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Providencia rettgeri and *Sporosarcina koreensis* as efficient cell factories for valorization of palm oil mill effluent to produce polyhydroxyalkanoates

Ezechukwu Precious Somtochukwu and Ugwuanyi Jerry Obeta

Department of Microbiology, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.

§**Corresponding author**: Ugwuanyi Jerry Obeta. E-mail: jerry.ugwuanyi@unn.edu.ng, Phone: +234 (0) 803 306 6518

Abstract

Polyhydroxyalkanoates (PHAs) are versatile carbon-neutral, eco-friendly biopolymers that can replace highly polluting petroleum plastics. Microorganisms accumulate PHAs in response to stress. Unfortunately, PHA is more expensive than plastics due to high cost of feedstock. Palm Oil Mill Effluent (POME) an abundant waste from oil palm production can support sustainable production of PHA using appropriate microorganism. Fresh and naturally acidified POME were used as negative-cost feedstock to produce PHA using novel bacteria. Six of 247 isolates obtained from POME / dumpsite soil efficiently accumulated between 19 and 72% PHA. Two isolates that accumulated the most polymers were identified as *Providencia rettgeri* and *Sporosarcina koreensis.* At optimum yield they accumulated respectively 4.2g/L (72% of 5.8g/L biomass) and 3.4g/L (66% of 5.15g/L biomass) in POME at 72hours. Optimization studies show that pH 6, C:N ratio, 25:1, titratable acidity, 0.39% for both isolates, and inoculum size, 10% v/v for *Providencia rettgeri* and 5% v/v for *Sporosarcina koreensis* gave maximum concentration of PHA at 72hours. Fourier transform infrared spectroscopy (FT-IR) and GC-MS analysis of the polymer accumulated by both isolates identified the products as a poly-hydroxyl-butyrate (PHB). This work is reporting for the first time the use of *P. rettgeri* and *S. koreensis* in sustainable processes to valorize major agricultural pollutant to value-added high-cost biochemical. The yield of PHA by these isolates on POME feedstock is promising enough to serve as basis for sustainable industrial process. The waste effluent is reduced for COD and pollution potential and may safely be discharged to environment.

Key Words: Palm oil mill effluent (POME), Polyhydroxylalkanoates (PHA), *Providencia rettgeri, Sporosarcina koreensis,* Waste Valorisation

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INTRODUCTION

Petrochemical plastics are currently the most potent and widespread eco-hazardous, non-biodegradable pollutants (Khatami *et al*., 2020, Leong *et al*., 2021). In addition to their association with emission of greenhouse gases, they accumulate in waterways, oceans and seas; damage the aesthetics and recreational value of water bodies and lead to significant fish kill (Harshvardhan and Jha, 2013). On land, they adversely affect flora, wildlife and human health (Yates and Barlow, 2013, Aires da Silva *et al*., 2014). Unfortunately, rather than decrease with increase in environmental consciousness, their use is increasing, with applications in nearly all facets of human activities (Bharti and Swetha, 2016). As at 2020 global annual production was estimated at over 380 million tons and growing (Mazhandu *et al*., 2020).

Methods currently adopted to manage plastic wastes include disposal into landfills, recycling, energy recovery, gasification and incineration as well as microbial degradation. These methods have drawbacks. High cost, decreasing space and pollution of underground water makes landfilling non-viable (Al-Salem *et al*., 2009). Recycling is challenged with high labour cost and production of altered low quality plastics. Incinerators and gasifiers are expensive to install and maintain, and release hazardous environmental pollutants; while the eco-friendly microbial degradation is very slow (Aires da Silva *et al*., 2014).

Search for suitable replacements for petroleum plastics has focused on biopolymers such as Polyhydroxyalkanoates (PHA) that have comparable physicochemical properties (Rodriguez-Perez *et al*., 2018). PHAs are versatile, structurally diverse, high molecular weight, chiral polymers with potential applications in variety of industries including sensitive medical uses where petroleum plastics are of limited value (Riaz *et al*., 2021). Also, blends with highly varied properties can be produced, depending on the side chain and producing bacteria. These features enable synthesis of fit-for-purpose, stereo-specific PHAs (Guerra-Blanco *et al*., 2018). Being biocompatible and carbon-neutral, PHAs can contribute significantly to the global green economy, if competitively produced (Raza *et al*., 2018).

They accumulate naturally in bacteria as energy storage lipid granules (Riaz *et al*., 2021) during unbalanced growth or stress conditions, such as commonly obtain in a variety of abundant and high carbon to nitrogen-ratio wastes, and the rhizosphere, among others (Kaur and Roy, 2015). Industrial production of PHAs currently uses *Ralstonia eutropha* or genetically modified *E. coli*, although species of *Aeromonas, Alcaligenes, Azotobacter, Burkholderia, Chelatococcus, Comomonas, Corynebacterium, Cupriavidus, Enterobacter, Methylobacterium, Pseudomonas, Rhodobacter, Rhodopseudomonas, Sinorhizobium, Thermus,* etc., have also been identified as potent producers (Kourmentza *et al*., 2017). This suggests that microbial mining for novel producers could lead to even more potent and novel isolates.

Despite their advantages, industrial scale production of PHAs is still limited and products remain non-competitive (prices are up to 80% higher than for petrochemical plastics (Kourmentza *et al*., 2017). This is due to cost of feedstock (up to 50% of production costs (Van den Oever *et al*., 2017), low yield and expensive down-stream process (Daly *et al*., 2018). Even with these challenges however, it is projected that global production of bioplastics could reach 2.6 million tonnes valued at over $100m in 2023 with annual growth rate of 5% (European Bioplastics, 2018). Conventional carbon sources used for PHA production comprise carbohydrates, fatty acids and derivatives, as well as methanol and alkanes (Aslan *et al*., 2016), with use of food-crop-based carbon sources raising concerns over sustainability of process and impact on food prices (Jiang *et al*., 2016). To sustainably produce PHA, use of wastes and cheap renewable biomass as feedstock has been advocated (Sharma *et al*., 2017, Koller *et al*., 2019, Tsang *et al*., 2019, Crutchik *et al*., 2020). Such feedstock includes paper mill wastewater, food waste and wash effluents, organic fraction of municipal waste, agro-industrial wastes, oil mill and vegetable oil waste, including palm oil mill effluent (POME).

