Original Research Article

Isolation and Screening of indigenous Polyhydroxyalkanoates producing bacterial strain

ABSTRACT

Exploration for promising strains of PHA producers is a continuous process as effort to mitigate the accumulation of synthetic plastic is on the rise. The present study was carried out to explore the potentials of indigenous bacteria strains capable of PHAs (Polyhydroxyalkanoates) production from three samples. Using standard microbiological techniques, the samples were cultured and PHA producing potential of the isolates was investigated by qualitative as well as quantitative method. The positive isolates were detected for the presence of phaC synthase gene and their ability to show resistance against selected antibiotics were monitored. Positive isolates were identified by conventional bacteriological methods. Results revealed that out of 106 isolates, 55 were suspected to accumulate PHA after preliminary screening on plate and slide method using Sudan Black B stain. Screening on a liquid medium indicates that all the isolates produced PHA in the range of 0.2-1.1g/l. ThephaCgenes were successfully amplified from genomic DNA of the six positive isolates tentatively identified as *Bacillus* sp,*Lysinibacillus*spand *Pseudomonas* sp. The result of the present study indicated that these strains have the potential of producing PHA and its potential harnessed for future purposes.

Keywords: Polyhydroxyalkanoates (PHA), Indigenous, PCR, phaC synthase gene, Sudan black B

INTRODUCTION

Plastics are employed in practically every industry and used in varied application owing to their inexpensive, light weight and durability [1,2,3]. Increase in market demand as a result of growing urbanization and industrialization has helped to drive up plastics production [4]. Plastics in the market are hydrocarbon-based and enormous use of these products resulted in approximately 44 percent of the million tons produced annually being introduced into the environment in the same year [5, 6, 7].

Unfortunately, Due to its non-degradable nature they build up in huge amounts in the environment causing serious threats to human health, wildlife, and the environment [8,9]. Movement in public perception in recent years have increased awareness of the negative effects caused by synthetic plastics in the environment [10]. However, it is difficult to decline the use of plastics products because of their numerous and wide spread applications [11]. This has inspired numerous investigators to pursue their quest and search for a non-petrochemical environmentally acceptable alternative to plastics [12].

Microbial biopolymer, which has become a viable replacement in recent years, has received substantial attention[13].Among existing biopolymers, PHA is on the priority list and among the top group of biopolymers that have been intensively investigated and commercialized [14]. These secondary metabolites accumulate in the cytoplasm as granules and provide carbon and energy sources under adverse environmental condition [15, 16]. Microorganisms builds up to about 90% Dry Cell Weight of PHB which aids in prevention of starvation when essential nutrients are scare and carbon sources are abundant [17, 18]. PHA has lot of unique qualities and multiple benefits including biodegradation in both aerobic and anaerobic environment. [19,20].

Microbial producers of PHA have been reported to reside in various ecological niches with PhaC synthases gene being the most significant indicator for PHA detection [21,22]. Different bacteria produce different PHA biosynthesis gene ranging from class 1 to IV [23]. Short chain length PHA are produced mainly by the action of I, II and IV class synthases and Medium chain length PHA produced mainly by the class II synthases harbored by the *Pseudomonas* genus [24].

The amount of PHA produced however is practically insufficient to meet the requirement of various industries and therefore limits the commercial production of bioplastics [25]. Hence the need to isolate and characterize PHA producing organisms with potentials for industrial application [26,27]. The present study focuses on isolating and screening of indigenous PHA accumulating bacteria from different localities.

2. MATERIALS AND METHODS

2.1 Collection of samples

Samples were collected from different locations within Obio-Akpor, LGA in Port Harcourt, Rivers State. The samples included soil (from garden, automobile workshop, refuse dump, poultry, gas station) waste water effluent (from sewage, poultry, kitchen, abattoir, market) and organic waste. Three replicates of each samples were aseptically collected using sterile zip-lock bags. They were marked according to their source, location, and transported to the laboratory, stored at 4°C until when required.

