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Research paper



Screening and Isolation of Polyhydroxyalkanoates (PHA)-Producing Bacteria from Landfill by using Cocoa Pod Husks as Carbon Source

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Abstract

Polyhydroxyalkanoates (PHA) are bioplastics, produced by various bacteria as food and energy reservoir. PHA is an alternative for synthetic plastic because they are environmentally friendly and can be degraded naturally by microorganisms. One of the important factors for the growth of PHA producing bacteria is an excess of carbon supply. In order to reduce the overall cost of PHA production, a low cost pure substrate, which is cocoa pod husks (CPH) was used as a carbon source. The objectives of this study were to isolate and screen PHA producing bacteria from landfill samples which are leachate and soil, to identify the PHA producing bacteria by using morphological characterization and *16s rDNA* gene sequencing and to determine the best percentage of CPH that can be used as a carbon source for PHA producing bacteria. PHA producing bacteria from leachate and soil from landfill in Jeram, Selangor were screened by using Nile Blue A staining method. Two potential PHA producers with the brightest fluorescence under UV light from each samples were isolated and characterized by using morphological and molecular identification. Results of morphological identification shows all bacterial isolates have a rod shape and have a capsule, three bacterial isolates (L4, S3, S5) have an endospore while the remaining does not have endospore (L1). Three out of four were Gram positive bacteria (L4, S3, S5) and the remaining was Gram negative bacteria (L1). These isolates were confirmed of their identity as *K. pneumoniae* (L1), *B. cereus* (L4 & S3) and *B. toyonensis* (S5) using *16s rDNA* gene sequencing. Different concentration of CPH, which are 2% (w/v), 5% (w/v), 7% (w/v) and 10% (w/v) were used to study the best percentage of CPH that can be used as carbon source. PHA accumulation was the highest at 7% (w/v) for all bacterial species tested and lowest at 10% (w/v) CPH except for *B. toyonensis.* Therefore, *K. pneumoniae*, *B. cereus* and *B. toyonensis* which isolated from landfill show the ability to produce PHA and the used of 7% (w/v) cocoa pod husks as carbon source give the highest PHA accumulation.

Keywords: *Polyhydroxyalkanoates; cocoa pod husk; landfill; bioplastics.*

1. Introduction

Millions of tons of plastic that have been extensively used in our daily lives can cause environmental problems. Since these non-degradable plastics cannot be degraded by natural processes, it will accumulate in the environment [1]. Overconsumption and accumulation of the non-degradable plastics has risen environmental concerns and aggravate the desire for production of biodegradable plastics from renewable sources [2]. Therefore, biodegradable plastics have received global attention since it can be naturally degraded by microorganisms [3]. Polyhydroxyalkanoates (PHA) is a biodegradable polymer that can decrease pollution caused by the rising worldwide polymer request [1].

Polyhydroxyalkanoates are storage materials accumulate by various bacteria as energy and carbon reserve materials. They are environmentally friendly, biodegradable and also biocompostible bioplastics. They are one of the alternatives for synthetic plastics their biodegradability characteristics [4]. They can be used in a variety of disposable packaging goods and may also have high-value applications in medicine and pharmaceutical industry [5]. Polyhydroxyalkanoates are produced by around 300 different bacterial strains, serve as carbon and energy storage material and have similar physical and chemical properties to synthetic plastics [6]. However, the cost of growing the PHA producing bacteria is high. Half of the production cost is the cost for carbon sources [7]. In order to cut high PHA cost, different sources of cheap substrates were tested for the production of PHAs [8]

As an alternative, agricultural waste can be used as a carbon source. According to Gomaa [7], Agro-industrial wastes and by-products, such as olive oil mill effluent, sugarcane molasses, or paper mill waste water could be used as the carbon source rather than refine organic substrate. One of the abundant agricultural waste is cocoa pod husk (CPH). Cocoa is an industrially important crop since cocoa beans and its processed products are the main ingredients of chocolate, one of the world's most popular foods [9]. Nonetheless, increase in the production and processing of cocoa beans resulting in million tons of cocoa waste, which is cocoa pod husks (CPH). Study by Cruz *et al.*, [10] showed that cocoa pod husk has high carbon content and can be used to produce activated carbon by chemical activation. Hence, cocoa pod husks can be used as a cheap carbon source to supply carbon for the growth of bacteria. The usage of cocoa pods as carbon source is advantageous since cocoa pods are renewable resource that can be replenished continuously and thus, will reduce environmental problem.

