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Isolation of Bacterial Species Capable of Producing Polyhydroxyalkanoate

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Abstract: The major threat posed by petroleum-based plastics is environmental pollution, which further leads to death of marine animals from the plastic toxicity. A continuous scientific research on plastic innovations would help solve the plastic menace. Hence, this study was carried out to isolate and characterize microbial strains from high organic deposit sites capable of polyhydroxyalkanoate (PHA) production. Samples of soil and organic wastes were collected from Olusosun dumpsite, Ojota, motor mechanic shop at Covenant University, Ota and kitchen sewage from Covenant University's Cafeteria 2. Twenty bacterial isolates were obtained and screened for the ability to utilize waste frying oil and cassava effluent as cheap carbon substrates to synthesize PHA polymers. Quantitative analysis was carried out after 72 hours by crotonic assay method using the UV spectrophotometer at 235 nm wavelength. The result showed that there is no significant difference ($p<0.05$) between the PHA accumulated when either substrates are utilized by the isolates. The quantitative analysis result for the identification of monomeric units of PHA using Gas chromatography-mass spectrometry showed four isolates with capacities to yield more PHAs than other isolates under the subjected growth condition. These four isolates selected were characterized, morphological and phylogenetic characterization results showed that all four isolates A1 (MK989593), A2 (MK989594), A4 (MK989595), A6 (MK989596) have similarity (>90%) to the *Alcaligenes* spp. respectively. The use of waste oils and cassava effluent to produce PHAs by isolates used in this study

can be considered good candidates for cheap substrates choices to further optimize PHA production industrially.

Keywords: Polyhydroxyalkanoate, Biopolymers, Crotonic acid, Waste frying oil, Cassava effluent

Introduction

Despite the wide range of application, plastics are non-biodegradable and therefore end up as waste litters with high staying power [1, 2, 3]. Synthetic plastics don't biodegrade [4], because they resist corrosion, they rather undergo fragmentation during photo-oxidation forming microplastics and nanoplastics [5, 6]. The accumulation on land creates pyramids of waste extending to the marine environment by various dispersal mechanism leading to disruption of the aquatic habitat [2, 7, 8, 9].

Putting into consideration endangered species of the marine habitat as well as a possible disruption of man's food chain through consumption of toxic sea products; there is the urgent need to reduce the impact of plastic pollution.

Polyhydroxyalkanoates (PHA) have been proposed as substitutes for petroleum-based synthetic polymers due to their biodegradability and biocompatibility [10]. It has been estimated that at least 30% or more of PHA cost is attributed to carbon, nutrients and aeration cost, thus, PHA is not cost competitive compared to fossil-derived products. The cost of synthetic plastics ranges between US$0.60-0.87/lb, while the cost of PHA production ranges between US$2.25-2.75/lb, the cost of commercially producing PHA is higher as opposed to synthetic plastics and not cost effective [11]. According to [12, 13], there is need for continuous scientific research for new innovations that would tackle the plastics menace, through exploration of microbial plastics as an alternative to the conventional synthetic plastics. Therefore, this study was aimed at utilizing cheap and sustainable carbon source to produce polyhydroxyalkanoate (PHA) as an alternative to the synthetic plastics

Materials and Methods

Sample collection

The isolates used for this research were isolated from four sources. Organisms were isolated from soils at Olusosun dumpsite, Ojota within latitude 6.591N to 6.594N and longitude3.372E to 3.377E, motor mechanic shop at Covenant University, Ota within latititude 6.673 N to 6.674N and longitude 3.162E to 3.163E and kitchen sewage from Covenant University's Cafeteria 2 within latititude 6.6735 N to 6.6736N and longitude 3.1621E to 3.1623E The carbon sources: cassava effluent, were obtained from Sango-ota market, Ogun state and waste-frying oil from Covenant University's Cafeteria 2. The soil samples were collected using a hand trowel and placed in a sterile sampling bag and then transported to the microbiology laboratory at Covenant University for further analysis.

Enrichment and Isolation of Pure Bacteria Culture

Five grams (5 g) of each sample was sieved using a sieve with a woven wire mesh and dispensed into 250 ml conical flask in triplicates containing 100 ml Minimal salt medium [10]. This was

homogenized by gently stirring to allow soil to evenly spread and mix with the medium. Aliquots of the cassava effluent and waste frying oil were added to supplement as carbon and energy source. The bioreactor was incubated in a mechanical shaker for 5 days at 30^0C. Following the end of the incubation period, 0.1 ml of the enrichment broth was pipette into nutrient agar medium by spread plate method, and then plates were incubated at 37^0C for 24 hours. The isolated colonies were sub-cultured again by streaking method on nutrient agar plates, incubated at 37^0C for 24 hours. The pure cultures obtained were maintained at 4^0C on nutrient agar slants. The cultures were maintained by sub-culturing once in 6 weeks.

PHA accumulation experiment using cheap substrates

Prior to the PHA accumulation experiment, the cells were grown in nutrient broth for 18 – 24 hrs and centrifuged afterwards at 4000 rpm for 20 minutes. Harvested cell pellets for each experiment was inoculated into 100 ml of nutrient limiting minimal salt medium in a 250 ml conical flask. 50 ml of carbon source were added to each flask. The pure isolates obtained were grown in different carbon source; in medium containing cassava effluent as excess carbon source and in medium containing waste frying oil. The culture was incubated in a mechanical shaker at 30^0C for 72 hours at 150 rpm. Aerobic conditions were constantly monitored. The optical density (600 nm) was measured at time 0 and 72 hour respectively.

