

## Original Research Article

# Genetic and phenotypic characterization of Polyhydroxyalkanoate producing bacterial isolates from hypersaline water body, Atlantic Ocean, Nigeria

Comment [H1]: Phenotypic and genetic

### Abstract

**Aim:** To compare bacteria identification methods of phenotypic and genetic identification for marine sourced effective polyhydroxyalkanoate (PHA) production.

Comment [H2]: Give a brief introduction

**Study design:** Randomised design was employed for PHA producing bacterial isolates.

**Place and duration of study:** Samples containing PHA-producing bacteria from the hypersaline water body of Nigerian Southern Atlantic Ocean (6.35° N 3.28° E -St1; 6.35° N 3.40° E -St2; and 6.36° N 3.47° E -St3); water depths (0.07m (top), 50m (middle) and 100m (bottom) were collected and investigated between August, 2016-September, 2019.

**Methodology:** Bacterial isolates was characterized using standard microbiological biochemical tests for the phenotypes and 16S rDNA for the genotypes. Isolates were also screened for PHA potentials using specific primers.

**Results:** Genetically identified *Bacillus cereus* strains LB17 and *Bacillus paramycooides* SA27 were phenotypically identified as *Bacillus badius*. Similarly, *Acinetobacter calcoaceticus* JL11, five strains of *Alcaligenes faecalis*, seven strains of *Bacillus* spp., *Enterobacter cloacae*, *Falsoleobacterium*, *Ochrobactrum ciceri*, *Providencia stuartii* including two species of *Pseudomonas* and *Bordetella pertussis* were all phenotypically identified as *Corynebacterium kutscheri*. Invariably, *Alcaligenes faecalis* (strains PSD10 and DEP8), *Pseudomonas aeruginosa* (H47921) and *Vagococcus fluvialis* (AWW1) were identified as *Staphylococcus* species. All 34 bacterial isolates expressed *phaC* genes for PHA potential, while *Alcaligenes faecalis* strains and *Bacillus humi* (NBPP9) expressed *phaC1* for short PHA chain.

**Conclusion:** Variations were recorded in the genetical identity of phenotypes; *Corynebacterium kutscheri*, *Enterobacter amnigenus*, *Micrococcus luteus*, *Micrococcus varians*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. while, synchronization recorded in the genotypic and phenotypic identity of bacillus species. Varietal differences of PHA potential from the same species are also seen. It is therefore pertinent to rely on the genetic identification of bacteria for the effective determination of PHA-producing character as displayed in *Bacillus* spp.

Comment [H3]: Rephrase to convey meaning

**Keywords:** 16S rDNA, bacteria-phenotype, hypersaline-water, Polyhydroxyalkanoates, Nigeria.

### Introduction

Polyhydroxyalkanoates (PHAs) are produced in nature through the fermentation of sugar or lipids. Living cells may store them as carbon or expend as utilized energy, they are also biocompatible and completely biodegradable (Albuquerque & Malafaia, 2018; Brigham *et al.*, 2011; Sun, 2018). PHAs exhibit bio-plasticity, thermo-plasticity or elastomer material characters with a temperature range between 140°C to 180°C (Bugnicourt *et al.*, 2014). There are over 150 monomers with the potential to combine within this family that results to materials of extremely different properties (Bugnicourt *et al.*, 2014; Dhandayuthapani *et al.*, 2011; Valappil *et al.*, 2007). However, organisms such as bacteria are

specific to the type of PHA they synthesize (Hawkinset *et al.*, 2021; Jendrossek & Pfeiffer, 2014; Steinbüchel & Lütke-Eversloh, 2003).

The preference for bioplastic over synthetic plastics is hinged on the fact that the latter is non-biodegradable with disposal methods of burning which results in air pollution through the emission of toxic fumes that are inimical to human and animal health. Consequently, this action results in the disruption of the ecosystems and eventual global warming (Bagheriasl, 2013). However, biopolymers (PHA) will not contribute to environmental pollution at the end of their use when recycled due to their elastomeric property (Margesin & Schinner, 2001; Sriroth & Sangseethong, 2005; Numata *et al.*, 2009). These characteristics make them superior to synthetic plastics. Thus, the present extensive use of plastics today is based on their durability which is pivoted on their thermal and mechanical properties. Plastics are malleable at certain temperatures and so are rapidly replacing metals, glass and wood in various engineering applications (Nawrathet *et al.*, 1994). PHAs are of immense importance in the applications of medicine, engineering, agriculture, telecommunications, transportation and household utilities (Aditi *et al.*, 2015; Zhang *et al.*, 2015; Manikandan & Senthilkumar, 2017).

The categories of PHAs are Short Chain Length (SCL) 3-5 carbons (R)-hydroxyalkanoates and characterised by their high degree of crystallization and stiff crystals thermoplastic (Kolleret *et al.*, 2013; Wu *et al.*, 2003); Medium Chain Length (MCL) are 6-14 carbon polyhydroxyalkanoates with low degree of crystallization, hydrophobicity, low melting temperature and biodegradable (Ashbyet *et al.*, 2002; Solaiman *et al.*, 2006); Short Chain Length copolymers with medium chain length (SCL-MCL - Copolymers) are 4 to 14-carbon monomers, and characterized by a range of physical properties that are based on the percentage molar composition of monomers that make up the polymer. Hence copolymers having a low percentage of monomers of SCL are more elastomeric (Greenet *et al.*, 2002; Gumel *et al.*, 2012; McChalicher & Srienc, 2007; Tsuge *et al.*, 2005). There are other complex categories PHA that differ from the aforementioned such as low chain length-medium chain length PHAs (LCL-MCLPHA), LCL-PHAs (copolymers produced by *Pseudomonas aeruginosa*) of SCL-PHAs, copolymers of SCL-MCL and SCL-LCL-PHAs (Saranya *et al.*, 2017).

The survival and optimal growth of halophilic bacteria in their environments necessitate the production of one or more PHAs, enzymes, metabolites, exo-polysaccharides, endo-polysaccharides, pigments and compatible solutes (Jayet *et al.*, 2008; Rothschild & Mancinelli, 2001; Krulwichet *et al.*, 2011; Mesbah & Wiegel, 2012) of which their products may be of high commercial value. Also, the bio-molecules produced by halophilic bacteria are stable (Oren, 2013); therefore, their metabolites exhibit significant potential in the needs or actual use by different industries such as agriculture, cosmetic, chemical, environmental, pharmaceutical, etc. Unfortunately, there have been reported cases of wrong or mis-identification of bacteria obtained from the extreme environment (Abdulaziz, 2013; Bryan-Thomaset *et al.*, 2018; Sharaha *et al.*, 2019), of which is noticed to be associated with their peculiar isolation requirement as well as their genetic response to their environment. In recent times man has improved in his genetic technological skills as compared with the former traditionally (phenotypic) identification methods. However, this research is charged with the characterisation of PHA-producing bacteria into their proper nomenclature by comparing the genetic relatedness and phenotypic (biochemical) techniques. Therefore, the expected results will help to determine and ease the search for environmentally-compatible alternatives to petrochemical-based plastics in extreme hypersaline environments.