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2. Methodology

2.1. Samples collection

Leachate and soil samples were obtained from landfill site in Jeram, Selangor, Malaysia. Soil samples of 6.0-8.0 cm depth from the surface was used for isolation of the bacteria. An amount of 20-30 g of the contaminated soil was scrapped by using sterile spatula and was placed in a sterile container [11]. Then, the samples were brought to laboratory and stored at room temperature. The cocoa waste (cocoa pod husks, CPH) was obtained from Malaysian Cocoa Board in Negeri Sembilan, Malaysia.

2.2. Preparation of cocoa pod husks (CPH)

Cocoa pod husks (CPH) was used as a carbon source for the growth of bacteria. The CPH was dried in an oven for 2 days at 50 ^{o}C. After 2 days, the CPH was ground using blender to get a fine powder. The CPH powder obtained was kept in a sterile container at room temperature for further used.

2.3. Preparation of different concentration of cocoa pod husks solution

Different concentration of cocoa pod husks were used to study the optimization of PHA accumulation by the PHA producers which were 2%, 5%, 7% and 10% . For preparing 2% of CPH solution, 2 g of CPH powder were mixed with 100 ml of distilled water. The mixture was stored in refrigerator for 24 hours. Then, the mixture was filtered using vacuum pump. The solution then was autoclaved at 121 ^{O}C for 15 minutes and capped for further used. The process above was repeated to prepare the CPH solution at concentrations of 2% (w/v), 5% (w/v), 7% (w/v) and 10% (w/v).

2.4. Nutrient Agar preparation

An amount of 23 g of NA powder (Difco, USA) was suspended into 1000 ml of distilled water. The mixture was heated on hot plate until it boiled to dissolve all components. An amount of 0.5 µg/ml of Nile Blue A was added for screening of bacteria [12] (Bhuwal *et al.*, 2013). After autoclaved, the media was cooled and poured into sterile Petri dish plates.

2.5. Isolation of bacteria from leachate and soil

In order to isolate bacteria from leachate sample, streak-plate technique by using quadrant method was done as described by Sanders [13]. The objective of this method was to isolate individual bacteria colonies on a nutrient medium. One loopful of sample was streaked on Nutrient Agar [12] and the plate then was incubated for 24 hours. After 24 hours, the method was repeated by streaking single colony to a new plate.

2.6. Screening of PHA producing bacteria

The bacterial isolates that has grown on Nutrient Agar containing Nile Blue A were exposed to UV light (250 nm) to screen for the presence of PHA. Bacterial isolates that produced PHA would appear bright orange fluorescence and the intensity of the fluorescence increased with the increase of PHA production [12]. Hence, two bacterial isolates from each sample that showed the brightest fluorescence colour were selected for further study. The selected bacterial isolates were further sub-cultured until a pure was obtained. *Escherichia coli* was used as positive control.

2.7. Morphological identification of bacteria

The colony morphological identification of bacteria on Nutrient Agar plate using naked eyes and microscopic characterization of Gram-stained samples by using Olympus compound microscope under 1000X total magnification [14].