Extraction of PHA

The polymer was extracted after 72 hours of culture. Extracted by treatment with several solvents as described by [14]. After 72 h of incubation at 37°C culture broth was centrifuged at 8000 rpm for 15 min. The pellet along with 10 ml sodium hypochlorite was incubated at 50°C for 1 h for lyses of cells. The cell extract obtained was centrifuged at 12000 rpm for 30 min and then washed sequentially with distilled water, acetone, and absolute ethanol. After washing, the pellet was dissolved in 10 ml chloroform (AR grade) and incubated overnight at 50°C and was evaporated at room temperature.

Quantitative analysis of PHA by crotonic assay

The extracted polymer was dissolved in 10 ml Sulphuric acid and allowed to boil at 100^0C for 10 minutes as described by [14]. In the presence of PHA, Crotonic acid would be formed afterwards. Crotonic acid gives maximum absorbance at 235 nm when measured with UV spectrophotometer. The amount of Crotonic acid was calculated from the molar extinction coefficient which is 1.55×10^4 as described by [15]. The concentration of the PHA extracted from the bacteria isolates was determined using the beer lambert law.

Data Analysis

Microsoft excel was used to calculate for T-Test for the statistically significant values. A P-value below 0.05 was considered significant.

Microscopic and Biochemical characterisation of pure isolates

Four isolates were selected based on the concentration of PHA accumulated from

the enrichment experiment. They were characterised based on cultural and morphological differences. They were analysed further using biochemical tests which includes; catalase tests, oxidase test, indole, spore test, Vogues-Proskauer, methyl red, sugar utilisation, citrate and starch hydrolysis.

Molecular characterisation of bacteria isolates

The isolates A1, A2, A4 and A6 were cultivated in nutrient broth for 24 h at 30^0C. Cells were harvested by 15 min centrifugation at 12,000 rpm and their genomic DNA was extracted using the DNA extraction protocol by [16]. The 16S rRNA gene was amplified by PCR using the universal primers for prokaryotes 907R (5'-CCGTCAATTCMTTTRAGTTT-3') and 1492R (5'TACGGYTACCTTGTTACGACTT-3'). The 16S rRNA gene sequencing using the Sanger method was carried out on ABI 3500XL Genetic Analyzer, POP7TM (ThermoScientific). The sequences were trimmed on the Geneious Prime software and compared with previously published sequences of bacterial strains using the BLAST command available on the National Center for Biotechnology Information (NCBI) database. Obtained sequences were trimmed and then compared against other sequences in the NCBI Genbank database using the BLAST program. Multiple alignments were performed using the Geneious algorithm, phylogenetic trees were built using the Neighbor Joining method [17]. All sequence analysis was conducted in Geneious 2019.1.3.

Polymer analysis using GCMS

For molecular analysis of the produced polymer, quantitative analysis was carried out on the dried extracted cell pellets. As described by [18], the cells were treated with a mixture of 0.2 ml chloroform, 1.7 ml methanol and 0.3 ml of 98% Sulphuric acid. The lower organic layer of hydroxyalkanoate (HA) was used for GC/MS analysis. The samples were injected in the splitless mode utilizing helium (He) as the carrier gas. The program cycle was at an initial temperature of 60 °C which was held for 1 min, then oven was ramped 4 °C/min to 110 °C. Oven was held for 3 mins and ramped to 260°C at 8 °C/min. Oven was then held for 5 mins and then ramped to 300°C at 10 °C/min, this was then held for 12 min. the total run time was 56.25 min. The mass spectra obtained were compared with the NIST-14 library.

Results and Discussion

Isolation of Bacteria and PHA accumulation experiment using cheap substrates Nwinyi and Owolabireported that utilizing molasses as carbon source under enrichment experiment, enabled the selection of competent organisms capable of producing PHA. In this study, the enrichment experiment was therefore used to isolate the bacterial species capable of utilizing waste frying oil and cassava effluent as the selected carbon source. From the enrichment culture, ten (10) pure bacteria isolates were isolated successfully. The optical density (OD) taken at time 0 hr and 72 hr as shown in Fig. 3.1 and Fig. 3.2 proved the organisms were able to utilize the carbon sources leading to increase biomass. During this phase, the

isolates utilised their respective carbon substrates in a nitrogen-limiting salt medium.

