ORIGINAL ARTICLE

Production of Polyhydroxyalkanoates (PHA) by Probiotic Bacteria *Bacillus tequilensis* for Potentially Used as Drug Carrier

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ABSTRACT

Introduction: Polyhydroxyalkanoate (PHA) is a biopolymer that can be produced by microorganisms from numerous low-cost carbon sources, making it an environmentally friendly material. This study was designed to utilize different food waste (household food waste, spent oils and spent coffee grounds) as nutrient source for the cultivation of microbes to produce polyhydroxyalkanoate (PHA). **Methods:** The bacterial strain Bacillus tequilensis was grown in 250 mL Erlenmeyer flask each containing 50 mL of mineral salt medium, 25 ml of nutrient broth inoculum and 20 g/L of household food waste, spent ground coffee and spent oils, respectively. The initial pH of the media was 7.0 and the cultured bacteria was incubated at 30 °C, 180 rpm for 72 h as a batch culture. The sample was then extracted and weight, and further analyzed using Fourier Transform Infrared Spectroscopy (FTIR) and High Performance Liquid Chromatography (HPLC). **Results:** *B. tequilensis* yielded PHA of 7 % to 8 % (g PHA/g dry cell weight) on average using medium containing household food waste, spent ground coffee and spent oils. FTIR analysis showed the peaks range between 1750-1730 cm-1 which belong to PHA functional groups such as C=O. HPLC chromatogram revealed that the retention time obtained from digested PHA was approximately 4.5 min which was similar to the standard of PHA. **Conclusion:** This enables the utilization of low-cost waste by probiotic *B. tequilensis* as a carbon source for the sustainable production of biodegradable PHA for a wide range of applications in medicine.
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Keywords: Drug carrier; Polyhyrdoxyalkanoate (PHA); Bacillus tequilensis; Household food waste; Spent oils; Spent coffee grounds; HPLC

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INTRODUCTION

Food waste is classified as food including drink and affiliated inedible parts, excluded from the human food supply chain in several segments such as food manufacturing, food retail, food service and households. Due to urbanization and population growth, Malaysia will produce over 25,000 tonnes of domestic waste per day (1). In response to growing public interest in the negative environmental impact of excessive food waste, especially from the household, several researches have been published on the use of food waste that can be developed into environmentally friendly biopolymers such as polyhydroxyalkanoate (PHA).

PHAs are water-insoluble polymers synthesized and stored in the cytoplasm of numerous bacteria and archaea. Typically, a microbe will produce PHA in the presence of nutrient-limiting concentrations of sulphur, phosphorus, nitrogen, or oxygen, as well as excess carbon obtained from food waste. PHA are biodegradable and eco-friendly, making them a potential replacement for petrochemically derived plastics (2). PHA have lately gaining popularity as a biofuel that can be produced from low-value substrates, such as wastes and wastewaters. Interestingly, PHA commonly provide a remarkable alternative to enhance the transport and release of drugs while the drugs being administered to patients due to the PHA characteristics which are biocompatibility, flexibility and biodegradability (3).

B. tequilensis is a thermostable bacteria that can produce a thermostable, solvent-resistant enzymes. Its resistance to temperature, pH, organic solvents, metal

ions, and surfactants promotes its usage in a variety of challenged processes. Additionally, it was discovered that *B. tequilensis* as a probiotic in biofloc promoted the expression of essential proteins, hence minimising the likelihood of developing diseases during culture. According to Sohail et al. (4), *B. tequilensis* was capable of producing PHA from animal fats and glycerol, which may have been the result of increased enzyme activity. Therefore, *B. tequilensis* will be utilized in this study to synthesize PHA from various types of household food waste. These results demonstrate the successful of bioconversion of waste into bioplastic materials by probiotic microorganisms and therefore can be intensified in order to increase environmental sustainability and increase the efficiency and biocompatibility of the drug delivery system.

MATERIALS AND METHODS

Sampling

The household food waste was collected from UTHM Pagoh Campus students' hostels consisting of spent cooking oils and various food waste and scraps. Spent coffee ground was collected from Kedai Kopi cap 1855 in Johor Bahru. Alshalif et al. (5) described the isolation of *B. tequilensis* from cement kiln dust (CKD) at Cement Industries Malaysia Berhad (CIMA).