2.8. PHA Production using Different Concentration of CPH

Different concentrations of cocoa pod husks were used to study the optimization of PHA production by the PHA producers which were 2%, 5%, 7% and 10%. All four selected bacteria were grown in nutrient broth at 30 ^{o}C for 24 hours. An amount of 25 ml minimal broth medium was added in four conical flask and 5 ml of 2 % of CPH that has been filtered and sterilized were added into it. An amount of 10 µl (0.03% inoculum size) of bacterial culture was added into respective conical flask and each flask was labelled. The steps were repeated by using 5%, 7% and 10% cocoa pod husks. The broth was incubated with shaking at 150 rpm for 2 days at 37 ^{O}C to allow the fermentation of the bacteria [12] since PHA-producing bacteria required long culture time for PHA granule to accumulate in the bacterial cells [15]. Statistical analysis of one way ANOVA was carried-out to determine the significant difference between isolated bacterial species for the production of PHA.

2.9. Extraction and Quantification of PHA production

The production of PHA was quantified by using chemical extraction method described by Raj *et al.* [16]. The bacterial cultures were centrifuged at 5000 rpm for 10 min and were filtered by using vacuum pump to obtain the cell pellet. The cell pellet was dried and weighed using weighing balance. Then, the pellet was suspended in sodium hypochlorite solution and incubated at 37 ^{O}C for 1 - 2 hours to complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules (sediment) and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHA granules in the sediment was washed twice with three portions of acetone, methanol and diethyl ether and centrifuged again (1:1:1). A volume of 10 ml of boiling chloroform was used to dissolve the polymer granule and the chloroform was left to evaporate for one day. The PHA recovered after evaporation of chloroform was weighed again. The residual biomass and percentage of PHA yield was calculated by using formula from Bhuwal *et al*. [12].

PHA accumulation: Dry weight of extracted PHA $^{(g/ml)}$ x 100%
Cell dry weight (g/ml)

Residual biomass (g/ml): Cell dry weight (g/ml) - dry weight of PHA (g/ml)

Yield (%): PHA produced (g)
Weight of cocoa pod husk (g)

2.10. Genomic DNA extraction

The selected bacterial isolates were grown in 5 ml of sterile Nutrient Broth at 30 ^{O}C for 24 hours in an incubator. Then, 1 ml of each selected bacterial isolates was placed in microcentrifuge tubes and was centrifuged at 10,000 rpm for 2 minutes to obtain cell pellet. The supernatant was discarded and the pellet was used for DNA extraction [12]. The extraction was proceeded by using Invitrogen genomic mini kit (Invitrogen, US) according to the manufacturer's instructions. The genomic DNA was stored at -20 ^{O}C for further use.

2.11. Preparation of Gel Electrophoresis for DNA Visualization

The genomic DNA was visualized by using 0.8% agarose gel to confirm the genomic extraction. DNA ladders of 1kb in premixed, ready-to-use form was purchased from Norgen (Canada) and was used in gel elctrophoresis. A volume of 5 µl of ladder was loaded

to the first well, serving as a marker for DNA size estimation. An amount of 10 µl DNA of each bacterium was mixed with 2 µl loading dye. The mixture was added into wells of the electrophoresis chamber and electrophoresis was run at 80 V for 90 minutes. Once completed, the gel was removed from the chamber and was visualized by using ChemiDoc XRS+ Imager (Bio-Rad, US).

2.12.16S rDNA gene sequencing and analysis

The gene of bacteria obtained was sequenced and analyzed by using method described by Norashirene *et al.*, [17]. 16S rDNA gene sequencing was used for the identification of the bacteria followed by 16S rDNA amplification by using two universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The purified PCR product were sent to the third party, which is Next Gene, Malaysia for sequencing. Sequence analysis and alignment were carried out using the NCBI blast tool [8].

3. Results and Discussion

3.1. Screening of PHA producing bacteria

PHA-producing bacteria from leachate and soil samples from landfill were screened and isolated. According to Naheed *et al*. [18], contaminated environments have high number of bacteria that can produce PHA. A total of 104 bacterial isolates from landfill were collected which were 79 isolates from leachate and 25 isolates from soil. Nine potential PHA-producing bacteria were selected from each leachate and soil sample. Two bacterial isolates producing the brightest fluorescence under UV light from each sample were selected for further study because the intensity of the fluorescence increased with the increased of production of PHA. This was an indication that the two bacterial isolates selected were strong PHA producer.