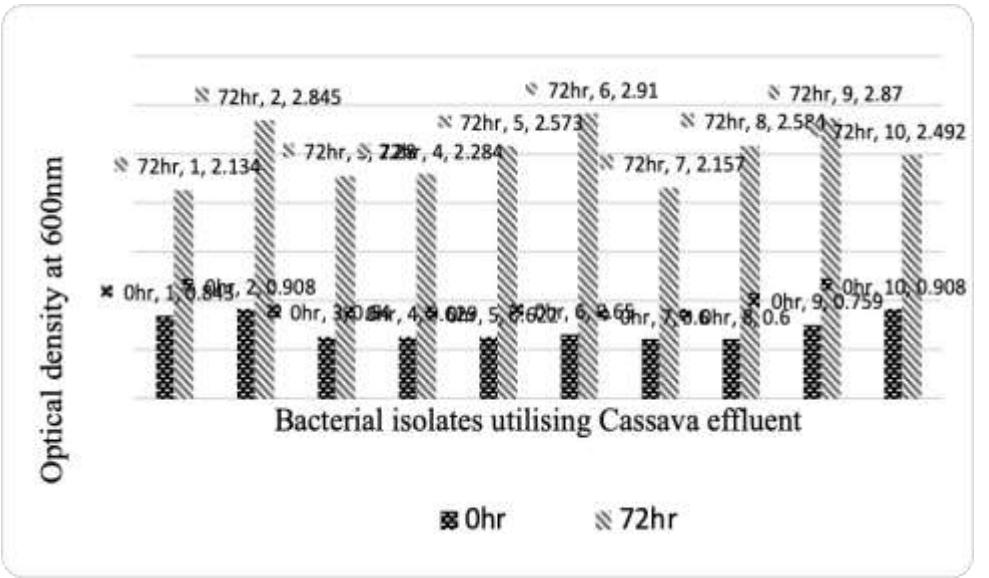

Fig 3.1: Optical densities of bacterial isolates against time measured at 600 nm during PHA accumulation phase utilizing excess cassava effluent as carbon source in a nitrogen-limiting medium

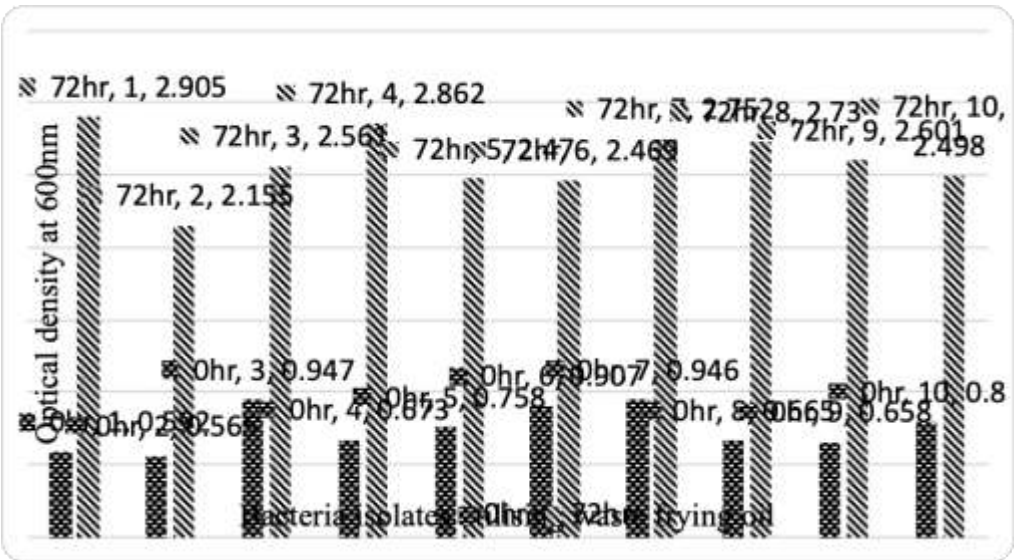

Fig 3.2: Optical densities of bacterial isolates against time during PHA accumulation phase utilizing excess waste frying oil as carbon source in a nitrogen-limiting medium

PHA extraction and quantification of PHA by Crotonic assay method

Although staining and genetic methods have been used to primarily detect the presence of intracellular PHA in organisms, they have not proven to successfully quantify PHA and give no information about the monomeric composition [19]. This study aimed at quantifying the PHA polymer after production. The Crotonic assay method has proven to be a reliable and convenient method to quantify PHAs extracted from microorganisms [15]. PHAs are converted into Crotonic acid in the presence of concentrated Sulphuric acid. Crotonic acid gave a maximum absorbance at 235 nm using the UV spectrophotometer. This procedure was described by [14]. At wavelength 235 nm, the absorbance of Crotonic acid from each isolate was read. According to beer lambert law, absorbance values are linear to the concentration. Fig 3.3 shows the different absorbance value which indicated PHA concentration.

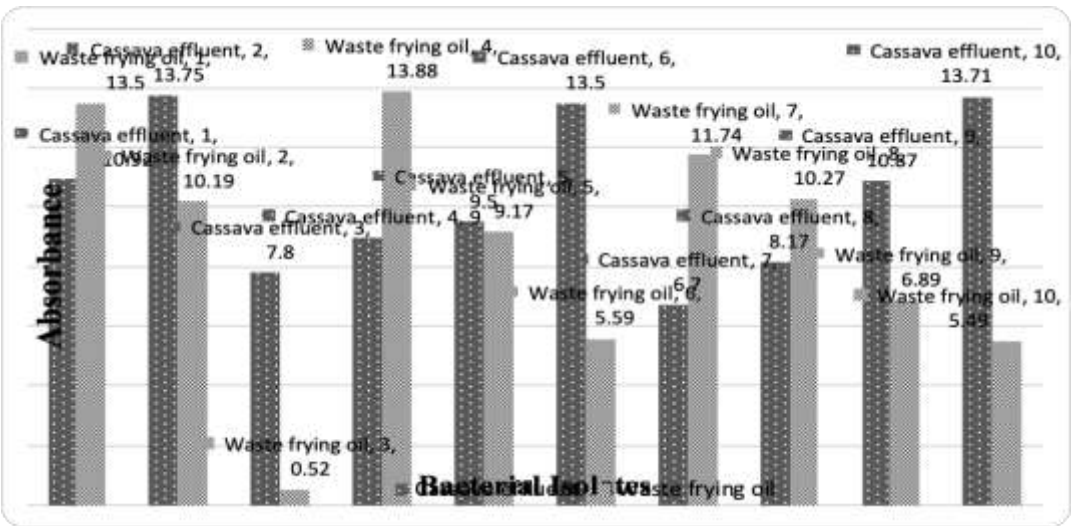

Fig. 3.3: Absorbance value taken after 72 hours from isolates grown in nitrogen limiting minimal salt medium in the presence of cassava effluent and waste frying oil as carbon source respectively