## Methodology

### Sample collection

Water samples from Lagos State, Nigeria marine water bodies were collected aseptically by modified sampling methods (Bugnicourt *et al.*, 2014). This was done with the aid of a water sampler, and at three different depths of the surface of the water (0-7cm), middle depth (50M) and bottom depth (100M) below the water surface. The points of the water collections were obtained and recorded from the Geographic Positioning System (GPS) as locations of (1) latitude 6.35°, longitude 3.28°; (2) latitude 6.35°, longitude 3.40°; and (3) latitude 6.36°, longitude 3.47°. The aseptically collected water was transferred into a 1-litre plastic container; the temperature was taken in situ before storage for transportation in a cooled box to the laboratory for further laboratory analysis.

### Culture and isolation of bacteria

The Plate Count Agar (PCA) medium was prepared according to the manufacturer's directions (Lab M) and poured into Petri plates, allowed to solidify while the sampled water was serially diluted from  $10^{-1}$  to  $10^{-7}$  and then cultured by spread plate method on the prepared agar plates; incubated at 35 °C within 24-48 hour.

### Enumeration and isolation of pure cultures from samples

The standard enumeration method was employed for the determination of microbial load. Bacteria with the same cultural characteristics were isolated and cultured on PCA by considering the following parameters of each growth colony which are; margin, smooth or entire of the surface, wavy, lobate, irregular, ciliate, branching, woolly, threadlike, 'hair look-like, elevations, flat, raised, convex, drop like umbonate, in-growing into the medium.

### Phenotypic identification

#### Identification of Bacterial isolates

The phenotypic identification was carried out according to the methods of Bergey's Manual of Determinative Bacteriology (Bergey *et al.*, 1939) which includes;

**Cultural characterization:** This was determined by a process known as plate reading by visual observation of a standard chart and observation with X10 hand lens on isolate culture plate to determine colony shape.

**Cellular characterization:** The cellular characterization was carried out by microscopy of simple Gram's stain preparations (Carter & Cole Jr, 2012) to determine the cellular structures and arrangements.

**Biochemical tests:** these were carried out by spore staining (Perez *et al.*, 2011), acid-fast staining (Koh *et al.*, 2003); catalase (Reiner, 2010), oxidase, citrate (Drahota *et al.*, 2004), nitrate reduction (Bhusal & Muriana, 2021), strict anaerobic (Dione *et al.*, 2015), starch hydrolysis (Agama-Acevedo, Islas-Hernández, Pacheco-Vargas, Osorio-Díaz, & Bello-Pérez, 2012), sugar fermentation (Barrett & Gibson, 2012), Vogues Prokauer (VP) (Cappuccino & Sherman, 2005) and 6.5% sodium chloride assay (Zhang *et al.*, 2021).

#### Deoxyribonucleic Acid (DNA) extraction

Quick DNA Fungi/Bacterial Mini prep kit was used for the DNA extraction according to the manufacturer's instructions. The isolates were grown overnight in plate count agar (PCA) medium prior to the DNA extraction.

#### Polyclonal Chain Reaction (PCR) Analyses

PCR technique was used to amplify the conserved region of the 16S rRNA gene using specific 16S rRNA Forward primer (5'AGAGTTTGATCCTGGCTCAG3') and 16S rRNA Reverse primer (5'ACGGCTACCTTGTACGACTT3'). The PCR mix for this analysis comprises of 1 µL of 10X PCR buffer, 0.4 µL of 50mM MgCl<sub>2</sub>, 0.5 µL of 2.5mM dNTPs 0.5 µL each of 5mM 16s rRNA Forward and reverse primers 0.05 µL of 5 units/µL of Taq polymerase enzyme and 5.05 µL of distilled water. The PCR mix was made up to 10 µL final volume with 2 µL of the DNA template. The MJ Research (PTC-200) thermal cycler was set to 30 cycles of 94°C for 60 seconds, 72 °C for 120 seconds and a final extension temperature of 72 °C for 5 minutes and 10 °C hold.

#### Gel Electrophoresis

Agarose gel electrophoresis was used to resolve the amplified fragments of the DNA using 1% agarose solution. Five microliters (5 µL) of each sample were resolved at 80V for 2 hours and viewed under UV trans illumination for appropriate documentation.

#### Genes encoding the synthesis of PHAs by halophilic bacteria

The multiplex PCR technique was used to determine the PHA classes of the isolates. The three primers specific for the 3 classes (*phaC*, *phaC1* and *phaC2*) of PHAs were used (Table 1). Primer sequence for the *phaC* gene was derived from *B. megaterium*, and *phaC1* primer was obtained from *Pseudomonas aeruginosa* (Conte *et al.*, 2006; Hoffmann & Rehm, 2004; Nayak *et al.*, 2013) while, *phaC2* primer was obtained from *Pseudomonas putida* (Sharma, *et al.*, 2017).

Each laboratory identification number of the pure bacterial isolates was maintained and assayed for PHA-producing potential using the primers as shown in table 1.

#### Multiplex PCR analysis

Multiplex PCR analysis was performed by preparing PCR master mix comprising 1 µL of 10X concentration PCR buffer mix, 0.4 µL of 50 mM of MgCl<sub>2</sub>, 0.5 µL of 2.5mM dNTPs, 0.5 µL of each of the forward and reverse *phaC*, *phaC1* and *phaC2* 100 µM primers (table 1) and 2 µL of 5 unit/µL of Taq polymerase before 1.1 µL of ultra-pure water and 2 µL of template DNA added. The preparation was prepared in PCR tubes and subjected to Thermocycler Model MJ Research (PTC-200) analysis using the following PCR conditions. The PCR condition used is initial denaturation temperature of 92°C for 3 minutes, followed by 25

Table 1: Sequence of Polyhydroxyalkanoate (PHA) primers

S/No.	Primer Name	Reaction	sequence	Base number
1	<i>phaC</i>	forward	3'CGTGCAAGAGTGGGAAAAT5'	20

2	<i>phaC</i>	reverse	5'TCGCAATATGATCACGGCTA3'	20
3	<i>phaC1</i>	forward	3'GGAGCGTCGTAGATGAGTAACAAGAA5'	26
4	<i>phaC1</i>	reverse	5'AGGTTGGCGCCGATGCCGTTGAA3'	23
5	<i>phaC2</i>	forward	3'TGCTGGCCTGGCGCATTCCCAA5'	22
6	<i>phaC2</i>	reverse	5'AAGTGGTAGTAGAGGTTGCC3'	20

cycles of 92°C for 60 seconds, 57 °C for 180 seconds and a final extension temperature of 57 °C for 5 minutes and the 10 °C hold.