Four selected bacteria that shows the most promising of PHA production based on their intensity of fluorescence were further characterized on the basis of microscopic appearance and characteristics [17]. The morphology of the isolates was observed microscopically using Gram staining, capsule staining and endospore staining. The observations based on the shape, size, elevation and opacity of the colonies after 24 hours of incubation.

Table 1 shows the colony morphology of bacterial isolates on Nutrient Agar plate. The isolate L1 colony have circular shape, convex elevation and entire margin. However, isolates L4 and S3 revealed the same colonies morphology. Both L4 and S3 isolates showed irregular shape, raised elevation and also entire margin. Isolate S5 colony, showed circular shape, raised elevation and entire margin.

Table 2 showed the results of microscopic morphological characterization of the selected bacterial isolates. The result shows that three out of four selected bacterial isolates were Gram positive which are L4, S3 and S5 while the remaining isolates was Gram negative which is L1. All four selected bacterial isolates were rod shape and encapsulated. The results for endospore staining showed that three out of four bacteria have endospore which is L4, S3 and S5, except for L1.

The bacterial isolates were divided into two groups, based upon their Gram characters. It has been estimated that over 300 bacterial species and more than 90 genera, including Gram-negative and Gram-positive organisms accumulate various PHAs [19, 20]. Even though there was diverse species of bacteria that can produce PHA, only certain bacterial species are capable to utilize low-cost carbon

Table 1: The colony morphology of bacterial isolates on Nutrient Agar

Bacterial isolate	Origin	Shape	Elevation	Margin
L1	Leachate	Circular	Convex	Entire
L4		Irregular	Raised	Entire
S3		Irregular	Raised	Entire
S5	Soil	Circular	Raised	Entire

L: Leachate
S: Soil

[21]. Gram-positive bacteria have been reported to dominate in the production of PHAs from waste products, for example, *Bacillus* sp. has the ability to secrete hydrolytic enzymes that allows the bacteria to utilize low-cost substrates such as agricultural and industrial wastes [22, 23].

Despite generally accumulating lower amounts of PHA, Gram-positive bacteria are advantageous over Gram-negative bacteria since they lack of lipopolysaccharide (LPS) in the bacteria's external cell membrane which may make them a better source of PHA raw material applications and makes the PHA extraction much simple [24, 25]. The LPS in external bacterial cell may co-purify with crude PHA polymer during the extraction process [25].

Table 2: Microscopic observation of selected bacterial isolates after Gram, endospore and capsule staining at total magnification of 1000X.

Bacterial isolate	Gram staining	Cell morphology	Presence of endospore	Presence of capsule
L1	Pink (-ve)	Rod	No	Yes
L4	Purple (+ve)	Rod	Yes	Yes
S3	Purple (+ve)	Rod	Yes	Yes
S5	Purple (+ve)	Rod	Yes	Yes

L: Leachate
S: Soil

3.2. Molecular Identification using 16S rDNA Gene Sequencing

In order to determine the identity of the selected isolates, *16S rDNA* gene sequence analysis was performed. The 16S rDNA gene was amplified by using primers 27F and 1492R [26]. When a gel stained with DNA-binding dye, the DNA fragments can be seen as bands and each representing a group of same-sized DNA fragments. The Fig. 1 shows the image of bands obtained from amplification of 16S rDNA gene using gel electrophoresis. The expected size of amplicon of all selected bacterial isolates is 1465 bp *E.coli* was used as the positive control.