2.2 Isolation and Purification of Bacteria Producing polyhydroyalkanoate (PHA)

Serial dilution and spread plate method [28]were applied for isolation of the organisms. One gram of each sample was obtained, diluted and prepared for cultivation. About 0.1 ml was transferred aseptically to Nutrient agar (NA) medium supplemented with 1% glucose and incubated for 24h at 28±2^{o}C. After incubation, distinct colonies were selected and purified by repeated streaking on Nutrient Agar medium to obtain pure culture which was maintained on an agar slant and preserved.

2.3 Screening microbial isolates for PHA accumulation

2.3.1 Qualitative test for PHA producing bacteria

The isolates were screen for PHA accumulators using Sudan black B on solid medium and microscope. The methods of [29,30]was adopted.

A) Solid medium screening: Purified isolates were screened for their relative PHA accumulating capacity on solid medium using Sudan black B solution. The isolates were grown on Nutrient Agar medium supplemented with glucose and incubated for 24hrs. After incubation, the plates were flooded with already prepared Sudan Black B solution and allowed to stand for 30minutes. The plates were distained using ethanol and excess Sudan black stain was drained off from the colonies. Single colonies identified as PHA accumulators was indicated by the retention of dark-blue coloration of colonies.

B) Microscopic Screening: Positive isolates obtained from solid medium screening was detected for presence of PHA granules using microscope. Loopful of each culture was smeared on a clean glass slide by heat fixing and stained with Sudan black solution for 10minutes. The slides were then immersed into xylene to decolorize the colonies and then counterstain using 5% w/v aqueous Safranin for 10s. The slide was washed with tap water, dried, and examined under light microscope for the presence of black stained granules with a pink background to confirm PHA positive isolates. Positive isolates were selected and used for quantity determination of PHA production in liquid medium.

2.4. Quantitative test for PHA and selection of working strains

Prior to the production of PHA, Inoculum was prepared by transferring a loopful of the different bacterial isolates positive for PHA accumulation into nutrient broth medium and the inoculum size was determined by comparison with readily prepared McFarland standard 0.5 and incubated at 30^{o}C for 24hours.

Production of PHA was carried out by submerged fermentation. Mineral salt medium containing (g/l); $(NH4)_2SO_4$ 0.2, KH_2PO_4 13.3, $MgSO_4$ 1.3, Citric Acid 1.7. glucose 20g/L, Trace element solution 10 mL/L, (g/L, $FeSO_4.7H_2O$ 10, $ZnSO_4.7H_2O$2.25, $CuSO_4.5H_2O$ 1, $MnSO_4.5H_2O$ 0.5, $CaCl_2.2H_2O$ 2.0,$Na_2B_4O_7.10H_2O$ 0.23, $(NH_4)_6Mo_7O_{24}$ 0.1, 35% HCl 10 mL); pH 6.8-7.0) as described by [31]. was used as basal medium and sterilized at standard temperature and time. A known volume of each Nutrient broth culture was cultivated in 50ml media prepared as described above and incubated at 30^{o}C for 72hrs.

2.4.3 Extraction and Estimation of PHA

Cells were collected from fermentation broth by centrifugation. After centrifugation, the supernatant was discarded and the residue (sediment) was collected, used for cell dry weight and PHA estimation. PHA extraction was performed using Sodium hypochlorite-chloroform method as described by [32]. The cells were suspended in equal volumes of sodium hypochlorite (NaClO) (4-6% w/v) and chloroform and the mixture incubated at room temperature for 1hr at 37^{o}C. After incubation, the mixture was centrifuged at 4000rpm for 15minutes to sediment the lipid granules. The supernatant was discarded, and the sediment was washed successively with acetone and methanol and centrifuged. The granules were dissolved in hot chloroform and allowed to dry leaving the PHA powder. The powdery mass on the test tube was collected and the percentage of PHA accumulated intracellularly was estimated gravimetrically using the formula;

$$\% \text{ PHA accumulation} = \frac{\text{dry weight of extracted PHA (g/L)}}{\text{weight of biomass(g/L)}} \times 100$$

2.4.4 Determination of Cell Dry Weight

The bacteria biomass was expressed as cell dry weight and was determined as described by [25]. Approximately10ml samples from fermentation broth was centrifuged and the supernatant discarded. The sediment obtained was washed with distilled water, dried in a hot air oven to constant weight. Thereafter, the total bacterial cell dry weight was weighed.