#### Gel electrophoresis

Three (3) microliters of each of the amplicons obtained from the multiplex analysis were mixed with 2 µL of 1X working concentration loading dye and then loaded into the wells created by the comb on the already prepared agarose gel. In addition, 3 µL of 1kbp ladder was also loaded into the first which acts as the molecular marker. The samples were electrophoresed at 80V for 2 hours after which the gel documentation unit was employed for viewing and photography in the presence of a UV light source embedded in the unit.

#### Gene Sequencing for Bacteria identification

##### Purification of PCR products for sequencing

The amplicons for each isolate were purified using 2M Sodium Acetate wash techniques (Oberacker *et al.*, 2019). Briefly, 10 µl of the PCR product was added to 1µl of 2M NaAct pH 5.2 and 20 µl absolute ethanol and incubated at 4 °C for 1 hr. The solution was centrifuged at 10,000 rpm for 10 minutes and washed with 70% ethanol and air dried. Then pellet formed after drying was re-suspended in 5 µl distilled water and stored at 4°C for sequencing.

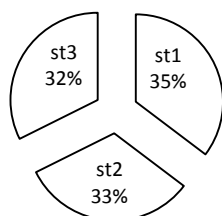
##### Preparation of sample for Gene Sequencer (ABI 3130xl machine)

The 10ulcocktail mix is a combination of 9 µl of Hi Di Formamide with 1µl of purified sequence. The samples were loaded on the genetic analyser and the chromatogram with the sequence was released(Pascucci *et al.*, 2014).

##### Nucleotide blast

A basic local alignment search tool (BLAST) program was used to align the nucleotide sequence with other sequences available on the sequence data bank of the National Centre for Biotechnology Information (NCBI) (Fleminger & Goldacre)) web site at <https://www.ncbi.nlm.nih.gov>, and the corresponding bacteria identity or similarity match for each set of sequences were recorded. Similarity percentage and accession number were also documented.

## Results



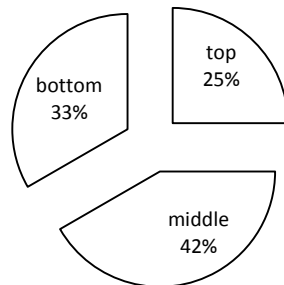
**Figure 1: Combined PHA amplified genes distribution from Bacterial obtained from Nigerian Southern Atlantic Ocean**

#### Keys

St1=6.35° N 3.28°E; st2=6.35° N 3.40° E; and st3=6.36° N 3.47° E

The combined distribution of PHA encoding gene amplification obtained from 3 different locations of st1 (6.35°N 3.28°E), st2 (6.35° N 3.40° E) and st3 (6.36° N 3.47° E) of Nigerian Southern Atlantic Ocean is shown in Figure 1. At locations st1, st2 and st3, 35%, 33% and 32% amplified genes that encode for PHA synthesis were recorded respectively. But in figure 2, at st1, 25%, 42% and 33% amplified genes that encode for PHA synthesis were recorded at the top (0.07 m below the water surface), middle (50 m below the water surface) and bottom (100 m below the water surface) respectively. While in figure 3, at st2, 46%, 36% and 18% were recorded for top, middle and bottom

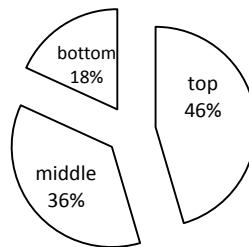
respectively. Also figure 4, at st3 shows 46%, 18% and 36% distribution records at the top, middle and bottom respectively.



**Figure 2: PHA amplified genes distribution from Bacterial obtained at 6.35° N 3.28° E (St1) location of the Nigerian Southern Atlantic Ocean**

Keys:

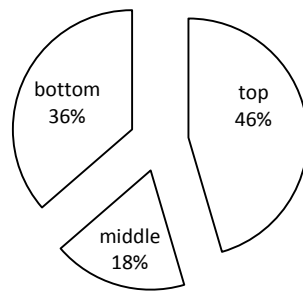
top= 0.07 m below water surface; middle= 50 m below water surface; and bottom= 100 m below water surface



**Figure 3: PHA amplified genes distribution from Bacterial obtained at 6.35° N 3.40° E (st2) location of the Nigerian Southern Atlantic Ocean**

Keys:

top= 0.07 m below water surface; middle= 50 m below water surface; and bottom= 100 m below water surface



**Figure 4: PHA amplified genes distribution from Bacterial obtained at 6.36° N 3.47° E (st3) location of the Nigerian Southern Atlantic Ocean**

Keys:

top= 0.07 m below water surface; middle= 50 m below water surface; and bottom= 100 m below water surface

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Table 2 (contd.): Assays for Phenotypic identification of Bacterial isolates from hypersaline water body of Lagos, Nigeria.

Characteristics Biochemical Tests (contd.)	← Isolates →																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sorbitol fermentation																		-ve
Motility									+ve				-ve					
Indole								-ve				-ve						
Lysin decarboxylate																		
Pigmentation									+ve									
Urease									-ve								-ve	
Ornithine decarboxylase																	-ve	
Hydrogen sulphide																		
Coagulase									-ve				-ve					
Probable bacteria	<i>B. badius</i>	<i>B. megaterium</i>	<i>B. maquariensis</i>	<i>L. delbrueckii</i>	<i>M. varians</i>	<i>M. luteus</i>	<i>M. smegmatis</i>	<i>M. delbrueckii</i>	<i>S. marcescens</i>	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. saprophyticus</i>	<i>K. Pneumoniae subsp pneumoniae</i>	<i>N. veillonella</i>	<i>E. intermedius</i>	<i>Y. pestis</i>	<i>C. kutscheri</i>	<i>E. amnogenus</i>