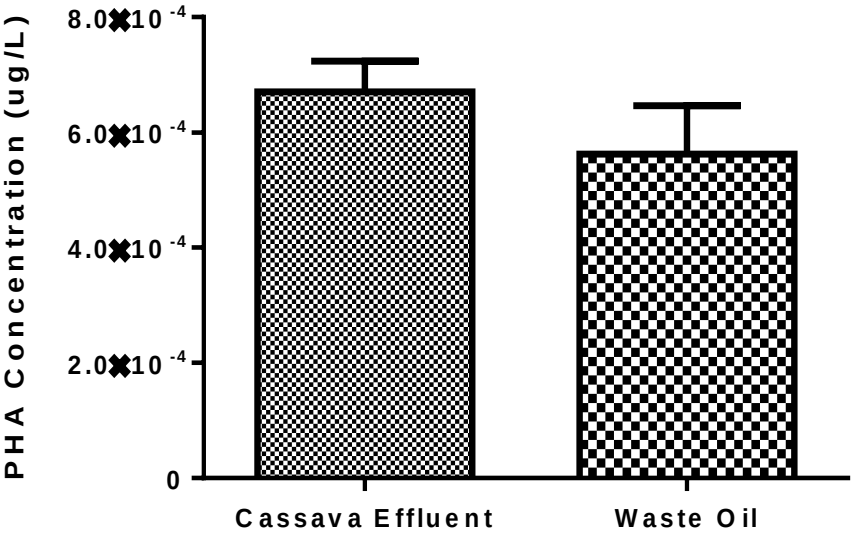

Fig 3.4: Cumulative concentration of PHA accumulated when cassava effluent and waste frying oil were used respectively as carbon source in a nitrogen-limiting medium

Data Analysis

The student t-test was used to determine the significant difference of concentrations obtained from both carbon sources. A P-value below 0.05 was considered significant. The t-test conducted on the concentration of PHA accumulated, proved that there is no significant difference ($P<0.05$) between the concentrations of PHA obtained. Hence, either carbon source can be used as one does not render an advantage over the other [20]. Fatty acid analysis conducted by [21] proved that the residual nutrients from foods present in the waste frying oil contribute to increased PHA production.

Characterisation of PHA producing isolates

From the PHA accumulation evaluation, two isolates that had the highest PHA accumulation were selected each from a utilised carbon source. The selected isolates were further characterised using biochemical tests and by microscopy. The table below shows the cultural and biochemical features of the isolates capable of yielding high amounts of PHA while utilising cassava effluent/ waste frying oil as excess carbon source. The microscopy results shown in table 3.1 shows that the isolates are Gram's negative short rods. Their ability to hydrolyze starch indicates the presence of the enzyme amylase hence their ability to utilize cassava effluent as a carbon source. Obtained sequences were trimmed and submitted to the Genbank database under the following accession numbers MK989593, MK989594, MK989595 and MK989596. The DNA sequence of the four isolates were aligned with the 16S rRNA gene of similar genes in the

NCBI database and were closely related to the Alcaligenes genus as adjudged by the phylogenetic trees in Figures 4.5 to 4.8. Sequence analysis showed that isolates Pairwise identity ranged from 96.1% to 100% for all isolates when compared with previously submitted sequences in the Genbank database. This confirms the PHA accumulation studies done on Alcaligenes spp. such as the study conducted by [22].

Table 3.1: Result showing the microscopic and biochemical characteristics of the selected bacterial isolates

CHARACTERISTICS	A1	A2	A4	A6
Gram's Reaction	-	-	-	-
Shape	Short rods	Short rods	Short rods	Short rods
Motility	Motile	Non-motile	Motile	Motile
Acid Fast Stain	-	-	-	-
Spore Stain	+	-	+	-
Citrate	+	+	+	+
	+	+	+	+
Catalase				
	-	-	-	-
Indole				
	-	-	-	-
MR	+	-	+	+
VP				
Starch Hydrolysis	+	+	+	+
Urease	-	+	-	+
	+	-	+	+
Oxidase				
	+	+	+	+
	+	-	+	-
Maltose				
Gas Production				
	+	+	+	+
Lactose	+	+	+	-
Gas Production				
	+	-	+	+
Sucrose	+	-	-	+
Gas Production				
	+	+	+	+
Growth at pH 6.0				
	+	+	+	+
Growth at 35˚C				
Growth in 5% NaCl	+	+	+	+