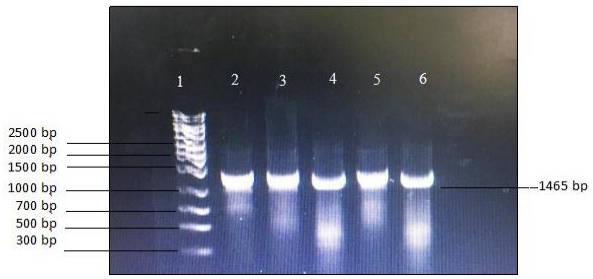

Fig. 1: Gel electrophoresis result for amplification of 16S rDNA gene (Lane 1: 1 kb Ladder, Lane 2: *E.coli* (positive control), Lane 3: L1, Lane 4: L4, Lane 5: S3, Lane 6: S5)

3.3. Molecular Sequencing of *16S rDNA* gene

DNA sequencing is a process of determining the nucleotide order from PCR product of given DNA fragment. The 16S ribosomal DNA nucleotide sequences for the four isolates were determined and compared with the databank contents by using Nucleotide BLAST [27](Higuchi-Takeuchi *et al*., 2016). BLAST analysis of nucleotide sequences for L1 showed a maximum of 99% identity towards *Klebsiella pneumonia* DSM 30104. The strain L1 belongs to Super kingdom: Bacteria, Phylum: Proteobacteria, Class: Gammaproteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae and Genus: Klebsiella. The bacterial strain belongs to phylum Proteobacteria, which is rod shape and Gram negative bacteria [28].

As for L4 and S3, BLAST analysis of nucleotide sequence showed high 99% and 83% similarity toward *Bacillus cereus* ATTC 1479, respectively. *B. cereus* is a Gram positive aerobic or facultatively anaerobic, rod-shaped bacterium that is widely distributed environmentally [29]. The strain L4 belongs to Kingdom: bacteria, Phylum: Fermicutes, Class: bacilli, Order: Bacillales, Family: Bacillaceae, Genus: Bacillus (National Centre Biotechnology Formation [NCBI]. *B. cereus* from leachate. Recently, Yu *et al.* [30] found that resistant bacterial strain *B. cereus* was found in landfill leachate. Study by Gomaa [9] showed that *B. cereus* was isolated from contaminated soil can produce PHA. According to the latest research by Motamedi [31], he found that *B. cereus* from oil contaminated soil also can produce PHA. Therefore, *B. cereus* mostly can be found in contaminated soil because contaminated soil contains high carbon, nitrogen and phosphorus ratio [31].

For the last bacteria which is S5, BLAST analysis of nucleotide sequence showed 89% of similarity towards *Bacillus toyonensis* BCT-7112. *B. toyonensis*, previously identified as *Bacillus cereus* var. *toyoi* is a member of the *B. cereus* group, which currently also comprises the species of *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. cytotoxicus.* All the members of the group are considered soilborne organisms, and some of the species are considered pathogens [32].

3.4. Identification of The Best Percentage of CPH for Production of PHA

The identification of the best percentage of CPH was done by extracting and recovering PHA using simple and effective methods which is cell lysis by chemical digestion of sodium hypochlorite. The use of sodium hypochlorite to extract PHA from biomass always results in severe degradation of PHA and yields PHA with a lower molecular weight [33]. Recovery of PHA from bacterial cells using organic solvents is often applied in industrial processes and in laboratory scale PHA extractions due to the recovery efficiency of the process, simplicity and rapidity [15].

Bacterial biomass is one of the methods used to study the quantification of PHA by bacterial isolates. Researcher assumed that the bacterial biomass consists of two components, which are residual biomass and intracellularly accumulated polymer (PHA). The residual biomass is the active part while accumulated polymer is metabolically inactive part [34]. Usually, dry weight of the microorganisms was used to calculate fluxes of carbon and energy of microorganisms [35]. The residual biomass was determined to study the details of metabolic intracellular fluxes of PHA. Percentage of PHA accumulation can be clarified as weight of PHA obtained to cell dry weight of bacteria [36]. Table 3 shows the result of the cell dry weight of bacteria, dry weight of PHA, residual biomass, percentage of PHA production and percentage yield obtained**.**

Table 3: Production of PHA by identified bacterial strains using different concentrations of CPH