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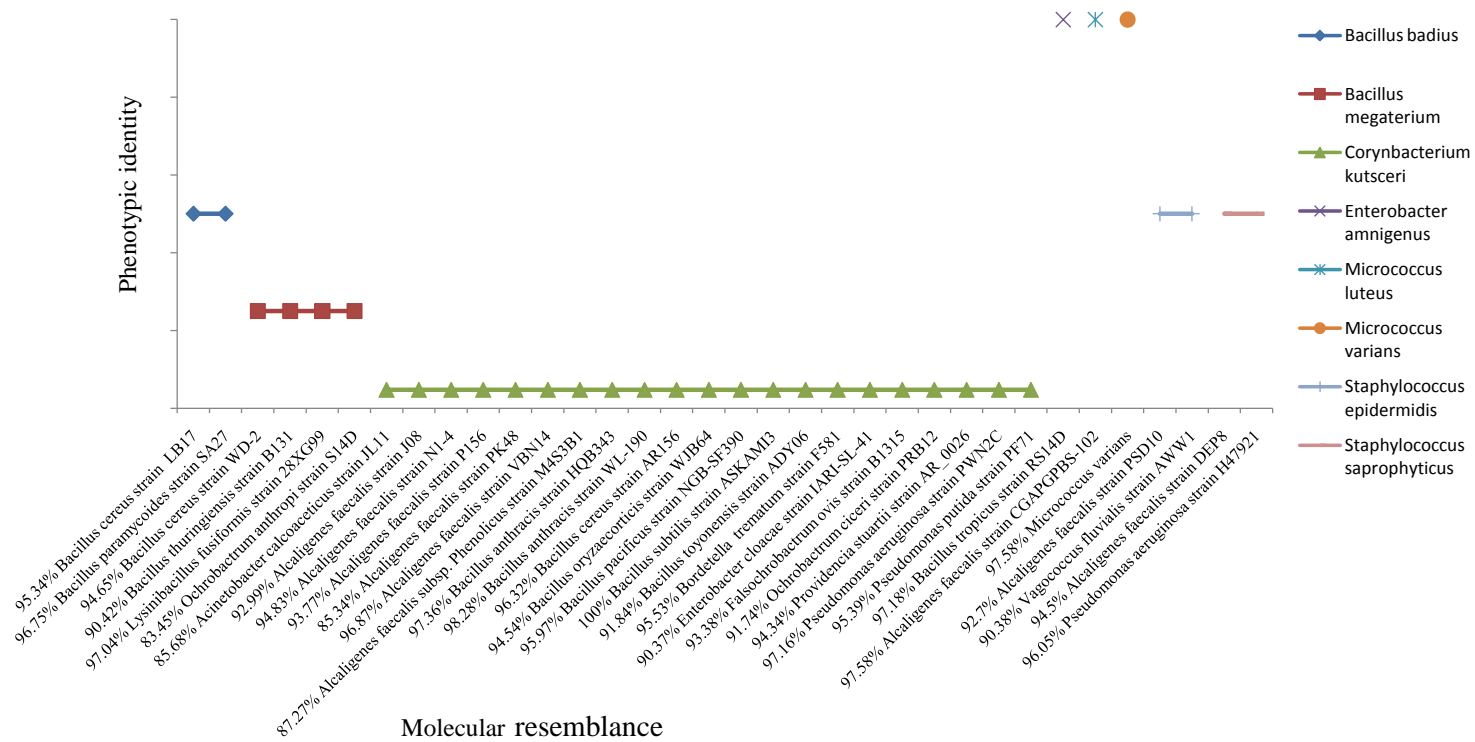


Figure 5: Comparison of Phenotypic and molecular resemblance of Bacterial isolates

**Table 3a:PHA gene potentials in bacteria obtained from Molecular and Microbiological identity assays in Nigeria hypersaline water.**

S/N	Lab No.	No. of Nucleotides	Phenotypic resemblance identity	Molecular identity resemblance	Level of Molecular resemblance	Accession No. resemblance	<i>phaC</i> status	<i>phaC1</i> status	<i>phaC2</i> status
1	102	1129	<i>Bacillus badius</i>	<i>Bacillus cereus</i> strain LB17	95.34%	MN087786.1	+ve	-ve	-ve
2	81	1107	<i>Bacillus badius</i>	<i>Bacillus paramycooides</i> strain SA27	96.75%	MN467588.1	+ve	-ve	-ve
3	163	1083	<i>Bacillus megaterium</i>	<i>Bacillus cereus</i> strain WD-2	94.65%	KJ526821.1	+ve	-ve	-ve
4	93	1076	<i>Bacillus megaterium</i>	<i>Bacillus thuringiensis</i> strain B131	90.42%	KX023374.1	+ve	-ve	-ve
5	166	1075	<i>Bacillus megaterium</i>	<i>Lysinibacillus fusiformis</i> strain 28XG99	97.04%	FJ174606.1	+ve	-ve	-ve
6	189	1151	<i>Bacillus megaterium</i>	<i>Ochrobactrum anthropi</i> strain S14D	83.45%	KT337520.1	+ve	-ve	-ve
7	187a	1063	<i>Corynebacterium kutscheri</i>	<i>Acinetobacter calcoaceticus</i> strain JL11	85.68%	EU418714.1	+ve	-ve	-ve

**Table 3b:PHA gene potentials in bacteria obtained from Molecular and Microbiological identity assays in Nigeria hypersaline water.**

S/N	Lab No.	No. of Nucleotides	Phenotypic resemblance identity	Molecular identity resemblance	Level of Molecular resemblance	Accession No. resemblance	<i>phaC</i> status	<i>phaC1</i> status	<i>phaC2</i> status
8	187b	1202	<i>Corynebacterium kutscheri</i>	<i>Alcaligenesfaecalis</i> strain J08	92.99%	GQ426313.1	+ve	-ve	-ve
9	89	1132	<i>Corynebacterium kutscheri</i>	<i>Alcaligenesfaecalis</i> strain N1-4	94.83%	MK972333.1	+ve	+ve	-ve
10	38	1171	<i>Corynebacterium kutscheri</i>	<i>Alcaligenesfaecalis</i> strain P156	93.77%	CP021079.1	+ve	-ve	-ve
11	190	1174	<i>Corynebacterium kutscheri</i>	<i>Alcaligenesfaecalis</i> strain PK48	85.34%	KU245532.1	+ve	+ve	-ve
12	130	1124	<i>Corynebacterium kutscheri</i>	<i>Alcaligenesfaecalis</i> strain VBN14	96.87%	MG027661.1	+ve	-ve	-ve
13	69	1171	<i>Corynebacterium kutscheri</i>	<i>Alcaligenesfaecalis</i> subsp. <i>Phenolicus</i> strain M4S3B1	87.27%	MH470268.1	+ve	+ve	-ve
14	80	1135	<i>Corynebacterium kutscheri</i>	<i>Bacillusanthracis</i> strain nHQB343	97.36%	KT758502.1	+ve	-ve	-ve
15	57	1156	<i>Corynebacterium kutscheri</i>	<i>Bacillusanthracis</i> strain n WL-190	98.28%	KJ210666.1	+ve	-ve	-ve
16	115	1125	<i>Corynebacterium kutscheri</i>	<i>Bacillus cereus</i> strain AR156	96.32%	CP015589.1	+ve	-ve	-ve
17	84	1086	<i>Corynebacterium kutscheri</i>	<i>Bacillusoryzaecorticis</i> strain WJB64	94.54%	KU877643.1	+ve	-ve	-ve
18	37	1145	<i>Corynebacterium kutscheri</i>	<i>Bacillus pacificus</i> strain NGB-SF390	95.97%	MK318260.1	+ve	-ve	-ve