2.7 Detection of PhaC gene of the selected isolates using PCR

The potent isolates obtained after Shake flask studies were subjected to PCR amplification to detect phaC gene encoding for PHA synthase. The method of [33].was adopted for the study. The isolated DNA samples were amplified using phaC gene specific primers; phaC1F1(5′ TGGARCTGATCCAGTAC3′) and phaC1F2 (5′ SATCAACCTGATGACCGA3′) on an ABI 9700 Applied Biosystems thermal cycler. PCR was carried out at a final volume of 25 microlitres for 35 cycles (95ºC for 5 minutes; 95ºC for 30 seconds; 58ºC for 30 seconds; 72ºC for 30 seconds) and a final extension, 72ºC for 5 minutes. The PCR product was finally subjected to electrophoresis and resolved on a 1% agarose gel visualized under a UV transilluminator.

2.8 Identification of potential bacterial strain by conventional phenotypic method

2.8.1 Macroscopic and Microscopic characterization: Isolates which showed efficient PHA production in shake flask screening were presumptively identified by their morphology and biochemical properties**.**Colony characters such as color, shape, size, opacity etc. of the isolates were observed based on bacteria growth on nutrient agar medium. Microscopy-based morphological studies including gram reaction, motility test was performed.

2.8.2 Biochemical characterization: Biochemical test such as catalase, oxidase, indole, methyl red /vogues Proskauer and sugar fermentation was carried out following method of [34].The results obtained were compared with standard description given in Bergey's Manual of Determinative Bacteriology [35].

2.9 Detection of antibiotic resistance of selected isolates

Antibiotics resistance of the selected isolates was determined using Kirby Bauer's disc diffusion in Mueller Hinton agar as described by [36]. 0.5 McFarland standard was prepared and used as a measure of turbidity for each inoculum. Approximately $1–2\times10^{8}$cfu/ml of the bacterial inoculum was applied using spread plate technique to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks were then placed on the inoculated agar surface. Plates were incubated for 16–24 h at 35°C prior to determination of results. The zones of growthinhibition around each of the antibiotic disks measured to the nearest millimeter. The zone diameters of each drug were interpreted using the criteria published by the Clinical and Laboratory Standards Institute.

3. RESULTS

3.1 Isolation

Various samples collected from diverse areas were used to isolate PHA accumulating bacteria. A total of one hundred and six distinct colonies which varied in size, shape and appearance were

picked and purified by streaking, thereafter transferred to nutrient Agar medium slant and maintained as pure cultures for further screening using Sudan black solution.

3.2 Screening of PHA producing bacteria

The purified isolates were inspected for qualitative production of PHA on a nutrient agar plate. Upon macroscopic evaluation, the isolates exhibited different ability to accumulate PHA in terms of color absorption pattern and were classified into four categories (high. Moderate, poor and weak). The chat in figure 1 reveals that from the 106 isolates, 55 colonies (52%) were highly stained by appearing dark black, 29 (27%) were moderately stained by showing pale black color, 6 (6%) were poorly stained while 16 (15%) showed no color absorption and appeared white or brown (Figure1). Colonies which showed dark black color as shown in figure 2 were selected and observed microscopically.