POME is brown oily, high COD slurry generated during the processing of oil palm fruit (Iwuagwu and Ugwuanyi, 2014, Mohammad *et al*., 2021). For each tonne of crude palm oil (CPO) produced, it has been estimated that 5.0-7.5 tonnes of water are required with over 50% of this ending as POME. This waste, commonly managed through slow, open ponding system, is the most potent environmental pollutant of agricultural origin in all oil palm producing nations (Lee *et al*., 2015). Thus, whereas microorganisms have the biochemical potential to convert this vastly abundant high polluting

waste to high value bioplastics, this has not happened due to limited efforts to find organisms that are efficient in the conversion of POME to PHA.

This study was implemented to isolate organisms with potential to efficiently convert POME to PHA while concomitantly reducing the pollution potential of waste. This should improve the economics of PHA production while greatly benefiting the environment. This work investigated POME management through conversion to PHA by use of local isolates of competent bacteria, directly and via organic acid pre-production, achieving in the process zero-cost, sustainable waste treatment with value creation. Use of novel eco-adapted isolates of *Providencia rettgeri* and *Sporosarcina koreensis* to degrade POME and achieve high accumulation of PHA as basis for economic and sustainable valorisation / management of this high volume strongly polluting waste is shown to have significant potential and is being reported for the first time.

MATERIALS AND METHODS

Collection and characterization of POME and soil samples

POME and contaminated soil were collected from different palm oil processing facilities and dump sites in Nsukka, Enugu State, an area representative of the large scale and expanding oil palm production in Nigeria. Ten samples each of POME and soil were collected in sterile universal bottles, transported to laboratory, and kept refrigerated until use.

Isolation of bacteria from POME and soil samples

POME samples were processed by ten-fold serial dilution in sterile distilled water with brisk manual shaking followed by plating (0.1ml) on POME-mineral salt agar (PMSA) by spread plate method. PMSA had the following composition (per L of muslin filtered fresh POME): $FeSO_4.7H_2O$, 20mg, KH_2PO_4, 1.5g, $(NH_4)_2SO_4$, 1g, $Na_2HPO_4.12H_2O$, 9g, $MgSO_4.7H_2O$, 0.2g, $CaCl_2.2H_2O$, 10mg, agar, 18g; trace element solution, 1ml. Composition of trace element solution ((per litre) was: H_3BO_3, 0.3g; $CoCl_2$ $6H_2O$, 0.2g; $ZnSO_4.7H_2O$, 30mg; $MnCl_2.4H_2O$, 30mg; $NaMoO.2H_2O$, 30mg; $NiCl_2.6H_2O$, 20mg, $CuSO_4.5H_2O$, 10mg). Medium pH was adjusted to 7.0 before sterilization (15 minutes at 121°C). Soil samples were processed by weighting 1g of soil into a universal bottle containing 9ml of sterile distilled water, allowed to stand for a few minutes and mixed by vortex. This was followed by a serial, ten-fold dilution and plating (0.1ml) on PMSA. Plates were incubated at room temperature (30°±2°C) for up to 96 hours. Visible colonies were sub-cultured on to fresh nutrient agar and PMSA and incubated for 24–96 hours as appropriate to obtain pure isolates. Stock of isolates were stored at 4ºC and sub-cultured weekly.

Rapid Sudan Black B Screening of Isolates for Production of PHA

Mineral salt medium used to screen isolates for accumulation of PHA was prepared following the composition reported by Fang *et al*. (1996). The pH of the medium was adjusted to 7.0 before sterilization for 15 minutes at 121°C. Carbon source (glucose) was separately sterilized (110°C/10mins) and added to medium to make $50gL^{-1}$ before pouring into petri-dishes. Inoculated plates were incubated at 30±2ºC for 72 hours.

Rapid screening of the isolates was carried out by flooding mineral salt agar colonies (48hr cultures) of isolates with 0.02% alcoholic Sudan Black B solution (Sigma-Aldrich, Germany) for 30 minutes at room temperature (Bhuwal *et al*., 2013). The dye was decanted, and plates were washed twice with absolute ethanol. Colonies that did not produce PHA failed to incorporate Sudan Black B and appeared white. Colonies that produced PHA appeared bluish black. Positive isolates were sub cultured and maintained for further studies. Six isolates that most retained the stain were further screened to quantify PHA accumulation.

Pretreatment and characterization of POME used for production of PHA

Fresh POME was sieved through muslin then cheese cloth to remove coarse particles. Sieved POME was analysed for Chemical oxygen demand (COD), total solids (TS) and total suspended solids (TSS) by standard methods (APHA, 1995). Total dissolved solid (TDS) and pH were determined as previously reported (Iwuagwu and Ugwuanyi, 2014). Ash content was quantified by ignition at 550°C (Gallenkamp Oven S-3, England). Lipid was quantified by chloroform/ methanol (2:1) extraction method (Folch *et al*., 1957) while total nitrogen was measured by Kjeldahl method (Horwitz and Latimer, 2010). Organic carbon was determined by dichromate oxidation (Walkley and Black, 1945). POME was dispensed into 100mL flasks and sterilized (121°C /20min) before use.

Preparation and standardization of bacterial isolates for PHA production

A 0.1ml volume of 18hour broth culture of each isolate was inoculated in 10ml sterile peptone water and incubated at 35°C for 24 hours. A 10-fold serial dilution of the bacterial suspension was made and 0.1mL of each dilution was used to count viable cells by pour plate method on nutrient agar following incubation at 35°C for 24 hours. Standard inoculum of each isolate adjusted to approximately 4.56 x 10^7 CFU/ml was obtained.