A subculture method was used to maintain cultures of microorganisms, which were kept at 4°C and refrigerated. In order to prepare food waste slurry, it was diluted 1:1 with water and then filtered. Spent ground coffee was dried at 60°C. All samples and apparatus were autoclave for 15 min. Nutrient broth and agar were used in this experiment and consist of 5 g/l peptone, 5 g/l NaCl, 1.5 g/l yeast extract. The nutrient agar was prepared to collect the *B. tequilensis* while the nutrient broth was for sub-culture to prolong life and increase the number of cells. A single colony of bacteria was inoculated with 25 ml of nutrient broth and incubated at 37 °C for approximately 24 h.

Batch fermentation process

Batch fermentation was carried out by preparing mineral salt (MS) medium as moistening media. The composition of the mineral medium in g/l: 3 g/l of $(NH_4)_2SO_4$, 1 g/l of KH_2PO_4, 11.1 g/l of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g/l of $MgSO_4 \cdot 7H_2O$, 1 ml of microelement solution. The composition of microelement solution in g/l: 9.7 g/l of $FeCl_3$, 7.8 g/l of $CaCl_2$, 0.156 g/l of $CuSO_4 \cdot 2H_2O$, 0.119 g/l of $CoSO_4.7H_2O$, 0.118 g/l of $NiCl_2$, 0.062 g/l of $CrCl_2$ and 3.646 g/l of H_3BO_3. As indicated earlier, cultivation was carried out in a 250 ml Erlenmeyer flask which contained 50 ml of MS medium, 25 ml of nutrient broth inoculum and 20 g/l of household food waste, spent ground coffee and spent oils individually, as the only carbon source as previously mentioned by Van Thuoc *et al.* (6). The flasks were incubated for 72 h at 30 °C in an incubator shaker (180 rpm) (7). Concentration of 0.1 mol/l NaOH was used to adjust pH to 7. All experiments were performed in triplicate.

PHA extraction

The pellets were centrifuged, dried, and weighed after 72 h. The hypochlorite method was modified slightly to extract PHA from *B. tequilensis*. Sodium hypochlorite was added to the dried pellet and incubated for 2 h at 37 °C for the lysis of cells (8). Undissolved debris was removed by centrifuging at 4200 rpm for 30 min after adding hot chloroform to extract PHA. A final centrifugation of the pellet at 4200 rpm for 30 min followed by a rinse with water and an ethanol-acetone (2:1) mixture was applied to the PHA pellets (9). Using pre-weighed filter paper as a filter, PHA was collected and at 70 °C the pellet was dried until achieved a constant weight (10). Calculating the weight of extracted PHA and quantifying PHA yield % was performed using the Eq. 1 below:

$$\text{PHA yield (\%)} = \frac{\text{PHA weight (g)}}{\text{Dry cell weight (g)}} \quad \text{Eq.1}$$

Statistical and analytical methods

The experiments were conducted in triplicate, and the means results were presented in table 3.1. At the p=0.05 level, a one-way analysis of variance (ANOVA) was conducted to determine the significance of the difference between mean values for comparison, and various letters indicate significance. FT-IR spectroscopy was used to analyze the functional groups in the sample structure and HPLC was used to determine the extracted PHA polymer. Direct FTIR scanning was performed on all samples, both before and after extraction. PHA can be detected using FT-IR spectroscopy (11). For HPLC analysis, the samples were eluted with 5 ml C18 110 column and performed in isocratic mode with water/ACN 70:30 at a flow rate of 0.6 mL/min at 25 °C. A combination of 700 ml distilled water and 300 ml acetonitrile is used for the mobile phase.

RESULTS

Percentage of PHA production

It is well-known that food waste from households has a high carbon content, which is ideal for PHA-producing bacteria because inclusion bodies are produced as energy reserves when carbon content is high (4). This study showed that the most PHA production for *B. tequilensis* was from spent oils and ground coffee at 8 % as carbon sources than with household food waste (7 %) after 72 h of incubation. It can be seen from Table I that *B. tequilensis* able to produce PHA with different types of carbon sources. According to Sohail *et al.* (4), among thirteen isolates, *Pseudomonas aeruginosa* and *B. tequilensis* were the only two

Table I : Cell dry weight and PHA production (%) obtained after cultivation of *B. tequilensis* using three different carbon sources

Samples	Spent oils			Household food waste			Spent coffee ground		
	Cell Dry Weight (CDW) (g/l)	Dry weight of extracted PHA (g/l)	% PHA	Cell Dry Weight (CDW) (g/l)	Dry weight of extracted PHA (g/l)	% PHA	Cell Dry Weight (CDW) (g/l)	Dry weight of extracted PHA (g/l)	% PHA
1	10.0	1.0	10%	13.8	0.8	6%	14.4	1.2	8%
2	14.5	1.6	11%	15.2	0.9	6%	14.8	1.4	10%
3	12.9	0.5	4%	9.3	0.7	8%	14.0	0.8	6%
Average	12.5	1.0	8%	13.0	0.8	7 %	14.4	1.1	8%