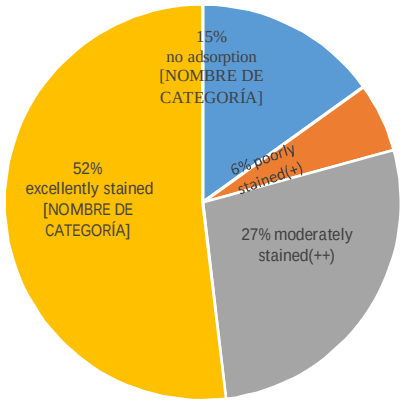

Figure 1. Intensity of staining on solid agar using Sudan black B solution

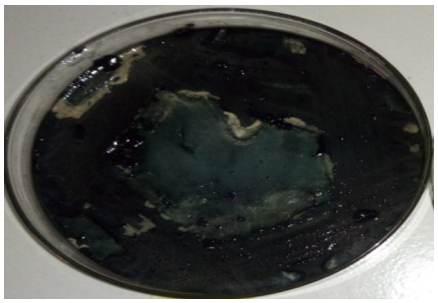

Figure 2. Dark black colored colony appeared after application of Sudan black B solution

3.3 Microscopic analysis

Microscopic observation revealed that the 55 isolates showed presence of dark black granule within pink colored cells as shown in fig 2

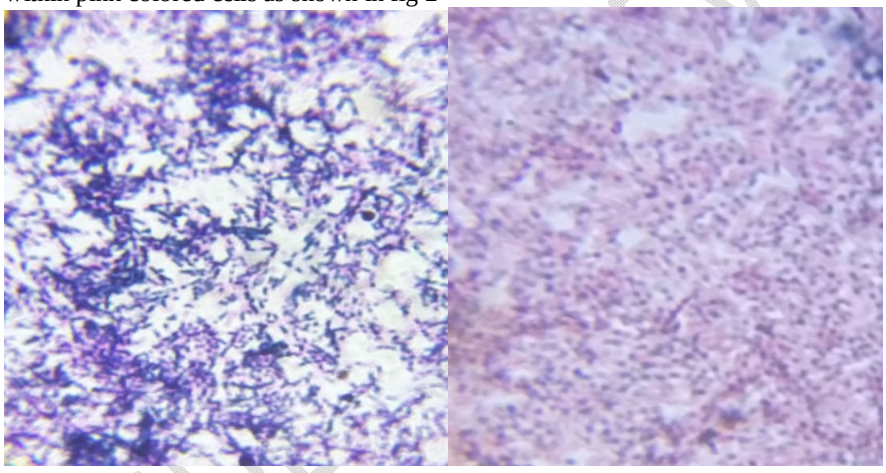

Figure 3: PHA granule (stained dark blue) in bacterial cell

3.4 Selection of the Best PHA producing Bacteria

Result of the quantitative screening of the 55 bacterial isolates are shown in Figure 3. The PHA yield of the 55 isolates ranged from 0.2-1.1g/l. Out of the 55 isolates studied, 56% (31) yielded less than 0.5g/L PHA and were grouped as weak producers. About 33% (18) of the total isolates were grouped as moderate PHA producers with yield at range of 0.6-0.8g/L while few numbers about 11% (6) were classified as strong PHA producers as they yielded greater than 0.9g/l of PHA. Among all isolates, six with designated codes OWO1, WW4, WW7, WW9, WW15, and RD 18 were able to produce high amount of PHA
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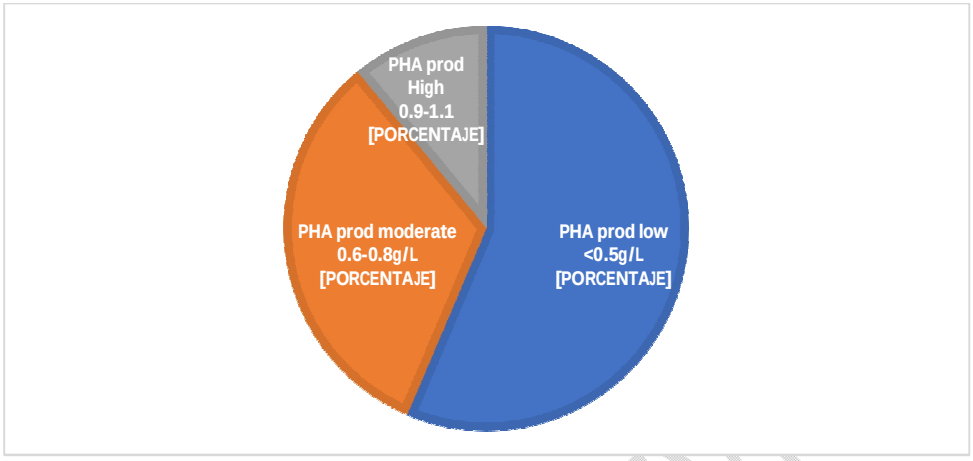

