2: Primary screening using staining method (Sudan B Black) on solid medium

Sample Source	Isolate Code	Reaction
Fermented Cow Milk	CW1	++
	CW2	-
	CW3	-
	CW7	-
	CW8	+
	CW10	+++
	CW14	-
	CW15	-
	CW19	-
	CW26	-
	CW32	-
Wastewater From Dairy Industry	WWD2	-
	WWD3	+++
	WWD12	+
	WWD14	-
Ogi	OG1	-
	OG4	-
	OG8	++
	OG9	++
	OG11	-

KEY: +++ High intensity ++ medium intensity + partial intensity - No intensity
CW: Isolated from fermented cow milk; WWD: Isolated from dairy wastewater
OG: Isolated from ogi

The two isolates (CW10 and WWD3) with the highest PHA production were selected for the optimization process. Since PHA production by bacterial strains depends on the carbon source used [17], the highest PHA yield in the two isolates was recorded using glucose as the carbon source, (16.5% and 16.1% respectively) followed by Lactose for isolate CW10 and galactose for isolate WWD3 while isolate CW10 recorded the lowest PHA yield using glycerol (3.3%) and isolate WWD3 with sucrose (4.9%) as the carbon source (Figure 1). Glucose being the best source of carbon for PHA production in this study is in line with the work of Song *et al*. [18] who reported glucose as the best carbon source for PHA production by *Pseudomonas spp*. In a similar research, Aljuraifani and others [19] found glucose and maltose to be the suitable source of carbon for improved PHA yield by *Pseudomonas* strain-P (16).

Table 3: Secondary screening of selected isolates using Liquid medium

Isolate code	Cell Dry Weight (g)	PHA Weight (g)	PHA Yield (%)
CW1	2.04	0.30	14.7
CW8	2.16	0.10	4.6
CW10	1.58	0.40	25.3
WWD3	2.12	0.34	16.1
WWD12	2.38	0.18	7.5
OG8	1.54	0.41	12.3
OG9	2.92	0.28	9.5

KEY:
CW: Isolated from fermented cow milk
WWD: Isolated from dairy wastewater
OG: Isolated from ogi

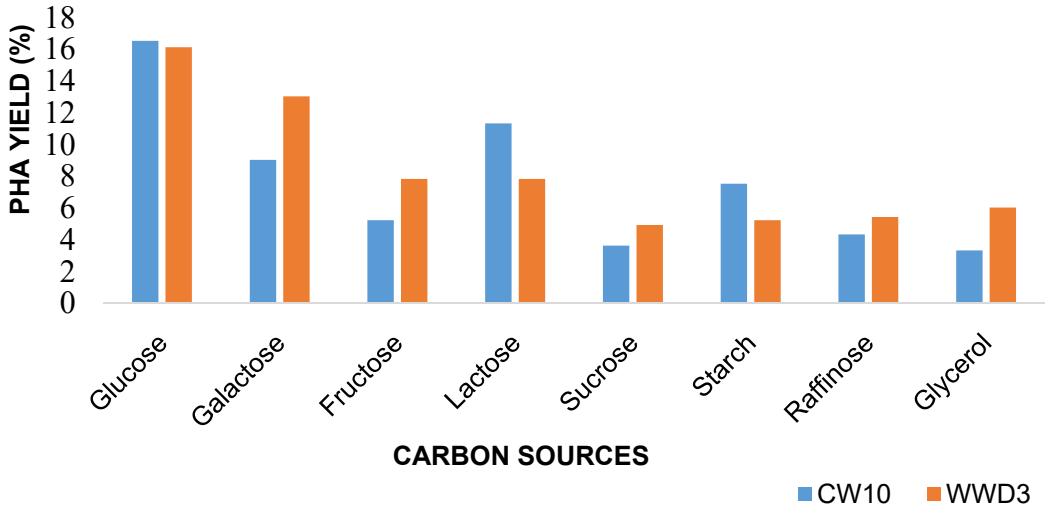


Figure 1: Effect of carbon sources on the PHA yield (%) by the two selected LAB strains, *L. plantarum* CW10 and *L. casei* WWD3

The amount of dry cell weight produced from the two isolates using the different carbon sources is shown in Figure 2. Both isolates CW10 and WWD3 had their highest dry cell weight of 21.2g/L and 20.6g/L respectively from the production media containing glucose as the sole carbon source. The least amount of cell dry weight of isolates CW10 (4.7g/L) and WWD3 (5.6g/L) was produced from the production media containing raffinose as the carbon source.

Figure 3 is showing the effect of the carbon sources on the weight of PHA produced by the two selected LAB strains, CW10 and WWD3. The highest PHA weight for both isolates was produced in the production media containing glucose as the carbon source with isolate CW10 producing slightly higher (3.5g/L) than isolate WWD3 (3.3g/L). However, the lowest PHA weight was recorded in the production media containing raffinose as the carbon source with 0.2g/L and 0.3g/L respectively.

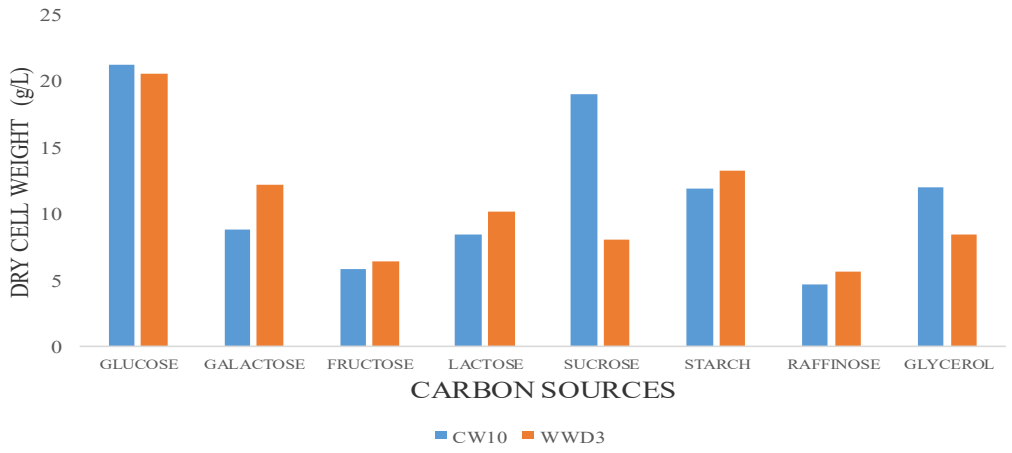


Figure 2: Effect of carbon sources on the dry cell weight (g/L) by the two selected LAB strains, *L. plantarum* CW10 and *L. casei* WWD3