Table 3c:PHA gene potentials in bacteria obtained from Molecular and Microbiological identity assays in Nigeria hypersaline water.

S/N	Lab No.	No. of Nucleotides	Phenotypic resemblance identity	Molecular identity resemblance	Level of Molecular resemblance	Accession No. resemblance	<i>phaC</i> status	<i>phaC1</i> status	<i>phaC2</i> status
19	137	1120	<i>Corynebacterium kutscheri</i>	<i>Bacillus subtilis</i> strain ASKAMI3	100.00%	FJ430566.1	+ve	-ve	-ve
20	129	1168	<i>Corynebacterium kutscheri</i>	<i>Bacillus toyonensis</i> strain ADY06	91.84%	MH084795.1	+ve	-ve	-ve
21	40	1081	<i>Corynebacterium kutscheri</i>	<i>Bordetella trematum</i> strain F581	95.53%	CP016340.1	+ve	-ve	-ve
22	33	1175	<i>Corynebacterium kutscheri</i>	<i>Enterobacter cloacae</i> strain IARI-SL-41	90.37%	JX645222.1	+ve	-ve	-ve
23	158	1134	<i>Corynebacterium kutscheri</i>	<i>Falschrochromovis</i> strain B1315	93.38%	NR_135736.1	+ve	-ve	-ve
24	68	1158	<i>Corynebacterium kutscheri</i>	<i>Ochrochromovis</i> strain PRB12	91.74%	MH685438.1	+ve	-ve	-ve
25	31	1150	<i>Corynebacterium kutscheri</i>	<i>Providencia stuartii</i> strain AR_0026	94.34%	CP026704.1	+ve	-ve	-ve
26	168	1163	<i>Corynebacterium kutscheri</i>	<i>Pseudomonas aeruginosa</i> strain PWN2C	97.16%	MK026852.1	+ve	-ve	-ve
27	45	1132	<i>Corynebacterium kutscheri</i>	<i>Pseudomonas putida</i> strain PF71	95.39%	MF838694.1	+ve	-ve	-ve
28	94	1135	<i>Enterobacter amnigenus</i>	<i>Bacillus tropicus</i> strain RS14D	97.18%	MK359039.1	+ve	-ve	-ve

Table 3d:PHA gene potentials in bacteria obtained from Molecular and Microbiological identities assays in Nigeria hypersaline water.

S/N	Lab No.	No. of Nucleotides	Phenotypic resemblance identity	Molecular identity resemblance	Level of Molecular resemblance	Accession No. resemblance	<i>phaC</i> status	<i>phaC1</i> status	<i>phaC2</i> status
29	96	1153	<i>Micrococcus luteus</i>	<i>Alcaligenes faecalis</i> strain CGAPGPBS-102	97.58%	KY495219.1	+ve	-ve	-ve
30	117	1117	<i>Micrococcus varians</i>	<i>Bacillus thuringiensis</i> strain NBPP9	88.4%	EM_PRO:FJ973539	+ve	+ve	-ve
31	73	1147	<i>Staphylococcus epidermidis</i>	<i>Alcaligenes faecalis</i> strain PSD10	92.70%	KP835577.1	+ve	-ve	-ve
32	105	1111	<i>Staphylococcus epidermidis</i>	<i>Vagococcus fluvialis</i> strain AWW1	90.38%	MH760800.1	+ve	-ve	-ve
33	2	1137	<i>Staphylococcus saprophyticus</i>	<i>Alcaligenes faecalis</i> strain DEP8	94.50%	KX118704.1	+ve	-ve	-ve
34	145	1132	<i>Staphylococcus saprophyticus</i>	<i>Pseudomonas aeruginosa</i> strain H47921	96.05%	CP008861.1	+ve	-ve	-ve

## Discussion

The vast attention towards polyhydroxyalkanoates (PHAs) is mainly attributed to their properties that resemble some petrochemical plastics. Several categories of PHA including low chain length-medium chain length PHAs (LCL-MCLPHA), LCL-PHAs (copolymers produced by *Pseudomonas aeruginosa*) of, SCL-PHAs, copolymers of SCL-MCL and SCL-LCL-PHAs have been reported (Saranya *et al.*, 2017). However, apart from structural and the expressed biochemical secretions, the bacterium may possess or harbour other sets of genes not yet reported (Kim *et al.*, 2010; Cabili *et al.*, 2011) that maybe triggered for expression under a completely different environmental factor (Zhu *et al.*, 2014). The phenotypic otherwise known as a traditional (cultural and biochemical) method of identification as one of the two methods (phenotypic and 16S rDNA probe) employed for each of the sample isolates in this research agrees with the earlier research findings which stated that morphological characteristics may be controlled by the expression of a set of similar genes situated at a particular locus in a sequence when triggered by specific environmental condition (Albert & Kruglyak, 2015; Govindaraj *et al.*, 2015). In this study, the genetically (16S rDNA) identified *Bacillus cereus* strains LB17 and *Bacillus paramycoides* SA27 are both phenotypically identified as *Bacillus badius* in this research. This is in agreement with Gaballa *et al.* (2021) reports. However, the report indicates that bacteria which belong to a group may be difficult to differentiate based on the similarities of their physiology and some vital biochemical reactions (Catoira *et al.*, 2019; McIntyre *et al.*, 2008) borne out the expressed genes. Similarly, *Bacillus cereus* strains WD-2, *Bacillus thuringiensis* strain B131, *Lysinibacillus fusiformis* strain 28XG99 and *Ochrobactrum anthropic* strain S14D also exhibit the phenotypic identity of *Bacillus megaterium*. However, this may also be associated with the fact that genes harboured and or acquired are expressed phenotypically while the genetic probes reveal the constitution of the bacterium identity (Albert & Kruglyak, 2015). Invariably, some individual isolates phenotypically identified as *Corynebacterium kutscheri* are genetically assayed as *Acinetobacter calcoaceticus* JL11, five strains of *Alcaligenes faecalis* (J08, N1-4, P 156, PK48 and VBN14) seven strains of *Bacillus* spp. (HQB343, WL-190, AR156, WJB64, NGB-SF390, ASKAMI3 and ADY06), *Enterobacter cloacae*, *Falsochrobactrum ovis*, *Ochrobactrum ciceri*, *Providencia stuartii* including two species of *Pseudomonas* (*P. aeruginosa* strain PWN2C and *P. putida* strain PF71) and *Bordetella trematum* (figure 5). Likewise, *Bacillus tropicus* strain RS14D is phenotypically identified as *Enterobacter amnigenus*. While *Alcaligenes faecalis* strains PSD10 and *Vagococcus fluvialis* strain AWW1 were identified as *Staphylococcus epidermidis*. *Alcaligenes faecalis* strain DEP8 and *Pseudomonas aeruginosa* strain H47921 are also identified as *Staphylococcus saprophyticus* in this research.