Figure 4: PHA production of selected isolate

3.5 Detection of PhaC gene

The six isolates with maximum PHA production were further subjected to PCR amplification for a rapid screening of the gene fragment of PHA synthasephaC subunit using specific primers.The results as shown in figure 3 reveals amplification of phaC gene fragments in all the six isolates by showing 550bp bands on electrophoresis

Comment [i1]: Maybe "5"

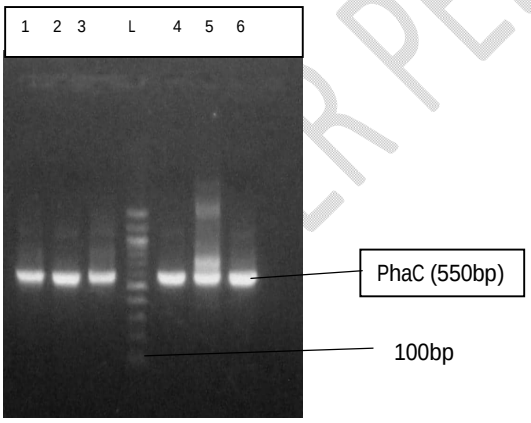

Figure 5: Agarose gel electrophoresis showing the amplified phaC synthase genes. Lanes 1-6 represent the phaC gene bands at 550bp while lane L represents the 100bp molecular ladder.

3.6 Characterization and Identification of selected bacterial isolates

Microscopic, Morphological and biochemical characterization of the promising bacterial isolates are summarized as follows in Tables 1 and 2. The colonies of the six isolates were creamy white with one exhibiting green pigmentation. Gram stain result reveals five of the isolates to be Gram positive and one Gram negative. Based on their biochemical characterization, the isolates were tentatively found to belong to four genera via *Bacillus*, *Pseudomonas* and *Lysinibacillus*

Table 1: Phenotypic characteristics of PHAs producing bacteria

Characteristics	**Observations**					
	OWO	WW4	WW7	WW9	WW15	Rd18
Size	Small	Small	Large	Medium	Small	Medium
Shape	Circular	Circular	1rregular	Irregular	Circular	Circular
Edge	Entire	Filamentous	Undulate	Wavy	Entire	Undulate
consistency	Smooth	Smooth	Smooth	Dry	Dry	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Translucent
Pigmentation	-	-	-	-	-	Green
Colour	Creamy white	Creamy white	Creamy White	Creamy White	Creamy white	Creamy white
Grams reaction morphology	+ve rod	+ve rod	+ve rod	+ve rod	+ve rod	-ve rod

Key -= Not present

Table 2 Biochemical characteristics of the isolates

Biochemical reaction	**OWO**	**WW4**	**WW7**	**RD18**	**WW9**	**WW15**
Catalase	+	+	+	+	+	+
Citrate	+	-	-	+	+	+
Indole	+	-	-	-	+	-
Motility	+	+	+	+	+	+
MR	-	-	-	-	-	+
VP	+	-	-	-	+	-
Oxidase	-	+	+	+	+	-
TSI						
Slant	K	K	K	K	K	K
Butt	A	A	A	A	A	A

H_2S	-	-	-	-	-	-
Starch hydrolysis	+	+	-	-	+	+
Urease	-	+	+	-	-	-
Sugar fermentation						
Glucose	+/A	+	+	+	-/A	+/A
Sucrose	+/A	+	+	+	-/A	-
Lactose	-	+	-	+	-/A	-
Maltose	+/A	+	+	-	-/A	A

+=Positive; -= negative; K=alkaline, A= acid; MR= methyl red; VP= Vogues Proskauer; TSI= Triple sugar iron