KEY: + indicates a positive Reaction
- indicates a negative Reaction

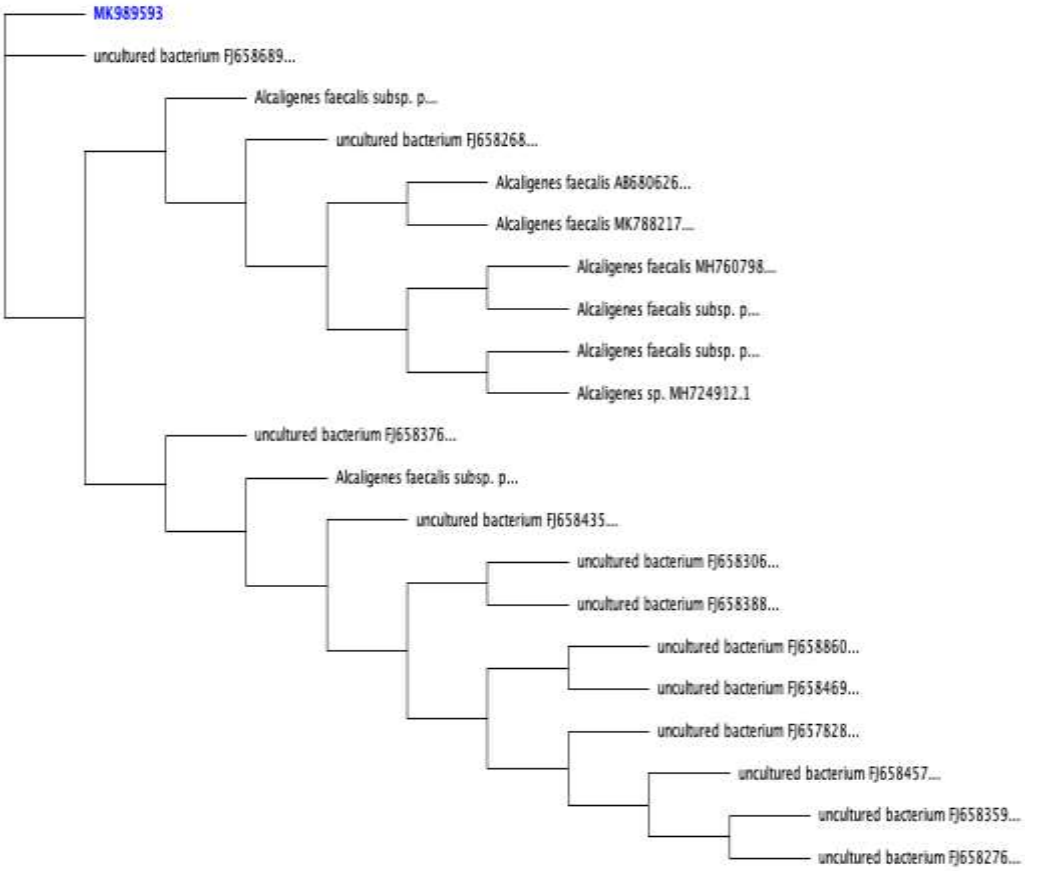

Fig 3.5: Phylogenetic tree of newly identified organism A1 with accession number MK989593 (source: Genious prime software). The DNA sequence of isolate A1 was aligned with the 16S rRNA gene of similar genes in the NCBI database and were closely related to the *Alcaligenesfaecalis* as well as other strains of *Alcaligenes faecalis* and various strains of uncultured bacterium. The genomic pairwise identity ranged from 96.1% to 100% when compared with previously submitted sequences in the Genbank database.