Production and assay of biomass and PHA in POME medium

PHA production was carried out in POME-mineral salt medium (PMSM) compounded as in Fang *et al*. (1996) using POME but without glucose and agar. A 2mL volume of the standardized inoculum was inoculated into 200mL Erlenmeyer flasks containing 38mL sterilized PMSM. Incubation was carried out in a shaker incubator at 80rpm and 30^{o}C, and 5ml pooled samples withdrawn at 24 hourly intervals for 96 hours were centrifuged (10,000rpm) for 15minutes. Biomass pellet, washed twice using distilled water was dried to a constant weight at 65^{o}C in a hot air oven. To lyse cells and digest cell components other than PHA, 10mL of sodium hypochlorite was added to cell pellet and incubated at 50°C for 1 hour. The lysate was centrifuged (10,000rpm /30min) and sequentially washed with 10mL distilled water, then acetone-methanol-diethyl ether (1:1:1) before resulting pellet was dissolved in 10mL chloroform and incubated overnight at 50°C to evaporate to dryness. Thereafter 10mL of concentrated H_2SO_4 was added and the solution heated in a water bath (10mins at 100°C) to convert PHA to crotonic acid. Poly-hydroxyl-butyrate (PHB) (Sigma-Aldrich, Germany) similarly crotonized, was used as standard. Absorbance was read at 235nm against concentrated H_2SO_4 blank in a spectrophotometer (Shimadzu UV-1800, Shimazdu Corp, Japan).

Molecular identification of selected isolates

Molecular identification of the two best PHA producers was based on PCR and Sanger Sequencing. DNA was extracted from 24 h culture of isolates in BHI broth harvested by centrifugation (14,000 x g/ 10min). The cells were washed thrice at 12,000 rpm for 5 minutes in 1ml of Ultra-pure water. DNA extraction and purification were done using ZR Fungal/ Bacterial DNA MiniPrep™50 Preps Model D6005 (Zymo Research, California, USA). A 50mg quantity of cells was re-suspended in 200µL of sterile water, transferred into a ZR Bashing-Bead™ tube and 750µL Lysis solution added. The solution was secured in a bead beater fitted with 2mL tube-holder and processed at maximum speed for 5minutes. Lysis tube was centrifuged (10,000 x g / 1min). 400µL supernatant was pipetted into a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged (7,000 x g / 1min), followed by the addition of 1,200µL of Fungal/Bacterial DNA binding buffer. 800µL of the mixture was then transferred into a Zymo-Spin™ IIC column in a collection tube and centrifuged (10,000 x g / 1min). The flow through was discarded and the process repeated to obtain the remaining products. 200µL DNA pre-wash buffer was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged (10,000 x g /1min). This was followed by addition of 500µL Fungal/Bacterial DNA Wash Buffer into the Zymo-Spin™ IIC column and centrifugation (10,000 x g /1min). The Zymo-Spin™ IIC column was transferred into a clean 1.5 ml microfuge tube and 100µL of DNA elution buffer was added directly to column matrix and centrifuged (10,000 x g /30sec) to elute the DNA. Resulting DNA filtrate (ultra-pure), used as a template during the assay was transported in ice to the laboratory for sequencing.

PCR was performed on the extracted DNA using universal degenerate primers 27F.1 Forward 5'AGRGTTTGATCMTGGCTCAG 3' and 1492R reverse 5'GGTTACCTTGTTACGACTT 3' that amplifies the entire 16S variable region at annealing temperature of 58°C (DeSantis *et al*. 2007). Each PCR reaction contained 5µL of 10 × Taq buffer, 2mM $MgCl_2$, 1.5U Super-Therm DNA Polymerase (Southern Cross), 0.25mM dNTP's, 0.1µM of each primer, 1µL of extracted DNA and Nuclease Free Water up to final reaction volume (50µL). PCR cycle started with an initial denaturation step at 94°C for 10 min. This was followed by 30 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1min and extension at 72°C for 1min, and a final extension at 72°C for 5min that was then followed by cooling to 4°C. Few microliters of the samples were run on 1% agarose gel (90V /30mins) to verify amplification. Entire PCR product was loaded onto 1% agarose gel and the correct band size (approx. 1500bp) was excised. DNA was recovered from gel slices using GeneJET™ gel extraction kit (Fermentas).

Sequencing was performed by Sanger (dideoxy) technique using automated PCR cycle-(Sanger Sequencer™ 3730/3730XL DNA Analyzers (Applied Biosystems)). Sequence analysis of nucleotides base pairs was performed by direct BLAST analysis on the NCBI database (http://blast.ncbi.nlm.nih.gov). For each isolate, a read was BLASTed and the top hits with minimum E-score for every BLAST result showing species name used to name the specific organism.

To carry out multiple alignments of sequences, ClustalW alignment Software was used after which the Molecular Evolutionary Genetic Analysis (MEGA) software was used to highlight the conserved domains in the aligned sequences. Construction of a Phylogenetic tree to depict the evolutionary relationship was carried out using the MEGA Software.

Nucleotide BLAST was carried out using the isolate genomic DNA as query sequence. The output description showed nucleotide sequences of organisms having a minimum of 93% query cover. 20 organisms with a minimum of 80% sequence identity with each query sequence were selected for the nucleotide multiple sequence alignment. Evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and optimal tree with the sum of branch length = 0.38891317 shown. Percentage replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale and branch lengths in units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were then computed (Tamura and Nei, 1993). Analysis involved 15 nucleotide sequences and codon positions included were 1st+2nd+3rd+Noncoding. Any ambiguous positions were removed for each sequence pair. A total of 1547 positions remained in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

Optimization of growth parameters for PHA production in POME medium

Optimization of process parameters for PHA accumulation was implemented using the one factor at a time (OFAT) approach. The factors considered were initial pH of the fermentation medium, inoculum size, carbon to nitrogen (C:N) ratio and POME titrable acidity.