This study shows that the *phaC* a gene for PHA detection potential was expressed in all 34 bacterial isolates (Table 2). However, it is worth noting that the manipulation of biological PHA synthesis can be carried out under the choice of PHA synthase that is, the enzyme responsible for the incorporation of (D)-3-hydroxyacyl-CoA substrate into PHA (Nomura & Taguchi, 2007; Zhuang, Wang, Liang, & Qi, 2014; Morya *et al.*, 2021). Therefore, the expression of *phaC* genes in the bacteria isolates does not give us an idea of the possible type of PHA due to these research isolates when optimum conditions are attained. Furthermore, it is also important to know that organisms that carry *phaC* gene synthase have the potential to synthesize homopolymer and copolymer and this is determined by the type of carbon source utilized (Chen, 2010; Mozejko-Ciesielska & Kiewisz, 2016; Mozejko-Ciesielska *et al.*, 2019) hence, the expression of this gene is only an indication of the potential of an organism to produce PHA and not to predict the type of PHA it might produce (Zinn *et al.*, 2001; Koller & Rodríguez-Contreras, 2015; Koller *et al.*, 2017)

The *Alcaligenes faecalis* strains (N1-4, PK 48, M4S3B1) in Table 2b and *Bacillus humi* strain NBPP9 (Table 2d) in this study expressed *phaC1* which is noted for short PHA chain potential. *phaC1* and some other related PHA synthase genes are harboured in *phaC* (Tan *et al.*, 2020; Lim *et al.*, 2021). Hence, the expression of this gene in any organism indicates the potential type of PHA it might synthesize. However, *phaC2* gene was not expressed on the bacterial isolates which indicate that none of the bacterial isolates can produce medium chain length PHA.

## Conclusion

The results from this study revealed that there are specie variations in the phenotypically identified isolates of *Corynebacterium kutscheri*, *Enterobacter amnigenus*, *Micrococcus luteus*, *Micrococcus varians*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* to that of their genetic identity. However; species is only distinctly synchronized in bacillus genotypic and phenotypic identity and not in the variety, while the varietal differences of the type of PHA produced from the same species are recorded. Observations from this study indicate that, of all the 16S rDNA BLAST

results obtained at NCBI website, only one (1) nucleotide sequence (*Bacillus subtilis* strain ASKMI3) matched at 100%; the remaining sequences resemblance are between 74.18 and 98.28% from the thirty-four samples. It is therefore pertinent to rely on the genetic identification of bacteria for the effective determination of PHA-producing character as displayed in *Bacillus* spp. Also, the non-synchronization of genetic identities noticed in the phenotypic method employed for *Corynebacterium kutscheri*, *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Bacillus* spp, *Enterobacter cloacae*, *Falschrobactrum ovis*, *Ochrobactrum ciceri*, *Providencia stuartii*, *Pseudomonas* spp. calls for further study that will be tailored to the order of standardization and validity of the identities of the aforementioned bacteria whenever a phenotypic method is to be used.

#### References

- Abdulaziz, S. *The microbiology and biogeochemistry of desert soils notably in relation to microbial phosphate solubilization*. University of Sheffield. 2013.
- Aditi, S., Souza Shalet, N., Pranesh, R., & Katyayini, T. Microbial production of polyhydroxyalkanoates (PHA) from novel sources: a review. *Int J RBS*, 2015; 4, 16-28.
- Agama-Acevedo, E., Islas-Hernández, J. J., Pacheco-Vargas, G., Osorio-Díaz, P., & Bello-Pérez, L. A. Starch digestibility and glycemic index of cookies partially substituted with unripe banana flour. *LWT-Food Science and Technology*, 2012; 46(1), 177-182.
- Albert, F. W., & Kruglyak, L. The role of regulatory variation in complex traits and disease. *Nature Reviews Genetics*, 2015; 16(4), 197-212.
- Albuquerque, P. B., & Malafaia, C. B. Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. *International journal of biological macromolecules*, 2018; 107, 615-625.
- Ashby, R., Solaiman, D., & Foglia, T. The synthesis of short-and medium-chain-length poly (hydroxyalkanoate) mixtures from glucose-or alkanolic acid-grown *Pseudomonas oleovorans*. *Journal of industrial Microbiology and biotechnology*, 2002; 28(3), 147-153.
- Bagheriasl, S. *Development and characterisation of polyhydroxybutyrate from selected bacterial species*. University of Birmingham. 2013.
- Barrett, J. S., & Gibson, P. R. Fructose and lactose testing. *Australian family physician*, 2012; 41(5), 293.
- Bergey, D. H., Breed, R. S., Murray, E. G. D., & Hitchens, A. P. Manual of determinative bacteriology. *Manual of determinative bacteriology*. 1939; Fifth Edn.
- Bhusal, A., & Muriana, P. M. Isolation and characterization of nitrate reducing bacteria for conversion of vegetable-derived nitrate to 'natural nitrite'. *Applied Microbiology*, 2021; 1(1), 11-23.
- Brigham, C. J., Kurosawa, K., Rha, C., & Sinskey, A. J. Bacterial carbon storage to value added products. 2011.
- Bryan-Thomas, J., Collins, K., Omoregie, J., & Levy, A. What is the Cost of Leptospirosis treatment in Jamaica? A Cross-sectional Study. *American Journal of Public Health*, 2018; 6(3), 166-172.
- Bugnicourt, E., Cinelli, P., Lazzeri, A., & Alvarez, V. A. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. 2014.
- Cabili, M. N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes & development*, 2011; 25(18), 1915-1927.
- Cappuccino, J., & Sherman, N. Microbiology: a laboratory manual (p. 507). San Francisco: Pearson/Benjamin Cummings. Sulentuss, JH, & Doyle, WJ (1999). Functions of phosphorus in plants. *Better Crops*, 2005; 83(1), 6-7.
- Carter, G. R., & Cole Jr, J. R. *Diagnostic procedure in veterinary bacteriology and mycology*: Academic Press. 2012.
- Catoira, M. C., Fusaro, L., Di Francesco, D., Ramella, M., & Boccafoschi, F. Overview of natural hydrogels for regenerative medicine applications. *Journal of Materials Science: Materials in Medicine*, 2019; 30(10), 1-10.
- Chen, G.-Q. Plastics completely synthesized by bacteria: polyhydroxyalkanoates *Plastics from bacteria* (pp. 17-37): Springer. 2010.
- Conte, E., Catara, V., Greco, S., Russo, M., Alicata, R., Strano, L., et al. Regulation of polyhydroxyalkanoate synthases (phaC1 and phaC2) gene expression in *Pseudomonas corrugata*. *Applied microbiology and biotechnology*, 2006; 72(5), 1054-1062.
- Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. Polymeric scaffolds in tissue engineering application: a review. *International journal of polymer science*, 2011.