Table II : ANOVA results between the means of the three types of food waste as carbon sources on the PHA production (%)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.666667	2	2.333333	0.355932	0.714372	5.143253
Within Groups	39.33333	6	6.555556			
Total	44	8				

that could grow efficiently on PHA detection media containing all carbon sources. Moreover, all samples used contain carbon-rich composition making it an ideal environment for bacteria that produce PHA. Meanwhile, in Table II, the p-value for ANOVA analysis was more than 0.05 which was 0.71, thus the null hypothesis was accepted where there is no difference between the means of the different samples in terms of PHA production using three different carbon sources. It clearly showed that there was no significant influence on PHA production by *B. tequilensis* either independently or in the interaction with the three different carbon sources. Thus, it proved that food processing waste can be used as sustainable carbon sources to synthesize the PHA using *B. tequilensis* in the bioplastics market. Further, no significant interactions were found regarding carbon sources used between groups, possibly because the drying period was too short to see the significant effects of their interactions. The F-value is a ratio of the squared deviation to the mean of the squared error. The groups F-values of 0.36 indicate factors that have a significant impact on the production of PHA. The higher the F-value, the stronger the impact on PHA production (12).

FT-IR analysis before and after sample extraction

FTIR spectroscopy was used to characterize the PHA extracted from *B. tequilensis*. Single peaks between 4000 cm^{-1} and 400 cm^{-1} were used for functional group analysis. Samples from before extraction and samples from the extraction were taken and analyzed. The peak was plotted using Agilent Resolutions Pro as shown in Figure 1. The extracted polymer and FTIR peaks obtained were compared to the different results from previous research together with FTIR peaks of the standard PHA (8, 11, 13). Starting off with the samples from before extraction, the first one was samples from spent oils (Figure 1A). Carbonyl (C=O) stretching in the ester group was responsible for the strong band observed at 1743 cm^{-1}. A band at 1461 cm^{-1} was the result of an asymmetrical deformation of the C-H bond in CH_2 groups, whereas the band at 1381 cm^{-1} represented CH^3 groups. The band at 3334 cm^{-1} and 2386 cm^{-1} was formed by terminal OH groups. In addition, it was determined that the bands at 2922 cm^{-1} corresponded to C-H stretching methyl, whereas the band at 2853 cm^{-1} corresponded to methylene groups. Alkyl halides can be detected at wavenumbers 1115 cm^{-1} and 987 cm^{-1}, confirming the polymer is scl-PHA (13).

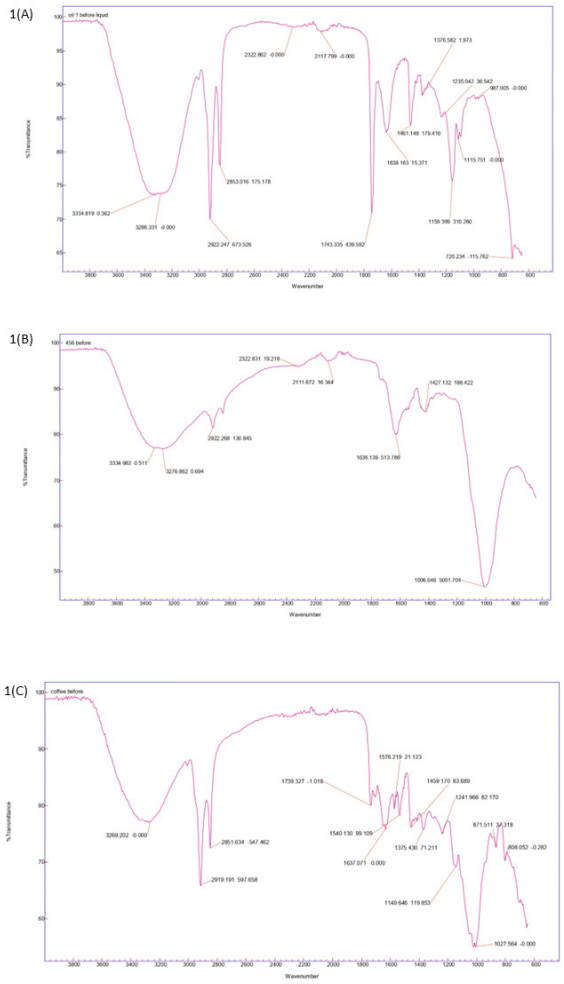

Figure 1 : FTIR analysis of PHA isolated from *B. tequilensis* cells before extraction was carried out: (A) Sample from spent oils (B) Sample from household food waste (C) Sample from spent coffee ground.