Effect of medium pH on PHA production

Acetic acid was used to adjust the pH of POME medium to 3.0 while 0.1M NaOH was used to adjust it to 5.0, 6.0, 7.0, and 8.0 before dispensing (38mL of each) into 200mL Erlenmeyer flasks and sterilizing at 121^{o}C for 20 minutes. A 2.0mL volume of the standard inoculum was used to inoculate each flask and incubated at 30^{o}C. Triplicate flasks were set up for each pH value. Samples were collected at 24 hourly intervals for assay.

Effect of inoculum size on PHA production

Inoculum sizes were varied at 1.0ml (2.5% v/v), 2.0ml (5.0% v/v), 3.0ml (7.5% v/v) and 4.0ml (10% v/v) of the standard inoculum, used to inoculate triplicate 200mL Erlenmeyer flasks containing 39ml, 38ml, 37ml, and 36ml respectively of sterilized POME medium. Samples were collected at 24 hourly intervals and assayed for PHA.

Effect of carbon to nitrogen ratio on PHA production

Glucose was used to adjust the C:N ratio of POME to 20:1, 25:1, 30:1, and 40:1. Unadjusted POME (C: N ratio equal to 6:0) was also used to set up reaction. Fermentation was set up as for inoculum size experiment, and for each isolate, inoculated with the volume of inoculum determined to give best performance. Samples were collected at 24 hourly intervals.

Effect of titratable acidity on PHA production

Raw POME in 1.0iter quantities was allowed to stand in air tight 1.0liter brown bottles for 0, 7, 14, 21 and 28 days to undergo spontaneous degradation / souring to accumulate organic acid (measured as titratable acid). The partially degraded POME was then used for fermentation as above. Fresh and degraded POME were titrated against 0.01M NaOH to determine titrable acidity calculated using the method of Hadid (2013).

Titratable acidity values of 0.18%, 0.22%, 0.30%, 0.39% and 0.32% were obtained for fresh POME, 7, 14, 21 and 28- day (degraded POME) samples respectively and were used as such for fermentation studies as described. Samples were analysed at 24hour intervals.

Characterisation of PHA

Fourier transform infrared (FT-IR) analysis

Infra-red analysis of PHA product was carried out as described by Bhuwal *et al*. (2013). A 2ml volume of crotonized sample was transferred to a cuvette and analysed on IR-Affinity-1S

Shimadzu FT-IR Spectrophotometer (Shimazdu Corp, Japan) in the range of 400 – 4000cm^{-1} for 10 - 64 scans at resolution of 4cm^{1}.

Qualitative determination of PHA content of biomass by GC-MS

The method of Tan *et al*. (2014) was used to qualitatively assess the accumulated polymer. The sample was methanolyzed using 50:50 chloroform: acidified methanol (15% (v/v) H_2SO_4). The preparation was then transferred to test tubes and incubated at 100°C for 3hours to depolymerize and methanolyse the polyesters. Methyl benzoate (5mg/L) was used as internal standard. Samples were analyzed with a gas chromatograph equipped with mass spectrophotometer (GC-MS) (7890/5975C, Agilent Technologies, US) and an HP-5MS capillary column (30m x 0.25mm x 0.25mm) (Agilent Technologies, Palo Alto, CA, USA) by the method of Tan *et al* (2014). A 1µL of methanolyzed sample was injected into the GC-MS. Temperature of the injection port was set at 250°C, interface at 280°C, quadrupole at 120°C, and ion source at 250°C. Oven temperature was programmed at an initial 40°C and subsequently raised at rate of 10°C/min to 280°C and held for 5min. Helium was used as carrier gas at flow rate of 1.2mL/min. Solvent delay was set at 2.5min. The MS detector using electron impact (EI) ionization at 70eV was operated in full scans (mass range of m/z 40-600 with a 0.1 mass accuracy). Results were compared with total ion chromatogram (GC-MS) for standard /reference PHA (PHB, Sigma-Aldrich, Germany) and the products were identified by comparing their retention times to the standard (Tan *et al*., 2014).

RESULTS

Physicochemical analysis of POME sample used for studies

The physicochemical parameters of pooled fresh POME used for study are as shown in Table1. POME is mildly acidic with high COD. The mean nitrogen content was 0.114% and the C:N ratio averaged 6:1. The COD is reflected in the high content of dissolved and suspended solids and is consistent with the high pollution potential of POME.

Isolation of bacteria from POME and dump sites and detection of PHA in cells

A total of 247 bacteria isolates were obtained on PMSA from POME and dump site soil samples. Of these, 101 isolates were obtained from POME, while 146 isolates were obtained from dumpsite soil samples. Thirty (30) isolates (12.15%) retained Sudan Black B on at least a portion of the colony (mostly the center, for those isolates that retained only limited amount of dye). Six (6) of the isolates (2.4% of the total) retained the dye uniformly over the entire colony and being considered prolific accumulators of polymer were subjected to quantitative analysis for PHA when grown in PMSM.

Peak biomass accumulated in PMSM was 2.99g/L for isolate 01 at 72hrs while isolates 29, 32, 33, 78 and 85 accumulated (per L) 2.77, 3.01, 3.67, 3.19 and 3.22 g respectively. Waste to biomass conversion appeared low as these figures translate to biomass accumulation on POME total solids of (g/g); 0.071, 0.065, 0.071, 0.086, 0.075 and 0.076 respectively (Table 2). Figure 1 shows the result for PHA production in PMSM by the six isolates. Isolate 01 accumulated 34.86% PHA on cell dry weight basis at the end of 72hr while isolates 29, 32, 33, 78 and 85 accumulated 25.51%, 32.10%, 72.25%, 19.63% and 36.10% respectively. Based on these, PHA accumulation in PMSM was (per L) 1.04, 0.71, 0.97, 2.65, 0.63 and 1.16 g respectively for isolates 01, 29, 32, 33, 78 and 85 (Table 2). Isolates 33 and 85, being the best producers, were identified by molecular methods and maintained for further studies.