3.7 ANTIBIOGRAM OF THE ISOLATES

Table 3 reveals the isolate were sensitive to Oflaxin (OFL) and Gentamicin (GEN). In addition to Oflaxin (OFL) and Gentamicin (GEN), Gram-negative bacteria (RD18) was sensitive to Ciprofloxacin (CPR). The Gram-positive bacteria isolates (OWO1, WW4, WW7, WW9 and WW15) were hundred percent resistance to Cefuroxime (CRX),Ceftazidime (CAZ), Augumentin (AUG), but for Erythromycin (ERY) all were resistant with the exception of WW7which showed an intermediate to the antibiotics used. The gram-negative bacteria were 100 percent resistance to five antibiotics used

Table 3: Antibiotics sensitivity test of the PHAs isolates

ISOLATE CODE	**ZONE OF INHIBITION (mm)**										
	CRX	**CAZ**	**AUG**	**CXC**	**CTR**	**ERY**	**GEN**	**OFL**	**CPR**	**CXM**	**NIT**
OWO	R	R	R	R	R	R	**S**	**S**	-	-	-
WW4	R	R	R	R	R	R	S	S	-	-	-
WW7	R	R	R	R	R	I	S	S	-	-	-
WW9	R	R	R	R	R	R	S	S	-	-	-
WW15	R	R	R	R	R	R	S	S	-	-	-
RD18	R	R	R	-	-	-	**S**	S	S	R	R

Key: ANTIBIOTIC DISK: CRX- Cefuroxime, CAZ- Ceftazidime, CXC-Cloxacillin, ERY-Erythromycin, CTR- ceftriaxone, AUG-Augmentin, GEN-Gentamicin, OFL- ofloxacin, CPR-Ciprofloxacin, CXM-Cefixime, NIT Nitrofurantoin, (-)-Not tested, S=sensitive, I= Intermediate; R=

4. Discussion

Environmental concerns have stimulated researchers to search for new biological methods to fabricate biodegradable plastics [37].Synthesis of PHA using bacteria is a novel approach and PHA producing bacteria are therefore in great demand [24, 38].In this regard, 106 bacterial isolates were obtained from soil, waste water effluent and organic waste from different localities. Manystudies have reported isolation of PHA producing bacteria from different natural environment such as soil, waste water effluent and sediments [39].[40] isolated two strains of *Clostridium bifermentans* from soil near gas station in Basrah city. Studies carried out by [41] screened for indigenous PHA producing bacteria from soil and organic wastes.

To assess the accumulating capacity of all the isolates, rapid screening by colony staining method followed by microscopic examination using Sudan black B solution was conducted [42]. These qualitative experiments using Sudan Black B a lipophilic dye, accredits dark blue colored colonies on plate assay and dark blue granules inside red cells when viewed microscopically as indicators for selecting PHA accumulating bacteria [43].Several authors have advocated this screening techniques for the identification of microorganisms with the potential to accumulate PHA. [44]and [45]in their studies, employed solid medium method of screening using Sudan black dye in alcohol. [46] used slide stained with Sudan black B solution viewed using oil immersion to confirm presence of PHA granule. [37] screened 26 isolates for PHA production by Sudan Black B slide method based on the presence of dark granules. [47]isolated one hundred and sixty bacterial from soil / water samples and thirty isolates showed positive for PHA accumulating capabilities based on dark black coloration on solid medium flooded with Sudan black. Sudan black B stain is a simple method not specific to PHA since it can bind to hydrophobic polymers such as lipids, therefore there is need to confirm the content of PHA of these isolates