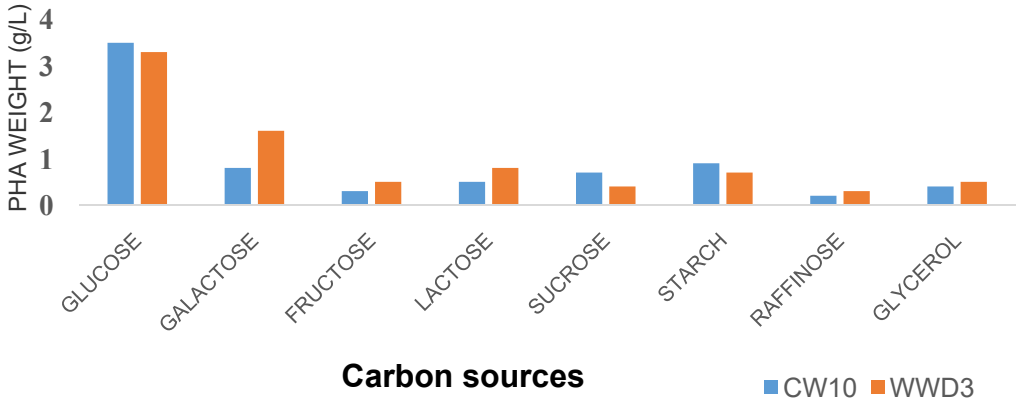


Figure 3: Effect of carbon sources on the PHA weight (g/L) by the two selected LAB strains (*L. plantarum* CW10 and *L. casei* WWD3)

However, research findings have also revealed that disaccharides and other complex carbohydrates such as starch are usually hydrolyzed first into monomeric units or their derivatives, before subsequently entering the various metabolic pathways.

Figure 4 is showing the effect of nitrogen sources on the PHA yield by isolates CW10 and WWD3. The highest PHA yield for both isolates CW10 and WWD3 was recorded in the production medium containing ammonium sulphate with 14.7% and 14.1% yield respectively. The observed maximum PHA yield recorded when ammonium sulphate was used as nitrogen source was in contrast to the findings of Urvija and Veena [20] who reported ammonium chloride and peptone as the best nitrogen source for PHA production by *Klebsiella pneumonia* but similar to the findings of Aljuraifani *et al*. [19] who obtained a maximum PHA production by *Pseudomonas* strain-P(16) using ammonium sulphate. The lowest PHA yield for isolate CW10 was recorded in the production media containing sodium nitrate with 8.2% yield while isolate WWD3 had the lowest yield of 6.7% in the urea containing medium.
The amount of dry cell weight produced by the two LAB strains using the selected nitrogen sources is as shown in figure 5. The production medium containing peptone supported the highest yield of cell dry weight in isolates CW10 (15.3g/L) and WWD3 (12.8g/L) while the lowest yield of 8.4g/L and 10g/L respectively was recorded in the production medium containing urea. However, the PHA weight was highest for both LAB strains in the production medium containing ammonium sulphate with a yield of 2.1 g/L and 1.8 g/L respectively (Figure 6). The lowest yield was recorded in the urea production medium with a yield 0.8 g/L and 0.7g/L respectively.

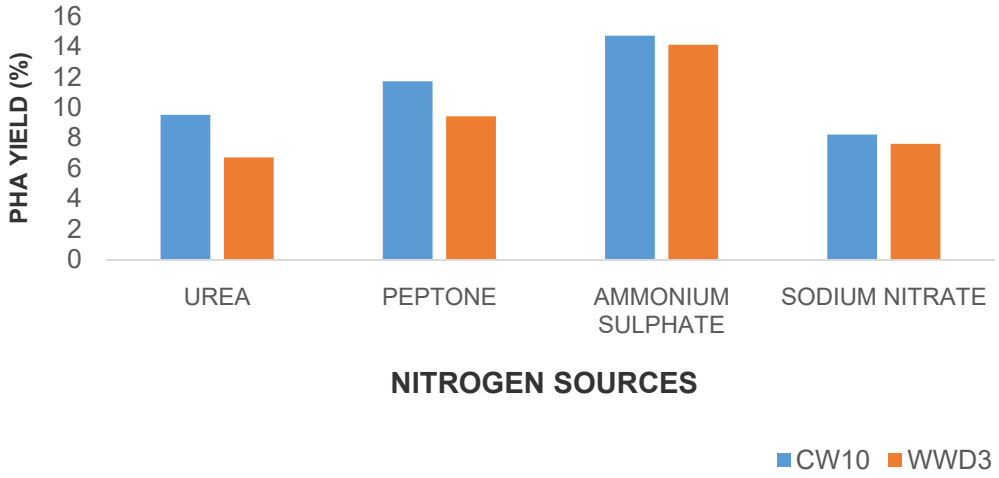


Figure 4: Effect of nitrogen sources on the PHA yield (%) by the two selected LAB strains, *L. plantarum* CW10 *and L. casei* WWD3

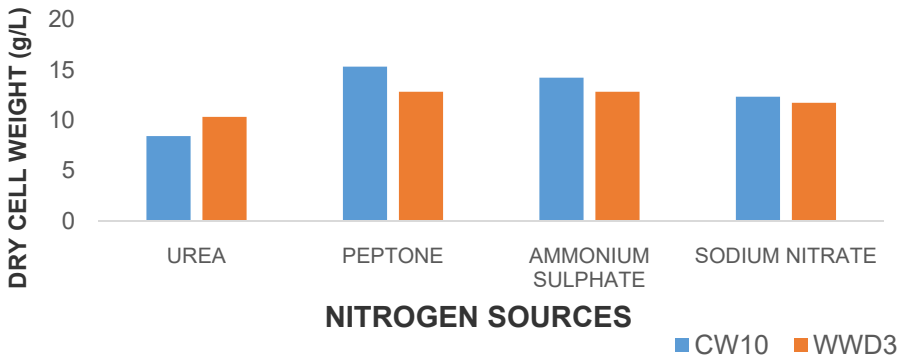


Figure 5: Effect of nitrogen sources on the dry cell weight (gL-1) by the two selected LAB strains, *L. plantarum* CW10 *and L. casei* WWD3