Table 1. Physicochemical characteristics of filtered raw pome sample

Parameter	Concentration (mg/L)
pH	4.3
Chemical Oxygen Demand	63,320
Total Solid	42,540
Total Dissolved Solid	32,000
Total Nitrogen (%)	0.1136
Total Organic Carbon (%)	0.6589
Available Phosphorus (PPM)	24,089
Total Lipid	3,580
Ash	400

Table 2 Accumulation of PHA by isolates as function of medium and dry cell biomass in POME mineral medium

Isolate	Biomass (g/l)	Biomass /POME Solid (g/g)	PHA (%, biomass DW)	PHA g/ L
01	2.99	0.071	34.86	1.04
29	2.77	0.065	25.51	0.71
32	3.01	0.071	32.10	0.97
33	3.67	0.086	72.25	2.65
78	3.19	0.075	19.63	0.63
85	3.22	0.076	36.10	1.16

Molecular identification of selected isolates

DNA sequence results

The nucleotide sequences (Supplemental Materials a & b) for isolates 33 and 85 (respectively) were used for the identification of the two isolates. Isolate 33 was identified as *Providentcia rettgeri* while isolate 85 was identified as *Sporosarcina koreensis.* The phylogenetic tree showing relatedness of the two isolates is as presented (Supplemental Material c).

Optimization of PHA production by *P. rettgeri* and *S. koreensis*

Effect of initial pH of medium

The effect of initial pH of medium on PHA accumulation by *P. rettgeri* (isolates 33) and *S. koreensis* (isolate 85) are shown in Figures 2a & 2b. Highest accumulation of PHA by *P. rettgeri* occurred in medium of initial pH 6.0 (3.55±0.1g/L), while pH 3.0 gave the least (0.50g/L) which is much lower than for other pH values. Total biomass produced (<2.0g/L) was also low at pH 3.0. At all pH values profiles of accumulation of PHA were similar and reach peak values at 48hr before declining progressively. The decline in PHA accumulation could be attributed to biomass turnover and reconversion of storage polymers. For *S. koreensis* highest accumulation of PHA was also obtained in medium of initial pH 6.0 (3.33±0.11g/L). This was followed, first by slight decline at 72hr, before dropping more rapidly to the end of the process (Fig. 2b). As with *P. rettgeri*, the least accumulation (0.48g/L) occurred in medium of initial pH 3.0. Both organisms thrived in medium of low pH (3.0), probably as a reflection of adaptation to the low pH of the isolation environments (≈4.3). Both organisms showed good growth and PHA production at all initial pH values from 4.3 to 8.0. Medium made from unadjusted POME (pH 4.3) consistently supported better polymer production than the nearest but adjusted acidic media (pH 3.0 and 5.0).

Effect of inoculum size

Highest accumulation of PHA (3.61±0.05g/L) occurred in *P. rettgeri* when the pH 6.0 medium was inoculated with 10% inoculum (figures 3a), while the least accumulation (1.99±0.12g/L) was obtained when the inoculum volume was set at 2.5%. In the case of *S. koreensis*, 5.0% inoculum resulted in the highest PHA accumulation (2.82±0.08g/L) while the least (2.06±0.15g/L) was recorded when inoculum volume was 2.5% (Figure 3b). Peak accumulation of PHA occurred at 48hrs in all cases.

Effect of carbon to nitrogen ratios

When the C:N ratios of PMSM (initial pH 6.0) were increased using glucose, the time of peak accumulation of PHA shifted from 48hr to 72hr for both isolates (fig. 4), suggesting that introduction of carbohydrate led to increase in the time to onset of stress condition in the isolates. Amount of PHA accumulated by *P. rettgeri* also increased. Highest accumulation, 6.08±0.25g/L, amounting to approximately 77% of the total *P. rettgeri* biomass (7.9g/L) occurred at ratio of 25:1 (fig. 4a). At C:N ratios of 20:1, 30:1 and 40:1 the amount of PHA accumulated were considerably lower. The least accumulation was obtained at ratio of 6:1 (unadjusted POME mineral medium) (Fig 4). As with *P. rettgeri* peak accumulation of PHA (3.20±0.074g/L) in *S. koreensis* was obtained at 72hrs in medium of C:N ratio 25:1. This amounted to approximately 45.85% of total biomass (6.98g/L) and was followed by ratios of 6:1, 20:1 and 30:1. Unlike with *P. rettgeri* increase in C:N ratio did not consistently improve accumulation of PHA by *S. koreensis* relative to unadjusted PMSM (cf. figures 4B and

2B), and actually negatively impacted it at 40:1. PHA accumulated at C:N ratio 25:1 (3.2g/L) was only marginally higher than in unadjusted PMSM (2.93g/L) (Figure 4B). In absolute terms, PHA accumulation in *P. rettgeri* (6.08g/L) was much higher than in *S. koreensis* (2.93g/L). Similarly, as function of biomass it was higher in *P. rettgeri* (77%) than *S. koreensis* (45.85%). This was accentuated further by the better accumulation of biomass recorded in *P. rettgeri* (7.9g/L) than in *S. koreensis* (6.98g/L).

Effect of titratable acidity of medium

After POME was allowed to undergo uncontrolled degradation/ souring, titrable acidity increased from 0.18% (0hour) to 0.22%, 0.30%, 0.32% and 0.39% in 7, 14, 21 and 28 days respectively. When such POMEs were used to prepare PSMS, accumulation of PHA increased with the amount of organic acid (fig. 5). For *P. rettgeri,* PHA increased from peak of 2.64g/L (66% of 4.0g/L biomass) in 0.18% acid medium to 4.2g/L (71.5% of 5.87g/L biomass) in 0.39% titratable acid medium (72 hours), representing 59.10% increase in PHA for 46.75% increase in biomass (Fig.5a). In *S. koreensis* PHA increased from 2.44g/L (76.3% of 3.20g/L biomass) in 0.18% acid medium to 3.4g/L (66% of 5.15g/L biomass) in 0.39% acid medium representing 39.34% increase in PHA for 60.94% increase in biomass (Fig. 5b). In both cases increase in titratable acid was approximately two-fold.