Quantitative screening was conducted to obtain efficient strain from the selected isolates which produces high amount of PHA. Hence Production medium containing 2% glucose as carbon source was used for quantitative estimation of PHA and cell dry weight. This was employed for selection of highest PHA producer from the group of 55 isolates. Result revealed that six bacterial isolates with the designated codes produced copious amount of PHA above 0.9g/L and was selected as outstanding PHA accumulators. [48]isolated five PHA producing bacteria and

recorded that *Bacillus cereus* NRRL-B-3711 produced an excellent biopolymer yield which serve as a criterion for its selection. [7]reported 19 bacteria strain of producing PHA ranging from 0.15 to 0.70g/l with highest yield obtained by *Burkholderia*sp B73 when PHA content was determined by gravimetric method. [49] also reported PHA accumulation with similar results from 48 bacterial isolates obtained from compost sample with 7 isolates having variant capabilities to consume glucose as carbon source by quantitative assay. Temitope and others in their study reported PHA production ranging from 0.04-0.30g/l in isolates obtained from sugarcane farm sites and cassava dumpsite by using production medium supplemented with 2% glucose [50].

In bacteria, three regulatory genes and enzymes are involved in the generation and synthesis of PHB. These enzymes include ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, which are all coded for by phbA, phbB, and phbC genes [51]. Specific primers namely phaC1F1 and phaC1F2 were used for amplification of PhaC gene in the DNA sequence of the selected bacterial species. The six bacterial isolates harbored PHA synthase gene (phaC) confirmed by PCR amplification indicating that phbC gene cluster was responsible for PHB production. The same set of primer was used in study conducted by [33] for detection of PhaC gene using PCR method. [52] screened for the presence of PHA synthase using PCR on different strains of bacteria and observed an amplification product of 540bp. Amplification of phaC gene has been reported for isolates such as *Bacillus cereus* SE-1 [53]; *Pseudomonas mendocina*PSU [54]; *Bacillus substillis* [55] and *Bacillus cereus* C113 [56] using PCR method.

Based on morphological and biochemical analysis, selected strains were identified to the genus level by comparing with the standard strains already described in Bergey's manual. The cultural (macroscopic) characteristics reveal the isolates to be creamy white with no diffusible pigment expect for isolate RD18 where green diffusible pigment was observed. Examination using Gram staining reveals all the isolates were rods, five Gram positive and one gram negative. Biochemical analysis revealed all the isolates were motile and able to ferment the carbohydrates such as glucose, sucrose etc. differently and were tentatively identified to belongs to three genera: *Bacillus*sp, *Lysinibacillus*p, and *Pseudomona*s sp. Many workers have reported that numerous bacteria ranging from Gram-positive to Gram-negative possess tremendous potentials of PHA accumulation in their cytoplasm [57]. Biochemical characterization was performed for preliminary identification of PHA bacterial isolated by [58]. Based on the study it was found to

be *Lysinibacillus* sp. [59] identified PHA producing bacteria belonging to the genera B*acillus*, while [60] used morphological and biochemical characteristics to identify PHA producing isolates and the seven potential isolates were identified as *Bacillus* sp, *Arthrobacter*sp and *Micrococcus* sp.

Agar diffusion technique was adopted and Antibiogram testing reveals that all the isolates showed resistance to Cefuroxime (CRX),Ceftazidime (CAZ), Augumentin (AUG). Among all the antibiotics used for Gram-positive bacterial, Oflaxin (OFL) and Gentamicin (GEN) was found to be sensitive than other antibiotics used.

Comment [i2]: The reason for the use of the antibiogram test is not clear. This topic must be discussed. Indicating only the sensitivity or resistance to the different antibiotics of the bacterial isolates without relation to the other aspects of this work does not provide arguments for the findings presented.

5. CONCLUSION

Based on the present study, fifty- five microbial isolates showed PHA producing ability during plate assay revealing a dark black coloration. Amongst them, six isolates were tentatively identified using morphological and biochemical characteristics as *Bacillus, lysinibacillus* and *Pseudomonas* sp. They were shortlisted as highest producers of PHA with efficient yield using submerged fermentation process. The PCR product confirms the presence of phaC gene in the six selected isolates. It can be concluded that the selected samples used for isolation serve as potential source for microorganisms and the isolated bacterial strains have PHA producing potentials. Screening of more microorganism from different sources with higher PHA producing potentials is needed to facilitate the discovery of novel PHA suitable for industrial applications. However, there is need for further study to enhance PHA production condition by optimization using the potent PHA isolates.

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