- Dione, N., Khelaifia, S., Lagier, J.-C., & Raoult, D. The aerobic activity of metronidazole against anaerobic bacteria. *International journal of antimicrobial agents*, 2015; 45(5), 537-540.
- Drahota, Z., Milerova, M., Stieglerova, A., Houstek, J., & Ostadal, B. Developmental changes of cytochrome c oxidase and citrate synthase in rat heart homogenate. *Physiological research*, 2004; 53(1), 119-122.
- Fleminger, J., & Goldacre, B. <https://www.ncbi.nlm.nih.gov/pubmed/23743517>.
- Gaballa, A., Cheng, R. A., Trmcic, A., Kovac, J., Kent, D. J., Martin, N. H., et al. Development of a database and standardized approach for rpoB sequence-based subtyping and identification of aerobic spore-forming Bacillales. *Journal of microbiological methods*, 2021; 191, 106350.
- Govindaraj, M., Vetriventhan, M., & Srinivasan, M. Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. *Genetics research international*, 2015; 2015.
- Green, P. R., Kemper, J., Schechtman, L., Guo, L., Satkowski, M., Fiedler, S., et al. Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid  $\beta$ -oxidation inhibited *Ralstonia eutropha*. *Biomacromolecules*, 2002; 3(1), 208-213.
- Gumel, A. M., Annuar, M. S. M., & Heidelberg, T. Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. *PLoS One*, 2012; 7(9), e45214.
- Hawkins, S., Fonseca, I. B. F. d. C., Lima da Silva, R., & Quirino, R. L. Aquaculture waste: potential synthesis of polyhydroxyalkanoates. *ACS omega*, 2021; 6(4), 2434-2442.
- Hoffmann, N., & Rehm, B. H. Regulation of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. *FEMS microbiology letters*, 2004; 237(1), 1-7.
- Jay, J. M., Loessner, M. J., & Golden, D. A. *Modern food microbiology*. Springer Science & Business Media. 2008.
- Jendrossek, D., & Pfeiffer, D. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly (3-hydroxybutyrate). *Environmental Microbiology*, 2014; 16(8), 2357-2373.
- Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahán, P., et al. Epigenetic memory in induced pluripotent stem cells. *Nature*, 2010; 467(7313), 285-290.
- Koh, Y., Nakata, H., Maeda, K., Ogata, H., Bilcer, G., Devasamudram, T., et al. Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus in vitro. *Antimicrobial Agents and Chemotherapy*, 2003; 47(10), 3123-3129.
- Koller, M., Bona, R., Chiellini, E., & Braunegg, G. Extraction of short-chain-length poly-[(R)-hydroxyalkanoates](scl-PHA) by the "anti-solvent" acetone under elevated temperature and pressure. *Biotechnology letters*, 2013; 35(7), 1023-1028.
- Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New biotechnology*, 2017; 37, 24-38.
- Koller, M., & Rodríguez-Contreras, A. Techniques for tracing PHA-producing organisms and for qualitative and quantitative analysis of intra-and extracellular PHA. *Engineering in life sciences*, 2015; 15(6), 558-581.
- Krulwich, T. A., Sachs, G., & Padan, E. Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology*, 2011; 9(5), 330.
- Lim, H., Chuah, J.-A., Chek, M. F., Tan, H. T., Hakoshima, T., & Sudesh, K. Identification of regions affecting enzyme activity, substrate binding, dimer stabilization and polyhydroxyalkanoate (PHA) granule morphology in the PHA synthase of *Aquitalea* sp. USM4. *International journal of biological macromolecules*, 2021; 186, 414-423.
- Manikandan, P., & Senthilkumar, P. K. On overview of saltpan halophilic bacterium. 2017.
- Margesin, R., & Schinner, F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied microbiology and biotechnology*, 2001; 56(5-6), 650-663.
- McChalicher, C. W., & Srienc, F. Investigating the structure–property relationship of bacterial PHA block copolymers. *Journal of biotechnology*, 2007; 132(3), 296-302.
- McIntyre, R. S., Nguyen, H. T., Soczynska, J. K., Lourenco, M. T. C., Woldeyohannes, H. O., & Konarski, J. Z. Medical and substance-related comorbidity in bipolar disorder: translational research and treatment opportunities. *Dialogues in clinical neuroscience*, 2008; 10(2), 203.
- Mesbah, N. M., & Wiegel, J. Life under multiple extreme conditions: diversity and physiology of the halophilic alkalithermophiles. *Appl. Environ. Microbiol.*, 2012; 78(12), 4074-4082.