Fourier transform infrared (FT-IR) analysis of PHA

Products showed all the bands characteristic of PHA on FT-IR analysis. For *P. rettgeri* product the spectra indicated the presence of marked peaks at wave numbers 3481.48cm^{-1} for the hydroxyl (-OH) bonding group, at 2933.36cm^{-1} for asymmetric methyl (C-H) stretching group, and 1721.32cm^{-1} to 1786.94cm^{-1}, corresponding to the C=O and aliphatic stretching of the carbonyl group of RCOA of the polymer (Supplemental Material d). The absorption bands at 1350.76cm^{-1} and 1493.58cm^{-1} were those of the aliphatic -CH_3, and -CH_2 groups. The bands at 1204.08cm^{-1} to 1289.00cm^{-1} are characteristic of carbon-oxygen stretch of esters (C-O-C bond) while the band at 918.44cm^{-1} is established to be of an alkyl stretch.

Similarly, the PHA of *S.koreensis* showed marked peaks at 3419.72cm^{-1} for the hydroxyl (-OH) bonding group, 2995.12cm^{-1} for the asymmetric methyl (C-H) stretching group, and at 1786.94cm^{-1}, corresponding to the C=O double bond and aliphatic stretching of the carbonyl group of RCOA of the polymer (Supplemental Material d). The bands at 1377.78cm^{-1} and 1470.42cm^{-1} were those of the aliphatic -CH_3, and -CH_2 groups, at 1258.12cm^{-1} it is characteristic of carbon - oxygen stretch of esters (C-O-C bond), while at 918.44cm^{-1} it is established to be that of an alkyl stretch.

Qualitative determination of PHA content of biomass by GC-MS

Total ion chromatograms (GC-MS) of PHA produced by *P. rettgeri* and *S. koreensis* are presented in Supplemental Material e. The chromatogram of the polymers showed peak with retention time of 10.780 mins for *P. rettgeri* PHA and 10.728mins for product of *S. koreensis*. (The reference standard showed peak for the monomer at 11.060mins). By comparing molecules in the GC-MS database, the identified peak represents an ester of tetradecanoic acid confirming the polymer as poly-hydroxyl butyrate (PHB).

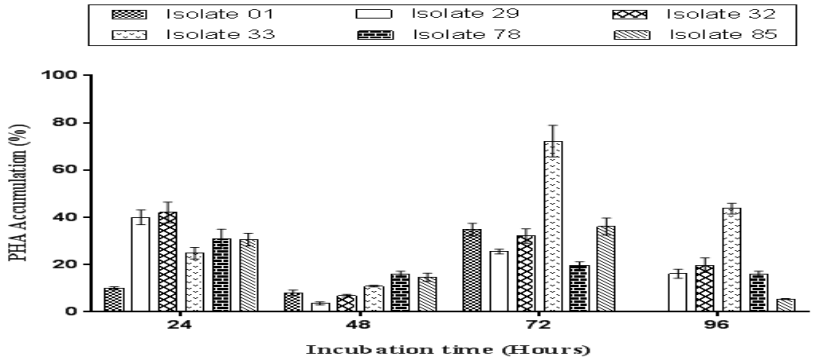

Figure 1: Accumulation of PHA (% of dry biomass) by six selected isolates

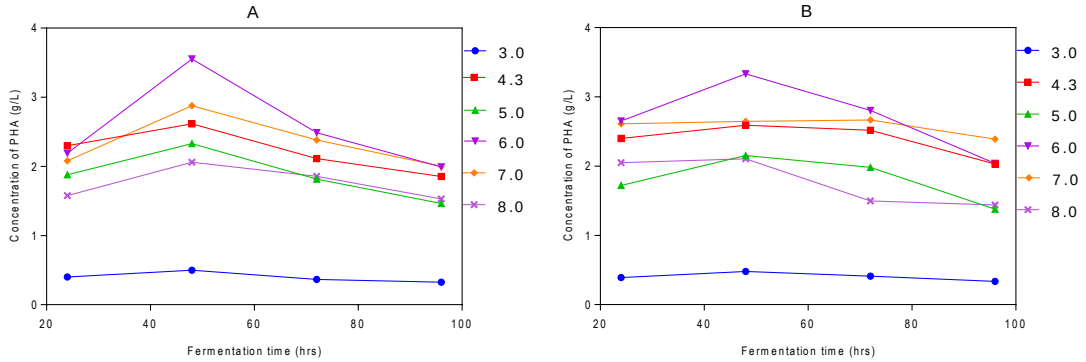

Figure 2. Production of PHA by *P. rettgeri* (A) and *S. koreensis*(B) at different pH values

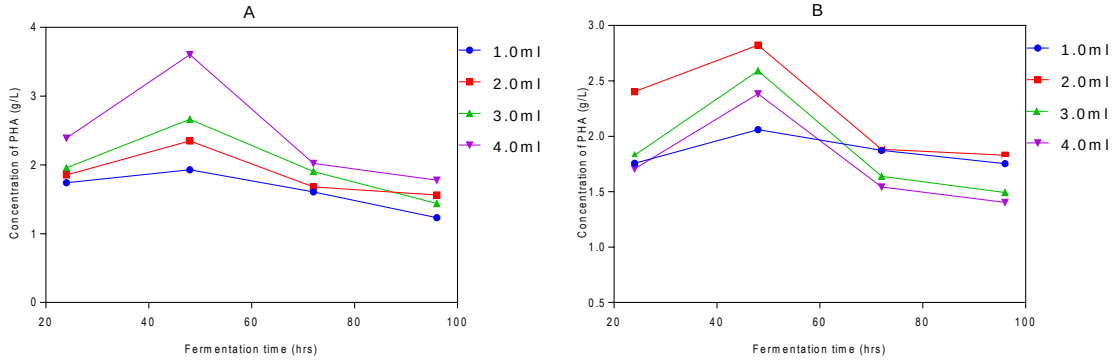

Figure 3. Effect of inoculum volume on production of PHA by *P. rettgeri*(A) and *S. koreensis* (B)