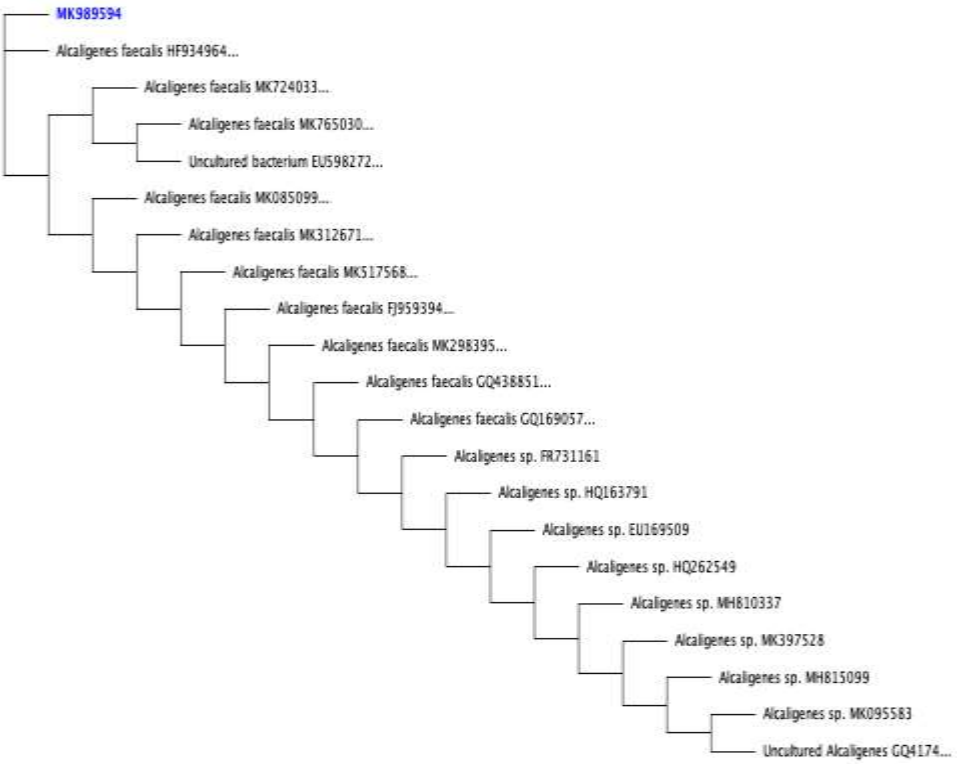

Fig 3.6: Phylogenetic tree of newly identified organism A2 with accession number MK989594 (source: Genious prime software). The DNA sequence of isolate A2 was aligned with the 16S rRNA gene of similar genes in the NCBI database and was found to be closely related to the *Alcaligenesfaecalis*, other species of *Alcaligenes* and various strains of uncultured bacterium. The genomic pairwise identity ranged from 96.1% to 100% when compared with previously submitted sequences in the Genbank database.

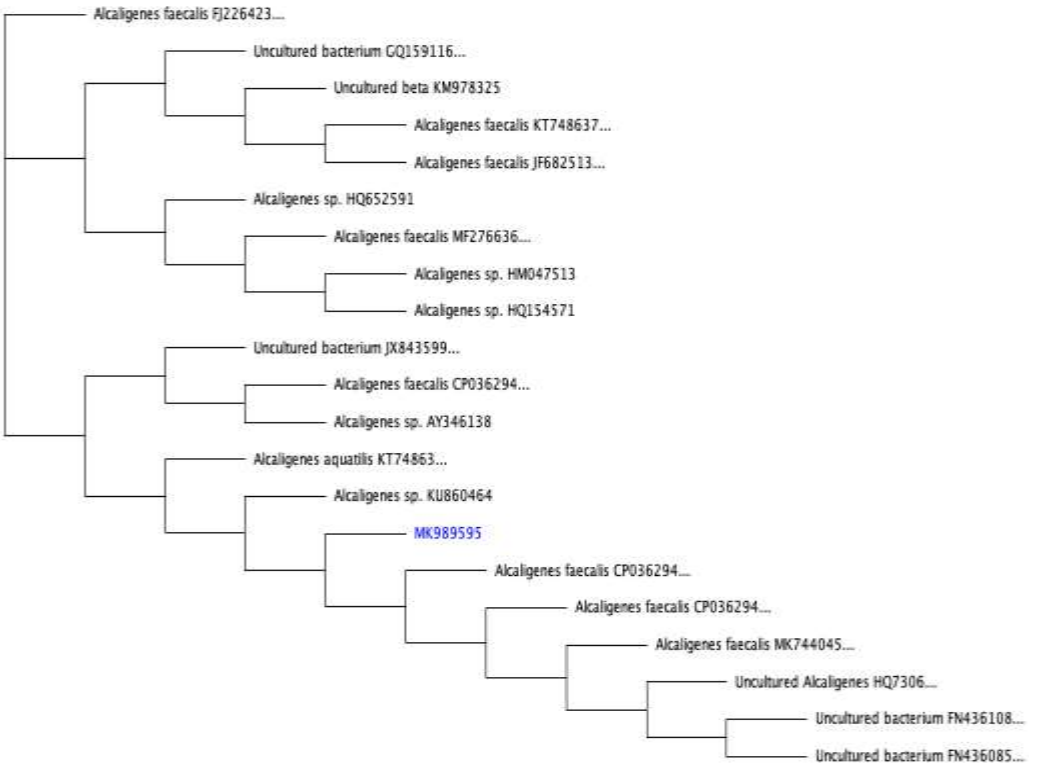

Fig 3.7: Phylogenetic tree of newly identified organism A4 with accession number MK989595 (source: Genious prime software). The DNA sequence of isolate A4 was aligned with the 16S rRNA gene of similar genes in the NCBI database and was found to be closely related to the *Alcaligenesfaecalis*, *Alcaligenesaquatilis* as well as other species of *Alcaligenes*and various strains of uncultured bacterium. The genomic pairwise identity ranged from 96.1% to 100% when compared with previously submitted sequences in the Genbank database.