- Morya, R., Sharma, A., Kumar, M., Tyagi, B., Singh, S. S., & Thakur, I. S. Polyhydroxyalkanoate synthesis and characterization: A proteogenomic and process optimization study for biovalorization of industrial lignin. *Bioresource Technology*, 2021; 320, 124439.
- Możejko-Ciesielska, J., & Kiewisz, R. Bacterial polyhydroxyalkanoates: still fabulous? *Microbiological Research*, 2016; 192, 271-282.
- Możejko-Ciesielska, J., Marciniak, P., & Szacherska, K. Polyhydroxyalkanoates synthesized by *Aeromonas* species: Trends and challenges. *Polymers*, 2019; 11(8), 1328.
- Nawrath, C., Poirier, Y., & Somerville, C. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proceedings of the National Academy of Sciences*, 1994; 91(26), 12760-12764.
- Nayak, P. K., Mohanty, A. K., Gaonkar, T., Kumar, A., Bhosle, S. N., & Garg, S. Rapid identification of polyhydroxyalkanoate accumulating members of Bacillales using internal primers for phaC gene of *Bacillus megaterium*. *International Scholarly Research Notices*, 2013.
- Nomura, C. T., & Taguchi, S. PHA synthase engineering toward superbio-catalysts for custom-made biopolymers. *Applied microbiology and biotechnology*, 2007; 73(5), 969-979.
- Numata, K., Abe, H., & Iwata, T. Biodegradability of poly (hydroxyalkanoate) materials. *Materials*, 2009; 2(3), 1104-1126.
- Oberacker, P., Stepper, P., Bond, D. M., Höhn, S., Focken, J., Meyer, V., et al. Bio-On-Magnetic-Beads (BOMB): Open platform for high-throughput nucleic acid extraction and manipulation. *PLoS biology*, 2019; 17(1), e3000107.
- Oren, A. Life at high salt concentrations. *The prokaryotes: Prokaryotic communities and ecophysiology*, 2013; 421-440.
- Pascucci, L., Coccè, V., Bonomi, A., Ami, D., Ceccarelli, P., Ciusani, E., et al. Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: a new approach for drug delivery. *Journal of Controlled Release*, 2014; 192, 262-270.
- Perez, J., Springthorpe, V. S., & Sattar, S. A. Clospore: a liquid medium for producing high titers of semi-purified spores of *Clostridium difficile*. *Journal of AOAC International*, 2011; 94(2), 618-626.
- Reiner, K. Catalase test protocol. *American society for microbiology*, 2010; 1-6.
- Rothschild, L. J., & Mancinelli, R. L. Life in extreme environments. *Nature*, 2001; 409(6823), 1092.
- Saranya, V., Rajeswari, V., Abirami, P., & Shenbagarathai, R. Polyhydroxy propionate-co-hydroxy dodecanoate-co-hydroxy octadecanoate for in vitro cell culture. *International Journal of Polymeric Materials and Polymeric Biomaterials*, 2017; 66(9), 425-431.
- Sharaha, U., Rodriguez-Diaz, E., Sagi, O., Riesenberger, K., Salman, A., Bigio, I. J., et al. Fast and reliable determination of *Escherichia coli* susceptibility to antibiotics: Infrared microscopy in tandem with machine learning algorithms. *Journal of biophotonics*, 2019; 12(7), e201800478.
- Sharma, P. K., Munir, R. I., Blunt, W., Dartailh, C., Cheng, J., Charles, T. C., et al. Synthesis and physical properties of polyhydroxyalkanoate polymers with different monomer compositions by recombinant *Pseudomonas putida* LS46 expressing a novel PHA synthase (PhaC116) enzyme. *Applied Sciences*, 2017; 7(3), 242.
- Solaiman, D. K., Ashby, R. D., Hotchkiss, A. T., & Foglia, T. A. Biosynthesis of medium-chain-length poly (hydroxyalkanoates) from soy molasses. *Biotechnology letters*, 2006; 28(3), 157-162.
- Srithong, K., & Sangseethong, K. *Biodegradable plastics from cassava starch*. Paper presented at the II International Symposium on Sweetpotato and Cassava: Innovative Technologies for Commercialization. 2005; 703.
- Steinbüchel, A., & Lütke-Eversloh, T. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical engineering journal*, 2003; 16(2), 81-96.
- Sun, Q., Qian, B., Uto, K., Chen, J., Liu, X., & Minari, T. Functional biomaterials towards flexible electronics and sensors. *Biosensors and Bioelectronics*, 2018; 119, 237-251.
- Tan, H. T., Chek, M. F., Lakshmanan, M., Foong, C. P., Hakoshima, T., & Sudesh, K. Evaluation of BP-M-CPF4 polyhydroxyalkanoate (PHA) synthase on the production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) from plant oil using *Cupriavidus necator* transformants. *International journal of biological macromolecules*, 2020; 159, 250-257.
- Tsuge, T., Yano, K., Imazu, S. i., Numata, K., Kikkawa, Y., Abe, H., et al. Biosynthesis of polyhydroxyalkanoate (PHA) copolymer from fructose using wild-type and laboratory-evolved PHA synthases. *Macromolecular bioscience*, 2005; 5(2), 112-117.

- Valappil, S. P., Peiris, D., Langley, G., Herniman, J., Boccaccini, A. R., Bucke, C., *et al.* Polyhydroxyalkanoate (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus* spp. *Journal of biotechnology*,. 2007; 127(3), 475-487.
- Wu, H.-A., Sheu, D.-S., & Lee, C.-Y. Rapid differentiation between short-chain-length and medium-chain-length polyhydroxyalkanoate-accumulating bacteria with spectrofluorometry. *Journal of microbiological methods*, 53(1),. 2003; 131-135.
- Zhang, M., Zhu, L., Zhou, G., Hao, T., Qiu, C., Zhao, Z., *et al.* Single-layered organic photovoltaics with double cascading charge transport pathways: 18% efficiencies. *Nature communications*,. 2021; 12(1), 1-10.
- Zhang, Y., Li, X., Bartlett, D. H., & Xiao, X. Current developments in marine microbiology: high-pressure biotechnology and the genetic engineering of piezophiles. *Current opinion in biotechnology*,. 2015; 33, 157-164.
- Zhu, H., Sandiford, S. K., & van Wezel, G. P. Triggers and cues that activate antibiotic production by actinomycetes. *Journal of industrial Microbiology and biotechnology*,. 2014; 41(2), 371-386.
- Zhuang, Q., Wang, Q., Liang, Q., & Qi, Q. Synthesis of polyhydroxyalkanoates from glucose that contain medium-chain-length monomers via the reversed fatty acid  $\beta$ -oxidation cycle in *Escherichia coli*. *Metabolic engineering*,. 2014; 24, 78-86.
- Zinn, M., Witholt, B., & Egli, T. Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Advanced drug delivery reviews*,. 2001; 53(1), 5-21.

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