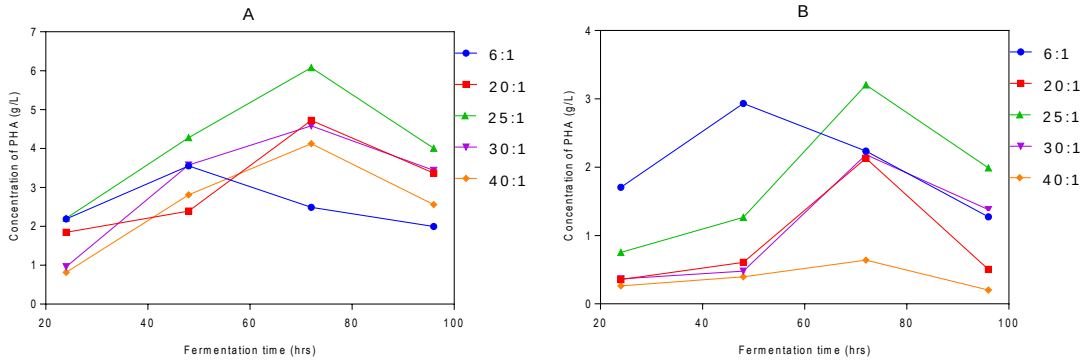

Figure 4. Effect of C:N ratios on production of PHA by *P. rettgeri* (A) and *S. koreensis* (B)

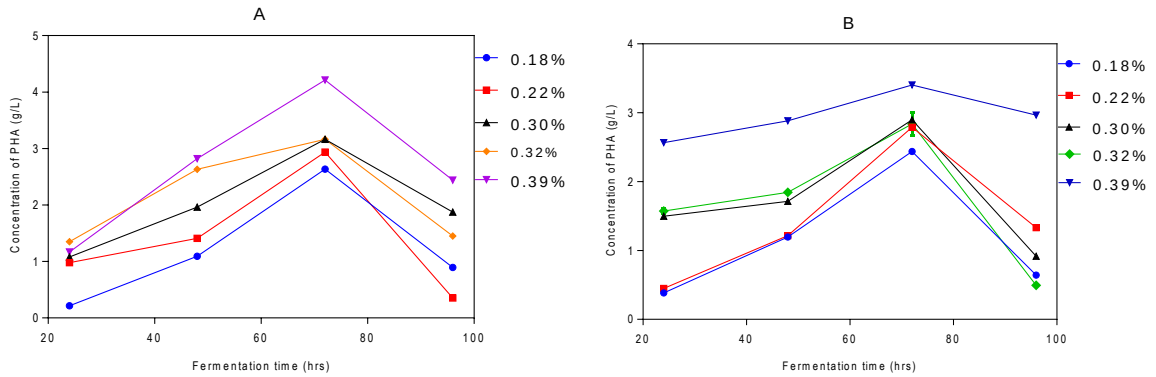

Figure 5: Effect of titrable acidity on PHA production by *P. rettgeri*(A) and *S. koreensis* (B)

DISCUSSION

Over the past few decades there has been sustained interest for production of eco-friendly plastics to reduce damage to environment due to expanding use of petroleum plastics. This has been boosted with the consideration of zero cost wastes and refuse as production feedstock. Use of such wastes in bioprocessing offers dual benefit of zero cost waste management / pollution control and reduced-cost value creation in bioproduct manufacture. This waste treatment-value creation nexus is given expression in this work in use of highly polluting POME as fermentation feedstock for production of high value biochemical (PHA), while achieving waste treatment. POME is abundant at negative value, has high carbon content and is amenable to in situ up-stream pre-acidification.

A total of 247 bacteria capable of growth on POME were isolated. This low number may be a reflection of the nature of isolation medium (PMSA) which was chosen to ensure selection of only organisms that are able to grow on, and use palm oil process waste components as carbon and energy sources to accumulate PHA. A high percentage (30 (12.15%)) of isolates accumulated some amount of PHA. Of these, six isolates appeared to be prolific producers when screened qualitatively in PMSM. Isolates 33 and 85 accumulated the most PHA of 72.25±6.68% and 36.10±3.63% of dry biomass respectively at 72 hours (see fig. 1). Although biomass accumulation (g/l) was modest at 3.67 and 3.22 respectively, these represent considerable accumulation (g/g) on medium waste solids at 0.086 and 0.076. Based on medium volume, PHA accumulation (g/L) by the two isolates was also high at 2.65 and 1.16 respectively. Considering that the medium was raw waste, this process represents considerable value addition with potential to be very competitive when compared with productivity figures reported in literature using defined and expensive media.

The two choice producers selected for further studies were identified as *Providencia rettgeri* (Isolate 33) and *Sporosarcina koreensis* (Isolate 85). To the best of our knowledge, neither of these organisms has been reported in studies of microbial production of PHA. Nor has any been used to achieve reprocessing of waste biomass. *Providencia* species are associated with disease situations (O'hara *et al*., 2000). However, by use of appropriate molecular tools the biotechnological potential of this organism may be harnessed to achieve value creation while managing the associated risk as is known with various applications of genetically engineered *E. coli* (Daly *et al*., 2018). As with *Providencia*, this work is reporting, for the first time, the use of *Sporosarcina* in waste biomass reprocessing with value creation. Although efficiency of PHA accumulation with this organism is lower than *Providencia*, it is attractive in being non-pathogenic and therefore readily deployable.