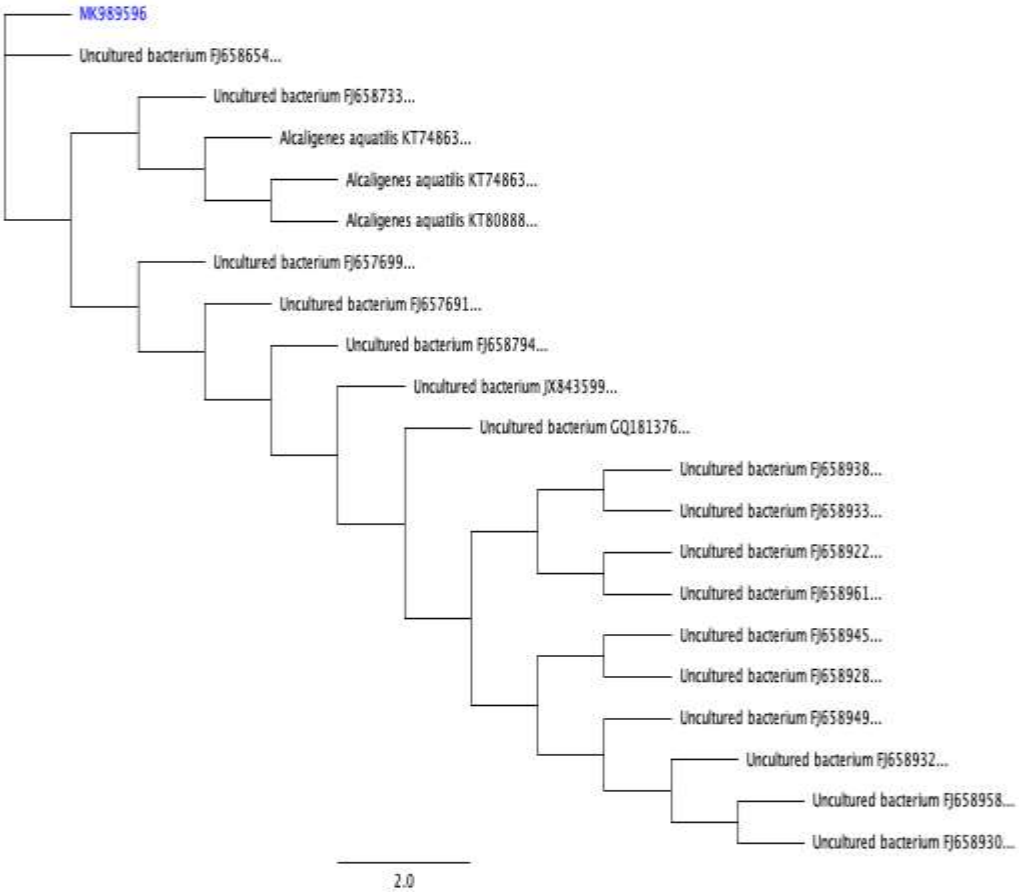

Fig 3.8: Phylogenetic tree of newly identified organism A6 with accession number MK989596 (source: Genious prime software). The DNA sequence of isolate A6 was aligned with the 16S rRNA gene of similar genes in the NCBI database and was found to be closely related to the Alcaligenesaquatilis and various strains of uncultured bacterium The genomic pairwise identity ranged from 96.1% to 100% when compared with previously submitted sequences in the Genbank database.

Polymer analysis using GCMS

The GC/MS was used to confirm the synthesis of PHA as proven by past studies [19, 23]. A study by [24] revealed the presence of phthalate produced from Bacillus spp, which is a type of plastic. Similarly, this present study revealed from the NIST-14 library of the GC/MS showed the presence of the polyhydroxyalkanoate called Methyl 2-hydroxy-heptadecanoate (Fig 3.9). It also revealed the presence of a type of plastic (phthalate) called isophthalic acid, di(6-ethyloct-3-yl) ester (Fig 3.10) and Bis(2-ethylhexyl) phthalate (Fig 3.11).

GC/MS Data File : Sample 1.D

Pk#	RT	Area%	Library/ID.	Ref#.	Cas#	Qual
94	21.372	1.60	C:\Database\NIST14.L			
			1-Docosene	167462	001599-67-3	93
			Heptacosyl acetate	253355	1000351-78-2.	83
			Methyl 2-hydroxy-heptadecanoate	159880	1000336-20-1	64
95	21.603	2.10	C:\Database\NIST14.L			
			Isophthalic acid, decyl phenyl est er	228549	1000344-36-3	35
			Terephthalic acid, decyl phenyl es ter	228553	1000324-06-8	27
			Terephthalic acid, 2-bromo-4-fluor ophenyl decyl ester.	262832	1000323-71-4	27
96	21.793	2.28	C:\Database\NIST14.L			
			3-Hydroxy-2,6,6-trimethyl-hept-4-e noic acid	52542	1000185-34-0.	46
			N-Carbethoxymethyliminomethylmalon ic acid methyl, ethyl ester	119715	1000223-03-2	44
			11-Methyl-octadecanoic acid, DMOX derivative	206508	1000336-95-2	44
97	22.122	0.94	C:\Database\NIST14.L			
			N-Carbethoxymethyliminomethylylmal onic acid dimethyl ester	106445	1000223-03-1	59
			2(1H)Naphthalenone, 3,5,6,7,8,8a-h exahydro-4,8a-dimethyl-6-(1-methyl ethenyl)-	81713	1000188-66-5	59
			1,1,3,5-Cyclohexanetetracarboxylic acid, 4-oxo-, tetramethyl ester	187606	051334-56-6	55
98	22.336	0.89	C:\Database\NIST14.L			
			N-Carbethoxymethyliminomethylylmal onic acid dimethyl ester	106445	1000223-03-1	76
			3,5-Dichloro-2',6'-difluorobipheny l-4-yl)methylamine	146702	1000306-72-7	55
			Butanedioic acid, 2,3-bis(8-nonen- 1-yl)-, dimethyl ester	235508	1000141-98-7	45

FIG 3.9: GC/MS NIST-14 library indicating the presence of Methyl 2-hydroxy-heptadecanoate