Bacterial strains and origin	Concentration of cocoa pod husks % (w/v)	Cell dry weight (g/ml)	Dry weight of PHA (g/ml)	Residual Biomass (g/ml)	% of PHA accumulation (g/ml)	Yield (%)
K. Pneumoniae, L1	2	0.0089	0.0027	0.0062	30.34	0.135
	5	0.0094	0.0053	0.0041	53.38	0.106
	7	0.0102	0.0055	0.0047	53.92	0.079
	10	0.0089	0.0010	0.0027	11.23	0.010
B.cereus, L4	2	0.0077	0.0034	0.0043	44.16	0.170
	5	0.0082	0.0038	0.0044	46.34	0.076
	7	0.0100	0.0057	0.0043	57.00	0.081
	10	0.0053	0.0018	0.0035	33.96	0.018
B.cereus, S3	2	0.0026	0.0011	0.0015	42.31	0.055
	5	0.0066	0.0031	0.0035	46.97	0.062
	7	0.0091	0.0049	0.0042	53.87	0.007
	10	0.0054	0.0017	0.0037	31.48	0.017
B. toyonensis, S5	2	0.0072	0.0024	0.0048	33.33	0.120
	5	0.0109	0.0058	0.0051	53.21	0.116
	7	0.0093	0.0051	0.0042	54.84	0.081
	10	0.0089	0.0040	0.0039	44.94	0.040

* Data obtained based on three replicates.

K. pneumoniae, L1 strain showed the highest PHA production at 7% (w/v) CPH concentration with 53.92% (g/ml) PHA accumulation followed by 5% (w/v) with 53.38 g/ml PHA accumulation, 2% (w/v) with 30.34% (g/ml) PHA accumulation and lowest at 10% (w/v) CPH concentration which is 11.23% g/ml. Previous research by Apparao and Krishnaswamy [28], showed that *K. pneumoniae* was capable of producing PHA.

B. cereus, L4 strain isolated from leachate showed the highest PHA production at 7% (w/v) of CPH with 57.00 g/ml of PHA accumulation, followed by 46.24% at 5% (w/v) CPH concentration, 44.16 g/ml at 2% (w/v) concentration and 33.96 g/ml at 10% (w/v) of CPH concentration. According to Sangkharak and Prasertsan [8], bacteria belonging to genera *Bacillus* is one of the species that had ability to produce high amounts (2–6.58 g/l) of PHAs and high levels of hydrolytic enzyme activities. Carbon substrates are metabolized by many different pathways in bacterial cell [37] and *Bacillus*, *Aeromonas* sp. and *Alcaligenes* sp. had great ability to utilize a variety of substrates [8].

For S3 which also *B. cereus*, the production of PHA is higher at 7% (w/v) CPH concentration which is 53.87 g/ml PHA accumulation, followed by 5% (w/v) which is 46.97 g/ml PHA production, 2% with 42.31 g/ml of PHA production and 31.48 g/ml at 2% (w/v) CPH concentration. The PHA production for *B. cereus* (S3) is lowest at 10% (w/v) which is 31.48% (g/ml). This finding was supported by Gomaa [7] who isolated *B. cereus* that produced a co-polymer PHB (organic polymer) up to 48.43% from trinitrotoluene contaminated soil.

For S5 which is *B. toyonensis*, the PHA production is higher at 7% (w/v) concentration with 54.84 g/ml of PHA production, followed by 5% (w/v) with 53.21 g/ml of PHA production, 10% (w/v) with 44.94 g/ml PHA production and 2% (w/v) which is 33.33 g/ml PHA production. There is no other research reported that *B. toyononsis* can produce PHA and this study was the first to prove that *B. toyonensis* can produce PHA.