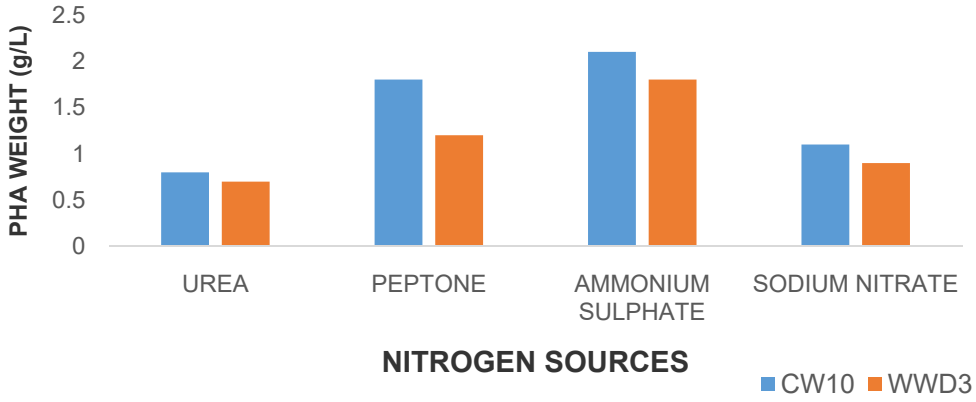


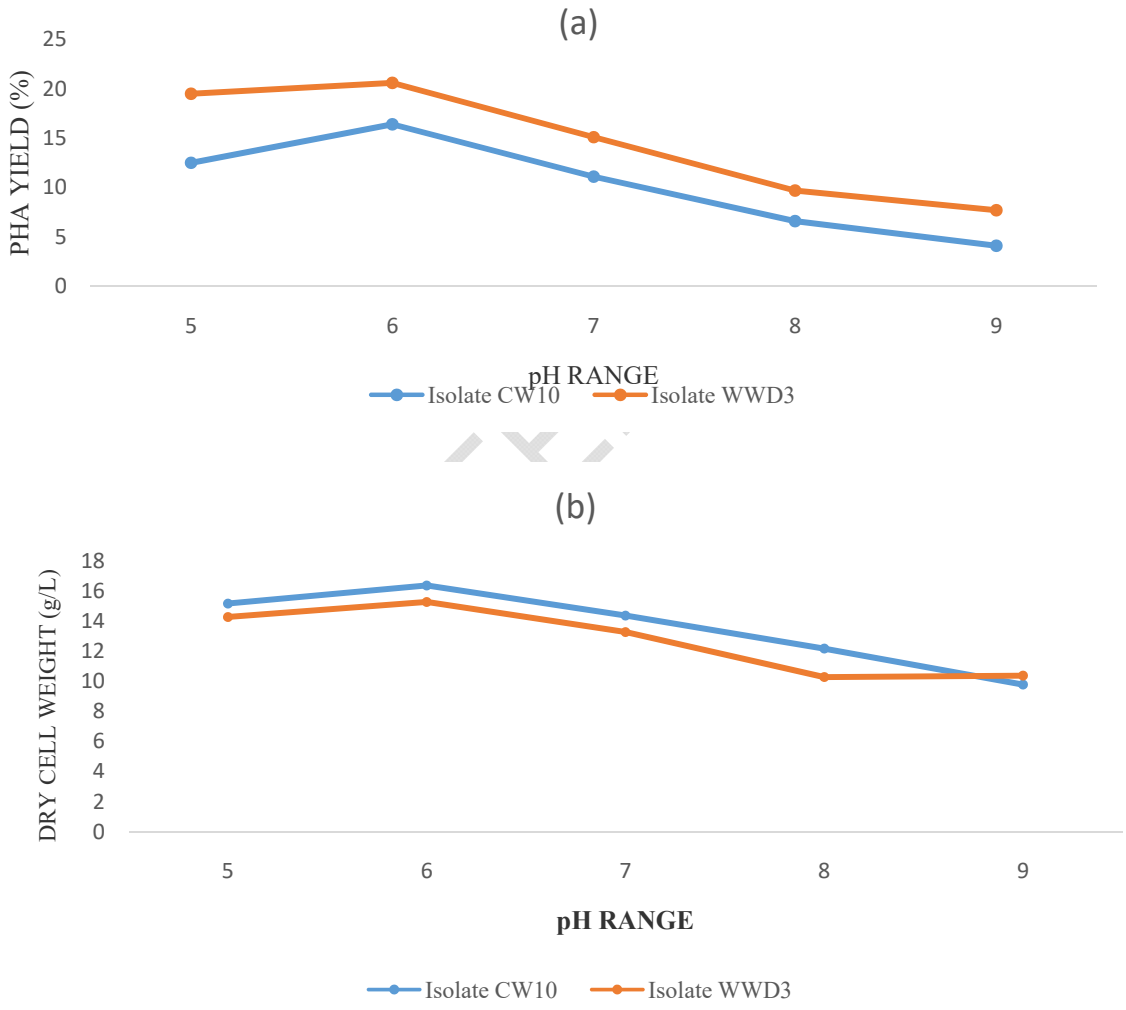
Figure 6: Effect of nitrogen sources on PHA weight (g/L) by the two selected LAB strains, *L. plantarum* CW10 *and L. casei* WWD3

Inorganic nitrogen sources gave higher PHA yield when compared to organic nitrogen sources and this is in agreement with the findings of Sangkharak and Prasertsan [21] who reported that the use of inorganic nitrogen sources leads to higher PHA yield than organic nitrogen sources in *Rhodobacter sphaeroides*.

To determine the best pH for the highest PHA yield, the effect of different pH of the production media containing glucose as the carbon source and ammonium sulphate as the nitrogen source was studied (Figure 7). The result shows that there was a progressive decline in the percentage PHA yield, dry cell weight and PHA weight as the pH increased from pH 6 to pH 9 for both isolates CW10 and WWD3.

For both isolates, the highest and lowest PHA yield, dry cell weight and PHA weight were recorded at pH 6 and pH 9 respectively. The highest PHA yield of 21.6% for isolate CW10 and 16.4% for WWD3 was recorded at pH 6 with the least yield observed at pH 9 (Figure 7a). The highest dry cell weight of 16.4g/L and 15.3g/L for isolates CW10 and WWD3 was observed at pH 6 while the least dry cell weight of 9.8g/L and 10.4g/L respectively was observed at pH 9 for both isolates (Figure 7b). The highest weight of PHA for isolates CW10 (3.3g/L) and WWD3 (2.7g/L) and the least weight of 0.8g/L and 0.4g/L were recorded at pH

6 and pH 9 respectively (Figure 7c).In the present study, The optimum pH 6 observed for maximum PHA yield and highest dry cell weight for both *Lactobacillus* strains was in contrary with the findings of some researchers who reported an optimum pH value of 7.5 for higher yield of PHA from *Bacillus* strains [22, 23].