GC/MS DataFile: Sample 1.D

Pk#	RT	Area%	Library/ID.	Ref#.	Cas#	Qual
92	21.452	0.49	C:\Database\NIST14.L			
			Isophthalic acid, di(6-ethyloct-3-yl) ester	255782	1000344-68-0	50
			Isophthalic acid, decyl 3,5-dichlorophenyl ester	256586	1000356-58-9	50
			Isophthalic acid, 2-chloro-5-methylphenyl decyl ester	250799	1000356-57-2	50
93	21.551	0.05	C:\Database\NIST14.L			
			2,4,7,14-Tetramethyl-4-vinyl-tricyclo[5.4.3.0(1,8)]tetradecan-6-ol.	150240	1000193-31-2	89
			N'-(5,7-Dibromo-2-oxoindolin-3-ylidene)-2-(2-isopropyl-5-methylphenoxy)acethydrazide	267225	327033-93-2	89
			Phthalic acid, 4-trifluoromethylbenzyl ethyl ester	206891	1000371-04-1	76
94	21.689	0.64	C:\Database\NIST14.L			
			N'-(5,7-Dibromo-2-oxoindolin-3-ylidene)-2-(2-isopropyl-5-methylphenoxy)acethydrazide	267225	327033-93-2	86
			2-t-Butyl-6-[2-hydroxy-2-(2,4,6-trimethylphenyl)ethyl]-[1,3]dioxin-4-one	177033	1000192-88-0	64
			Phthalic acid, decyl 3-iodobenzyl ester	268733	1000378-07-4	64
95	22.024	0.51	C:\Database\NIST14.L			
			Cyclopentanecarboxamide, 3-ethenyl-2-(3-pentenylidene)-N-phenyl-, [1.alpha.,2Z(E),3.alpha.]-	141080	136091-23-1	56
			Furosemide tri-methyl derivative	221810	1000137-05-3	45
			1,2-Bis[4-quinolyl]ethene N-oxide	157964	1000211-32-3	44
96	22.163	0.48	C:\Database\NIST14.L			
			Cyclopentanecarboxamide, 3-ethenyl-2-(3-pentenylidene)-N-phenyl-, [1.alpha.,2Z(E),3.alpha.]-	141080	136091-23-1	90
			Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	170048	1000192-89-2	64
			2,2-Dimethylpropanoic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester	111719	1000299-33-6	64

Fig 3.10: GC/MS NIST-14 library indicating the presence of isophthalic acid, di(6-ethyloct-3-yl) ester

5/13/2019 GC/MS DataFile: Sample 6.D

79	19.500	0.12	C:\Database\NIST14.L		
			Nonadecane	128835	000629-92-5 95
			Hexacosane	217890	000630-01-3 94
			Heptadecane, 9-hexyl-	182660	055124-79-3 91
80	19.853	0.04	C:\Database\NIST14.L		
			Bis(2-ethylhexyl) phthalate	233372	000117-81-7 68
			Diisooctyl phthalate	233366	000131-20-4 64
			Phthalic acid, di(2-propylpentyl) ester	233419	1000377-93-5 58
81	20.141	0.15	C:\Database\NIST14.L		
			Eicosane	142238	000112-95-8 98
			Nonadecane	128835	000629-92-5 95
			Hexacosane	217890	000630-01-3 94
82	20.592	0.01	C:\Database\NIST14.L		
			Octacosane	235614	000630-02-4 90
			Friedelan-3-one	249550	000559-74-0 59
			1-Hexacosene	216562	018835-33-1 59
83	20.759	0.11	C:\Database\NIST14.L		
			Eicosane	142238	000112-95-8 98
			Nonadecane	128835	000629-92-5 95
			Heptadecane	102600	000629-78-7 94
84	20.852	0.01	C:\Database\NIST14.L		
			Androst-5,15-dien-3ol acetate	173513	1000251-88-0 38
			Pregna-5,16-dien-20-one, 3-hydroxy -, (3.beta.)-	173537	001162-53-4 38
			Benzoic acid, 4-(1,3-dioxolan-2-yl)-, methyl ester	72031	142651-24-9 35
85	21.135	0.02	C:\Database\NIST14.L		
			17-Pentatriacontene	265112	006971-40-0 90
			Octacosane	235614	000630-02-4 59
			Eicosane	142238	000112-95-8 58
86	21.354	0.13	C:\Database\NIST14.L		
			Eicosane	142238	000112-95-8 98
			Octacosane	235615	000630-02-4 98
			Heptadecane, 9-octyl-	207503	007225-64-1 95
87	21.556	0.03	C:\Database\NIST14.L		
			2,4,4-Trimethyl-3-hydroxymethyl-5a -(3-methyl-but-2-enyl)-cyclohexene	85797	1000144-10-5 40
			trans-Sesquisabinene hydrate	85739	145512-84-1 38
			Cyclopropanecarboxylic acid, hydra zide, N2-cyclooctylideno-	72387	1000264-07-5 38

Fig 3.11: GC/MS NIST-14 library indicating the presence of Bis(2-ethylhexyl) phthalate

Conclusion

From this study, organisms isolated from sites with high organic content, showed ability for PHA production on pilot scale. Isolates A1, A2, A4 and A6 were identified as *Alcaligenes* spp. and were the high PHA yielders. Hence, this study also paves way for further studies on the newly characterized species and for commercialization of PHA using waste frying oil and cassava effluents as substrates. Understanding the biosynthetic pathways of these organisms to producing PHAs and further manipulating the pathways would be key to achieving low and competitive production cost, as well as product design of the desired polymer. Other cheap substrates should be explored using these already characterised PHA producing strains.

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