Palm fruits are handled differently between processors and resulting POME often differ in chemical characteristics. The POME used in this study had chemical features comparable to reported figures (Madaki and Seng, 2013, Ebana *et al*., 2017). Breakdown of oils readily leads to accumulation of various fatty acids during fruit processing and POME handling. When samples were allowed to stand over periods of up to 28 days, the titratable acidity increased progressively and impacted accumulation of PHA by both isolates (Figure 5). Optimum performance (obtained at pH 6) compares with or exceeds results reported in literature (Kourmentza, and Kornaros, 2016)

and as variously reported, declines as pH increased or decreased further from neutral (Gomaa, 2014). Maximum polymer yields for both organisms were obtained at C:N ratio of 25:1, decreasing at higher or lower ratios. Gumel *et al.* (2012) reported maximum PHA accumulation (70% of biomass) at ratios of 25:1 and 30:1.

Inoculum size impacted the time to onset of high-biomass related stress and varied between 5 and 10% for peak PHA accumulation at 48 hours. This is in consistent with some published reports (Yamane *et al*., 1996, Santhanam and Sasidharan, 2010) (high inoculums favour PHA production) while differing from Fang *et al.* (1996) who reported no difference in biomass yield and PHB synthesis when inoculum volume was varied from 10% v/v to 20% v/v. It is noted however, that unless inoculums volumes are tied to actual bacterial numbers comparability of data may be difficult.

In this study, fresh and stale (7, 14, 21 and 28 days) POME samples were used to constitute fermentation media with acidity levels of 0.18%, 0.22%, 0.30%, 0.32% and 0.39% respectively. Yield of PHA by both organisms increased with titratable acidity to 0.39% (figure 5). As the percent increase in PHA may not be accounted for solely by the increase in amount of titratable acid, it is likely that staling of POME led to pre-digestion of complex organic matter content; such that even without extensive accumulation of titratable acid it enabled improved utilization of POME. This may explain and support the significant increase in total biomass from 4.0g/L at 0.18% titrable acid to 5.87g/L at 0.39% titrable acid, and from 3.2g/L at 0.18% titrable acid to 5.15g/L at 0.39% titratable acid for *P. rettgeri* and *S. korensis* respectively. In addition, the increase in titratable acids (two-fold) could also lead to increased accumulation of PHA by stimulating the pathways of PHA metabolism, explaining the increase in PHA accumulation as percentage of total biomass. This precursor /pathway effect appears plausible than just increase in usability of medium, given that increase in PHA was more dramatic than increase in biomass as a function of total acid. The application of in-situ pre-digestion or souring of oily waste as a means of enhancing PHA accumulation through generation of volatile acids offers promise since holding raw waste may form part of the upstream pre-process. Acidogenic pre-fermentation of food waste has earlier been used to attempt to modulate quality /quantity of PHA (Wang *et al*., 2019, Vu *et al*., 2012). Although *P. rettgeri* is metabolically versatile having been reported in dye decolorization (Shi *et al*., 2021), this is the first time, to our knowledge that it is being used to accumulate PHA.

The distinctiveness of FT-IR spectra is determined by the chemical structure of the component and also directly related to the concentrations of components of the sample (Kansiz *et al*., 2000). FT-IR spectra of the polymer produced by both organisms indicated strong characteristic bonds at the regions of the functional groups present in PHA. PHA produced by *P. rettgeri* showed absorption at 3481.48cm^{-1} for hydroxyl (O-H) bonding group of the polymer chain, at 2933.36cm^{-1} for asymmetric methyl (C-H) stretching group, and at 1721.32cm^{-1} for carbonyl (C=O) stretching of an ester group, which is a PHA marker band. *S. koreensis* showed absorption at 3419.72cm^{-1} for hydroxyl (O-H) bonding group of the polymer chain, at 2995.12cm^{-1} for asymmetric methyl (C-H) stretching group, and at 1786.94cm^{-1} for the carbonyl (C=O) stretching group. Comparable results have been reported in the literature for PHA produced by other organisms (Costa *et al*., 2018). Mass spectrometry showed that the hydroxyalkanoic acid monomer obtained for both extracts was tetradecanoic (C14:0) acid with retention time of 10.780 mins for *P. rettgeri* and 10.728 mins for *S. koreensis* (11.060 min for reference standard). Costa *et al.* (2018) reported 6.48mins for methyl hydroxytetradecanoate from *Spirulina sp* while He *et al.* (He *et al*., 1998) reported a retention time of 12.280 mins for methyl ester of 3-hydroxy tetradecanoic acid from *Pseudomonas stutzeri* 1317 grown in a soybean oil medium. Data from GC-MS indicates therefore that crystals produced by both organisms was a PHB.

CONCLUSION

PHAs as eco-friendly polymers with diverse potential applications can be more than just replacement for petroleum plastics. The possibility of tailoring their composition and properties make them even more fascinating. There has been considerable progress in the search for new bacterial strains capable of affecting reduction in production cost to achieve sustainability. A combination of strains capable of utilizing waste biomass feedstock and efficient fermentation strategies is the way forward. The organisms used in this study and the choice of raw materials present viable fermentation options with clear environmental

and economic benefits. POME is clearly a viable raw material for sustainable production of PHA and its accumulation in oil palm producing nations need not remain a source of environmental concern and economic anxiety for the industry. Further process optimization can take PHA production using this feedstock to industrial scale and reduce cost of PHA. Use of modern molecular techniques can also obviate any challenges that may arise from the non-GRAS status of *P. rettgeri* enabling biotechnologists to harness its good productivity. PHAs will become the leading biodegradable polymers in the next decades and this could be fast tracked by optimizing fermentation parameters with waste streams as feedstock.

Conflict of Interest

Authors have no conflict of interest to declare.

The PDF copy of the supplementary data is downloadable from https://www.ajol.info/index.php/br/article/view/283815 **OR** https://bio-research.com.ng/index.php/home/article/view/199

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