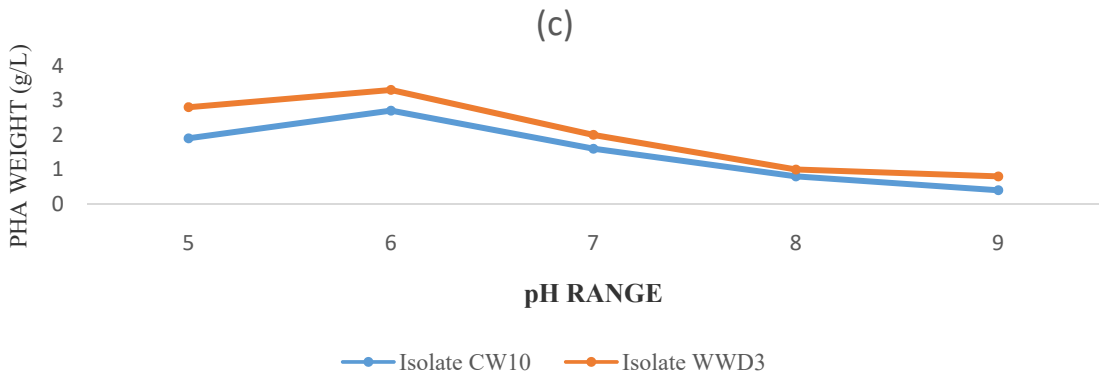


Figure 7: Effect of pH on (a) PHA production, (b) dry cell weight and (c) PHA weight by isolate CW10 (*Lactobacillus plantarum*) and isolate WWD3 (*Lactobacillus casei*)

Figure 8 is showing the effect of incubation temperature on PHA production by the two *Lactobacillus* isolates. For isolate CW10, the highest yield was recorded at a temperature of 35^0C (24.4%), followed by 30^0C (16.5%) and lowest yield at 40^0C (9.7%). Isolate WWD3 had its highest yield at 40^0C (15.7%) followed by 35^0C (14.7%) and least at 30^0C (13.4%) (Figure 8a). The optimum temperature of 35ºC recorded for PHA production by *Lactobacillus plantarum* CW10 is in agreement with the findings of Aslim *et al.* [16] who reported 30ºC-37^{o}C as the optimum temperature for PHA production by *Lactobacillus plantarum and Lactobacillus casei* isolated from different dairy and food products produced in Turkey. Similarly, Desouky *et al.* [23], recorded maximum PHA yield by *B. thuringiensis* at 35^0C incubation temperature. On the contrary, 40^0C incubation temperature was found to be the optimum for *Lactobacillus casei* WWD3. This might be as a result of variation in growth temperature from organism to organism. The highest and least dry cell weight for both isolates was observed at a temperature of 35^0C and 40^0C respectively (Figure 8b). The highest dry cell weight of 16.5g/L and 22.1g/L and least weight of 12.3g/L and 10.2g/L respectively was recorded for isolates CW10 and WWD3 at 35^0C and 40^0C. Highest PHA weight for isolate CW10 (4.0g/L) and isolate WWD3 (3.2g/L) was recorded at a temperature of 35^0C and the least weight of 1.2g/L and 1.9g/L respectively was observed at 40^0C (Figure 8c).