Bacterial biomass and PHA production are related with growth condition of particular strain of bacteria. From the result, as the biomass increased, the PHA production was reduced. Bacteria starts to accumulate PHA when the bacterial biomass increased. The PHA accumulation varied, which highest accumulation obtained at 7% (w/v) concentration and lowest at 2% (w/v). The PHA yield (gram of PHA per gram of substrate) also varied for all four concentrations of carbon source. The PHA yield with respect to amount of carbon source ranging from 0.007 (g/g) to 0.170 (g/g). The yield of PHA was highest at 2% (w/v) of CPH in *B. cereus* from leachate which is 0.170 (g/g), followed by 2% of CPH in *K. pneumoniae* which is 0.135 (g/g). This shows that PHA yield decrease when the concentration of CPH increase. This study was supported by Abid *et al.* (2016), which shows that PHA yield was highest at 2% of soybean oil but PHA accumulation was highest at 3%. According to Basak *et al*. [38], this can be due to the increase in cell biomass under nitrogen limitations.

Statistical analysis of one way ANOVA showed that there is significant difference between isolated bacterial species for production of PHA. This result was supported by Blamire [39], which is each bacterial species has its own characteristic and have different range of values in which it grows and reproduces best [39]. The size and the number of PHA per cell vary depending on the different species [15]. For example, the PHA production of *K. pneumoniae*, is highest at 7% (w/v) CPH concentration and lowest at 10% (w/v) CPH concentration. Meanwhile, *B. toyonensis* have highest percentage of PHA production at 7% (w/v) CPH but lowest at 2%

(w/v) of CPH. This can be concluded that different bacteria have different percentage of PHA production and have different growth ability.

Fig. 2 shows the effect of different concentrations of CPH solution against percentage of PHA accumulated by isolated bacterial strains. The PHA production were highest at 7% (w/v) of CPH solutions, followed by 5% (w/v) of CPH solution and 2% (w/v) CPH solution. The percentage of PHA production is lower at low concentration, which is 2% (w/v) of CPH. This is because, bacteria cells tend to alter their physiology when they were exposed to a medium with very little nutrient for a long time [37]. Hence, the PHA storage polymer starts to degrade when the bacteria lack of carbon source [40].

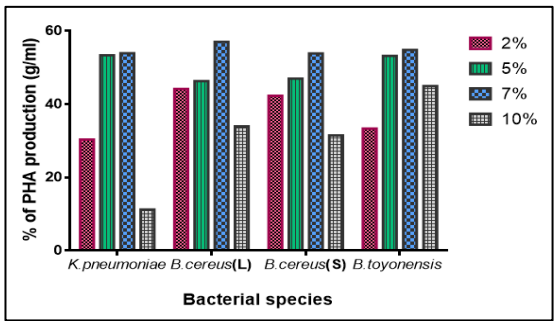

Fig. 2: Percentage of PHA accumulation at different concentrations of CPH solution

The production of PHA is lowest at 10% (w/v) of CPH solution except for *B. cereus* (S3). Even though the percentage of PHA accumulation increased as the percentage of CPH solution increased, the PHA accumulation tend to decrease when CPH concentration is 10% (w/v). This may due to inhibitory effect of high concentration of PHA. When bacteria starts accumulating PHA to maximum level, PHA tend to decrease after the highest biomass production. The PHA production decrease after the maximum biomass production because nutrient limitation causes the bacteria consume the PHA as a carbon source [41]. As a result, at 10% (w/v) cocoa pod husks solution, the PHA accumulation of bacterial isolates was decreased. Verlinden *et al.* [37] stated that, carbon substrates are metabolized by many different pathways in bacterial cell. Since L4 and S5 are same which is *B. cereus*, both of the bacteria reproduce best at 7% (w/v) CPH concentration followed by 5% (w/v), 2% (w/v) and 10 % (w/v) of CPH. Result of statistical analysis data of one ways ANOVA showed that there is a significant difference between different concentrations of cocoa pod husk used for optimization of PHA production since the P value is less than 0.05.

4. Conclusion

In conclusion, this study has led to the preliminary finding of bacterial strains that can produce PHA which are *K. pneumoniae*, *B. cereus* and *B. toyonensis*. In future, continuous PHA production should not only aim to increase the volumetric productivity, but also to open the door for tailor-made material properties by fine-tuning the polyester composition. This preliminary comparative analysis of three types of bacteria has led to the novel invention of a biodegradable environment friendly polymer with a high potential for future use.

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