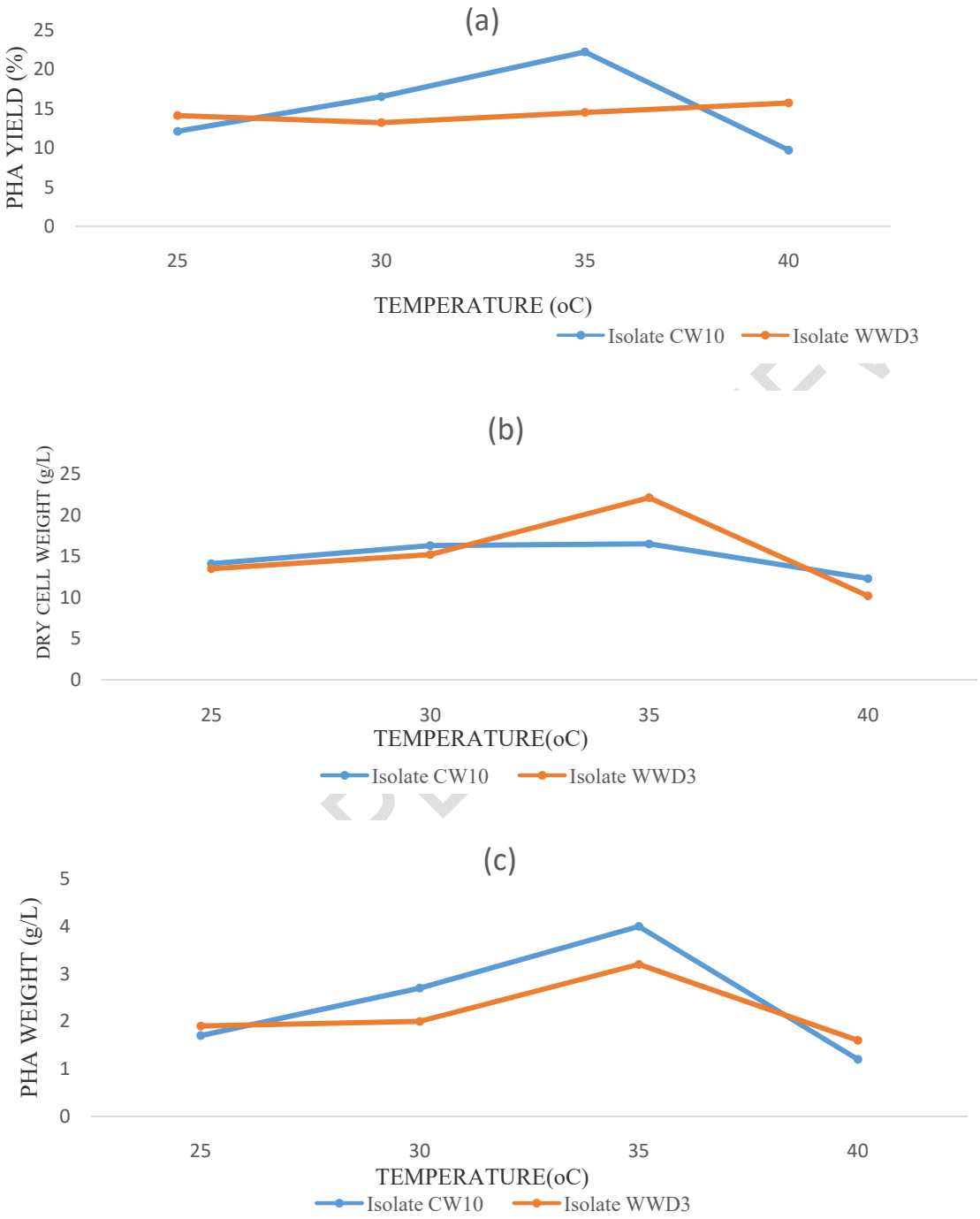


Figure 8: Effect of incubation temperature (^{0}C) on PHA production by isolate CW10 (*Lactobacillus plantarum*) and isolate WWD3 (*Lactobacillus casei*)

Figures 9 is showing the effect of agitation speed on PHA production by both isolates. From Figure 9a, the highest yield of PHA for isolate CW10 was observed at 200 rpm (20.5%)

followed by 150 rpm (16.5%) and the least at 100 rpm (14.1%). Highest PHA weight was recorded at 200 rpm (4.5g/L) and least at 100rpm (2.3g/L). The highest dry cell weight was observed at 150 rpm (23g/L) followed by 200rpm (21.9g/L) and lowest at 100 rpm (16.3g/L). For isolate WWD3 (Figure 9b), the highest production was observed at 200 rpm (19.7%) followed by 150 rpm (16.1%) and the least at 100 rpm (12.6%). The highest PHA weight was recorded at 200 rpm (3.8g/L) and lowest at 100 rpm (2.1g/L). Highest dry cell weight (19.8g/L) was recorded at 150 rpm and the least (15.4g/L) at 100 rpm. The best yield of both isolates observed at an agitation speed of 200 rpm is in line with the work of Yu-Hong *et al.* [24] who obtained the best yield at 200 rpm while producing PHA from local indigenous strains of *Cupriavidus taiwanensis* and *Pseudomona oleovorans.* However, Desouky *et al.* [23] obtained the best yield at an agitation speed of 150 rpm for PHA production by *B. thuringiensis*.

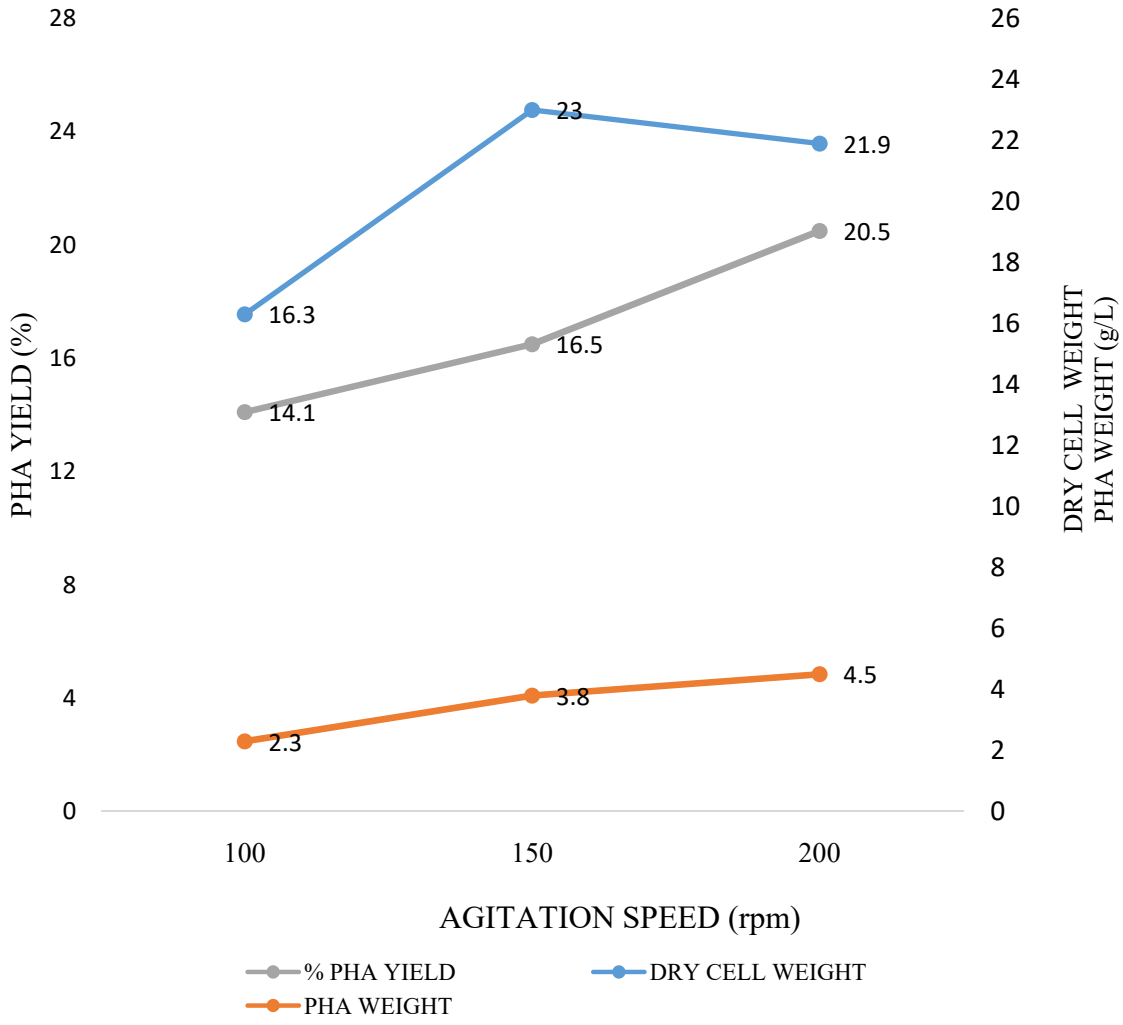


Figure 9a; Effects of different agitation speed (rpm) of isolate *Lactobacillus plantarum* CW10 optimized media on percentage PHA yield

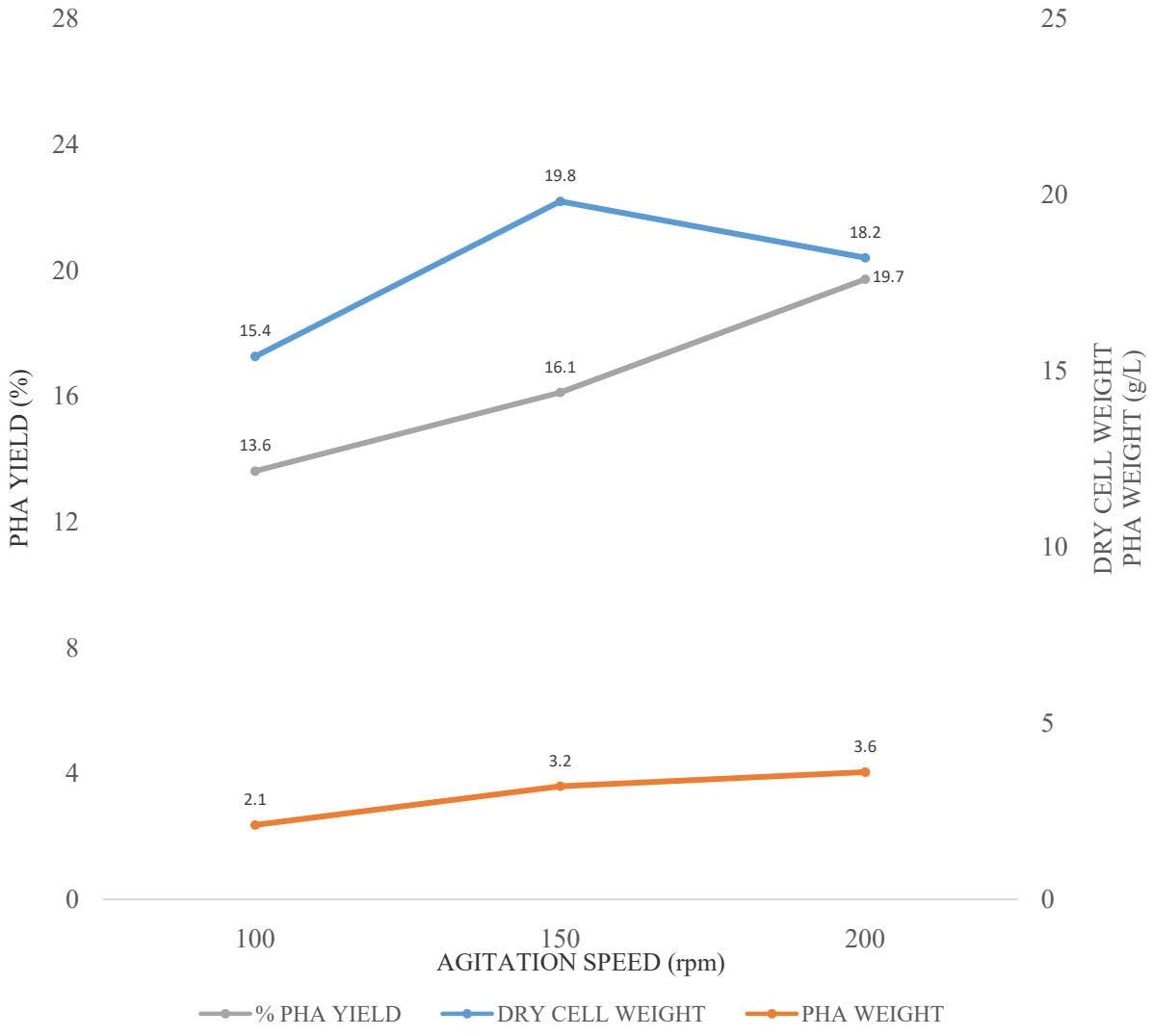


Figure 9b; Effects of different agitation speed (rpm) of *Lactobacillus casei* WWD3 optimized media on percentage PHA yield

The FTIR spectrum of the PHA produced by isolate CW10 is as shown in Figure 10. From the spectrum, the band at 3338 cm^{-1} showed the presence of OH group. The lower value of frequency and the broad nature showed that the OH is hydrogen bonded and free. The presence of C-CH_3 was confirmed by the wavelength at 2141 cm^{-1}. The band at 1638 cm^{-1} is as a result of the presence of carbonyl (C=O) of ester which is further confirmed by the presence of C-O of alkoxy group at 1363 cm^{-1}. The C=C of the alkene was found at 1543 cm^{-1}. It is also worthy to note that CH=CH_2 occurred in this sample at 1012 cm^{-1}. These peaks were also observed by other researchers [13, 19].

Figure 11 is showing the FTIR spectrum of the PHA produced by isolate WWD3. The band at 392 cm^{-1} stands for the presence of hydrogen bonded O-H, the band at 653 cm^{-1}

represents *cis* RCH═CHR bending vibration. This showed that this polyene plastic has one or more of its alkenes arranged in a *cis* isomeric form around the Sp^2-hybridized carbon of alkene. In addition, the aliphatic linked -CH_3 and -CH_2 were also present in this plastic type at the frequency of 2935 cm^{-1} and 2141 cm^{-1} respectively. The band at 1646 cm^{-1} which is a bit higher than normal C=C (at 1600–1620 cm^{-1}) showed that there is presence of at least one non-conjugated alkene in the plastic bond type in the sample. The absorption band at 1373 cm^{-1} confirms the bending vibrational mode, the presence of CH_3 at the terminal end of the chain or as a substituent along the chain. The C=C-H was an out of plane bending at 890 cm^{-1} while the *cis*-RC=CHR appeared in the PHA at 578 cm^{-1}. This conformation showed that the PHA is a highly unsaturated polyene.

Figure 10: FTIR Spectrum of produced PHA from isolate *L. plantarum* CW10

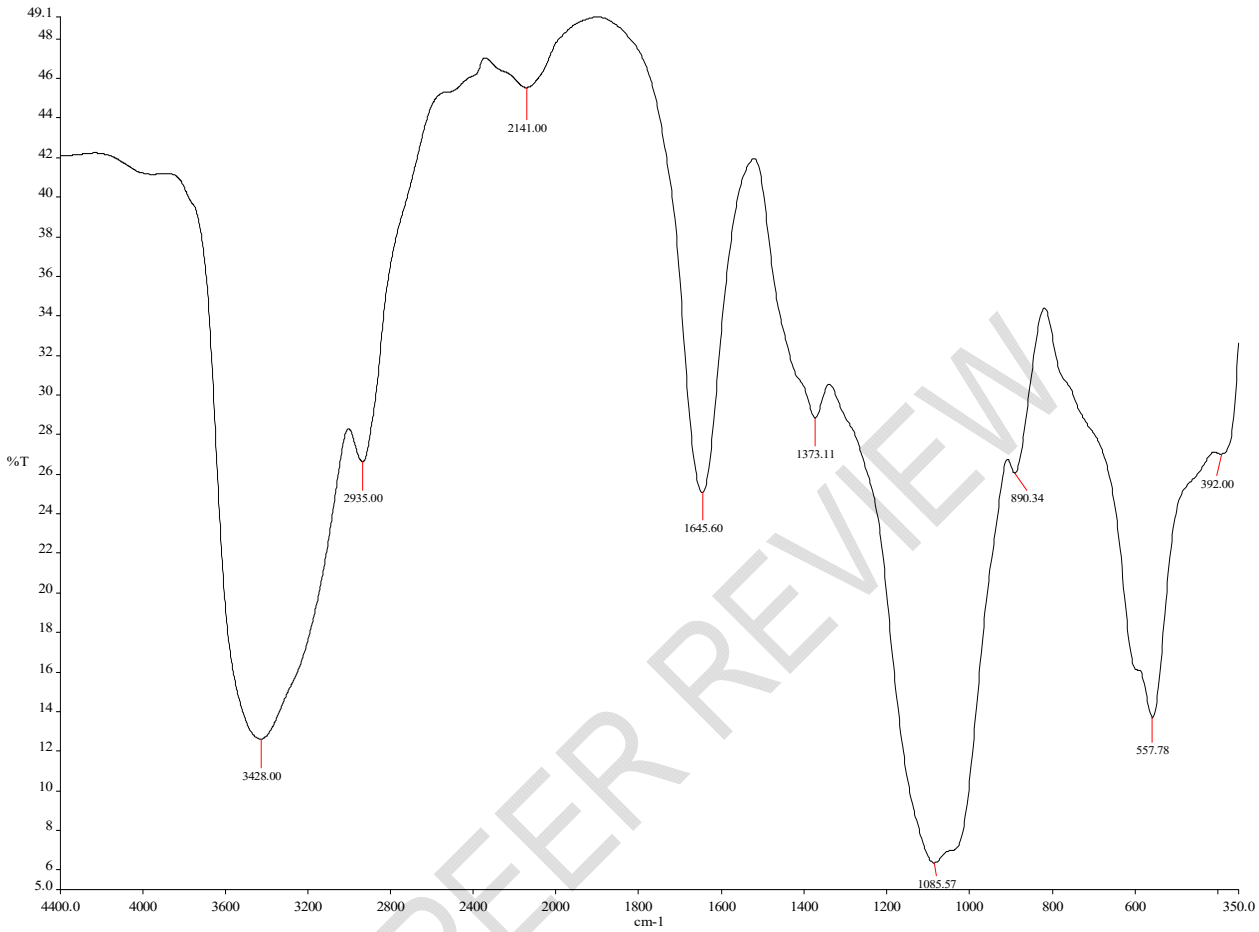
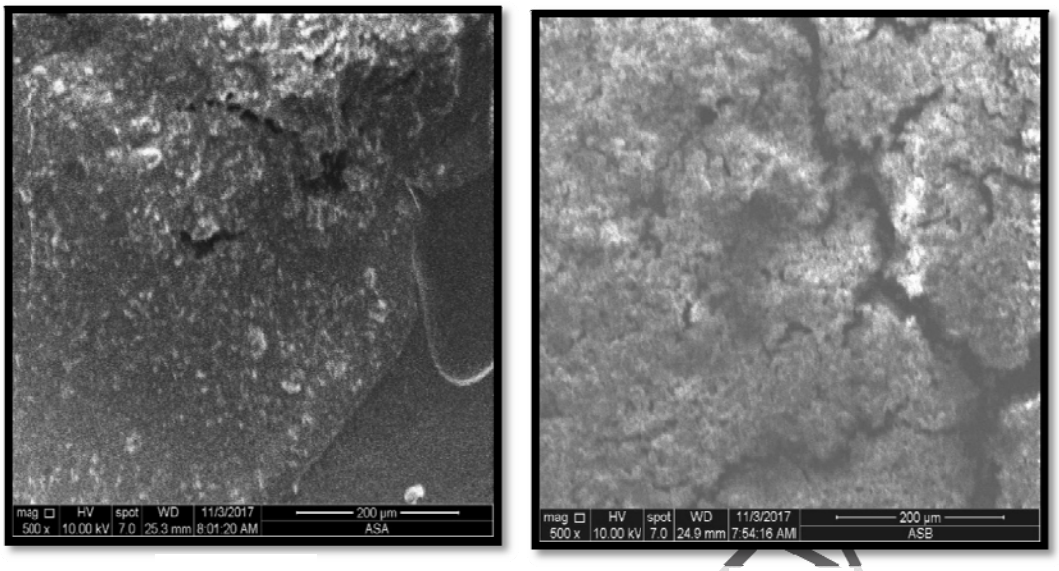


Figure 11: FTIR Spectrum of produced PHA from isolate *L. casei* WWD3

Figure 12 (a) and (b) is showing the scanning electron micrograph of the PHA produced from isolates CW10 and WWD3 at X500 magnification. The similar microstructure gives a fairly porous with fine grains that are interlinked and a propensity to form multigrain clusters. The morphology shows grains that are pseudo-spherical in shape with fairly regular distribution. This observation is in agreement with the work of Nwinnyi and Owolabi [13]and Tabassum *et al.* [25].

The two selected PHA producing isolates (CW10 and WWD3) were identified based on their morphological and biochemical characteristics as well as sugar fermentation pattern. Both isolates are gram positive rods, catalase negative, oxidase negative, casein hydrolysis negative, nitrate reduction positive and starch hydrolysis positive among others. However, isolate CW10 tested positive to ribose, raffinose and sorbitol sugar fermentation test while isolate WWD3 tested negative to this same test. The probable identity of isolate CW10 is *Lactobacillus plantarum* and WWD3 is *Lactobacillus casei*.

The molecular characterization results confirm that isolate CW10 is 99% identical to *Lactobacillus plantarum* strain E7304 and isolate WWD3 is 97% identical to *Lactobacillus casei* strain ATCC 334.



(b)

Figure 12: Scanning electron micrograph of PHA produced by (a) Isolate CW10 and (b) Isolate WWD3 at X500 magnification

4. CONCLUSION

An appreciable production of PHA from *Lactobacillus plantarum* CW10 and *Lactobacillus casei* WWD3 was recorded when glucose and ammonium sulphate was used as carbon and nitrogen sources, and at pH 6, agitation speed of 200 rpm and incubation temperature of 35^0C and 40^0C respectively.

REFERENCES

1. Bugnicourt E, Cinelli P, Lazzeri A, Alvarez V. Polyhydroxyalkanoate (PHA): review of synthesis, characteristics, processing and potential applications in packaging. eXPRESS Polymer Lett. 2014; 8, pp. 791-808.
2. Marang L, van Loosdrecht MCM, Kleerebezem R. Enrichment of PHA- producing bacteria under continuous substrate supply. New Biotech. 2018; 41, 55–61. https://doi.org/10.1016/j. nbt.2017.12.001
3. Santiago RP, Antonio S, Alba AP, Bernabe AF. Challenges of scaling-up PHA production from waste streams. A review. J Environ. Management. 2018; *205*, 215–230.
4. Muhammadi S, Muhammad A, Shafqat H. Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: production, biocompatibility, biodegradation, physical properties and applications. Green Chem. Lett Rev. 2015; 8 (3-4), pp. 56-77.
5. Zibiao L, Jing Y, Xian Jun, L. Polyhydroxyalkanoates: Opening doors for a sustainable future. NPG Asia Mat.. 2016; 8. e265. doi:10.1038/am.2016.48.
6. Verlinden RAJ, Hill DJ, Kenward MA, Williams CD, Radecka I. Bacterial synthesis of biodegradable polyhydroxyalkanoates. J Appl. Microbiol. 2007; 102; 1437-1449.

7. Chen GQ. Introduction of Bacterial Plastics PHA, PLA, PBS, PE, PTT, and PPP. *Plastics from Bacteria: natural functions and applications*. Microbiol. Mono.2010; 14; 1-16.
8. Chen GQ, Jiang XR. 2018. Engineering microorganisms for improving polyhydroxyalkanoate biosynthesis. Curr. Opin. Biotechnol. 2018; 53, 20–25. https://doi.org/10.1016/j.copbio.2017.10.008
9. Koller M, Niebelschutz H, Braunegg G. Strategies for recovery and purification of poly [(*R*)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. Eng. Life Sci. 2013; 13, 549–562.
10. Madkour MH, Heinrich D, Alghamdi MA, Shabbaj II, Steinbuchel A. PHA recovery from biomass. Biomacromolecules, 2013; 14, 2963–2972.
11. Baptista SDA. Screening of polyhydroxyalkanoates producing bacteria isolated from marine ecosystems. Masters dissertation, University of Interior Biera; 2013.
12. Amrita R, Ibrahim V, Monica D, Sekar KV, Yogesh BJ, Bharathi S. Screening, optimization and characterization of polyhydroxyalkanoates produced from microbial isolates. *Inter. J. Curr. Microbiol. Appl. Sci.* 2014; 3(4): 785-790.
13. Nwinyi CO, Owolabi, TA. Scanning electron microscopy and Fourier transmission analysis of polyhydroxyalkanoates isolated from bacteria species from abattoir in Ota, Nigeria. *Journal of King Saud University – Science.* http://dx.doi.org/10.1016/j.jksus.2017.08.003
14. Bergey's Manual of Systematic Bacteriology, 2nd edn, In: Brenner D.J., Kreig N.R. and Staley J.T., Springer, New York; 2009.
15. Natarajan K, Subashkumar R, Minu Venugopal, Rathinamala J. 2017. Biosynthesis of Poly-β-Hydroxybutyrate and distribution of *phbC* Gene in *Lactobacillus plantarum***.** Int. J Adv.Res. 2017; 5(2), 1847-1855.
16. Aslim B, Fikret C, Yavuz B, Ufuk G. Poly-β-Hydroxybutyrate production from lactic acid bacteria. FEMS Microbiol. Lett. 1998; 159, 293-297.
17. Raza Z, Abida S, Banat IM. Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *Inter. Biodeter. Biodegrad.* 2018; *126*, 45–56. https://doi.org/10.1016/j.ibiod.2017.10.001
18 Song JH, Jeon CO, Choi MH, Yoon SC, Park W. Polyhydroxyalkanoate (PHA) production using waste vegetable oil by *Pseudomonas* sp. strain DR2. J. Microbiol. Biotechn. 2008;18, 1408–1415.
19 Aljuraifani AA, Berekaa MM, Ghazwani AA. Bacterial biopolymer (polyhydroxyalkanoate) production from low-cost sustainable sources. *Microbiol. Open*. 2019; 8: e755. https://doi.org/10.1002/mbo3.755
20 Urvija A, Veena G.K. Production of Polyhydroxyalkanoate (PHA) by a Moderately Halotolerant Bacterium *Klebsiella pneumoniae* U1 Isolated from Rubber Plantation Area. *Int. J Environ. Biorem. Biodegrad*. 2015; 3(2), pp 54-61. http://pubs.sciepub.com/ijebb/3/2/3
21 Sangkharak K, Prasertsan P. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electronic J Biotech. 2008; 11 (3).
22 Nisha VR, Sudheer KS, Carlos RS, Ashok P. 2009. Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. Brazilian Arch. Biol. Technol. 2009; 52: 1-12.
23 Desouky SE, El-Shiekh HH, Elabd MA, Shehab AM. Screening, Optimization and Extraction of Polyhydroxyalkanoates (PHAs) *Bacillus thuringienesis.* J Adv. Biol. Biotechnol. 2014;1(1): 40-54.

24 Yu-Hong W, Wei-Chuan C, Chin-Kuei H, Ho-Shing W, Yi-Ming S, Chi-Wei L, Om-Murugan, J. Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains. Inter. J Molecular Sci.2011; 12: 252-265

25 Tabassum M, Suraini A, Phang LY, Wan Md Zin WY, Yoshihito S, Mohd AH. Synthesis, Characterization, and Structural Properties of Intracellular Copolyester Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Produced by *Comamonas sp.* EB 172 from Renewable Resource**.** Inter. J Polymer Analysis Charact. 2010; 15(6): 329-340.