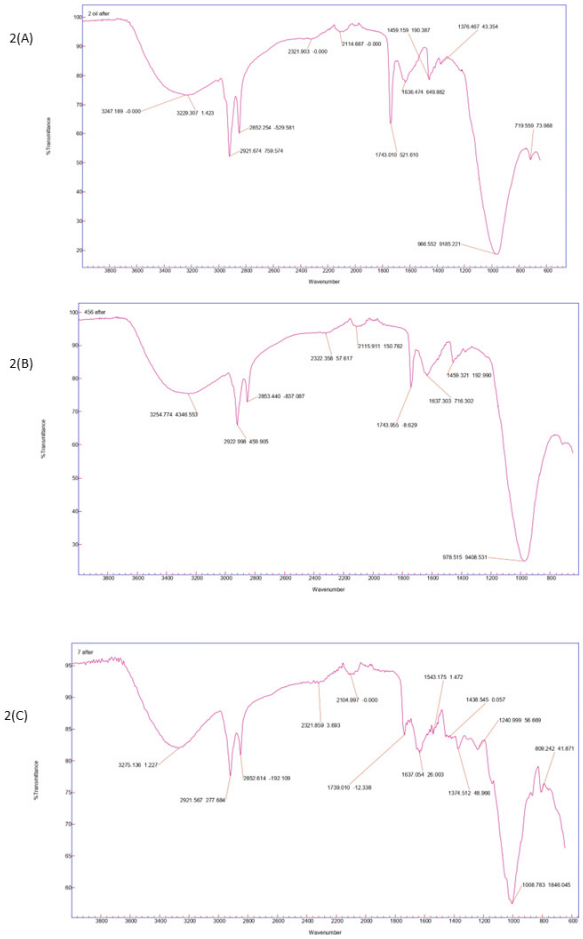

Figure 2 : FTIR analysis of PHA isolated from *B. tequilensis* cells after extraction was carried out: (A) Sample from spent oils (B) Sample from household food waste (C) Sample from spent coffee ground.

Figure 1(B) showed the strongest peak was observed at 1006 cm^{-1}, which corresponds to C-O-C groups in the polymer. There are four absorption bands near 3334 cm^{-1}, 2922 cm^{-1}, 1638 cm-1 and 1450 cm^{-1} related to CH_3, CH_2 and -CH groups, respectively. The absorption band had around the same peaks and confirmed that it was a short chain length (scl)-*co*-medium chain length (mcl) PHA (8). Meanwhile, Figure 1c shows representative spectra absorp the major cellular constituent which was found to have very similar peaks compared to previous research (14). In addition to the intense ester carbonyl stretch at 1739 cm^{-1}, PHB shows many bands with wavenumbers ranging from 1460 cm^{-1} to 1000 cm^{-1}, corresponding to methylene (CH_2) and methyl (CH_3) deformations and C-O stretching. In the lower peaks of the spectral spectrum, protein absorbance was evident as strong features due to the organism itself (14). A band of amide I at 1637 cm-1 was mainly caused by stretching vibrations of the amide carbonyl, while a band of amide II at 1540 cm^{-1} was mainly caused by N-H bending vibrations. Multiple C-C stretches and medium length bands were observed in the extracted polymers at 966.5 cm^{-1}, indicating alkane groups.

On the other hand, FTIR result for sample after extraction showed a small peak of C-O bond at 2921 cm^{-1} which expressed a same peak at 2923 cm^{-1} as previously mentioned by Javaid et al. (8). A scl-PHA, mcl-PHA or scl-mcl-PHA were observed at 1743 cm^{-1} based on characteristic of ester carbonyl band (13). Compared to this study, at least one FTIR analysis from every sample has the same wavenumber as mentioned in Hong et al. (15). Figure 2(A) and 2(B) has a signal at 1743 cm^{-1} that demonstrated its ester carbonyl bands which was a characteristic of scl-*co*-mcl PHA (16). Due to the stretching vibrations of the amide carbonyl, the amide I band ranges between 1637 - 1639 cm^{-1}. The amide II band ranges between 1459-1560 cm^{-1}. Protein content was the only factor affecting the amide I and II bands at all PHA concentrations (14). It showed the sharpest peak was at 978 cm^{-1}. Similarly, this peak had an extremely sharp definition, which indicated that the distance between C-C stretches (8).

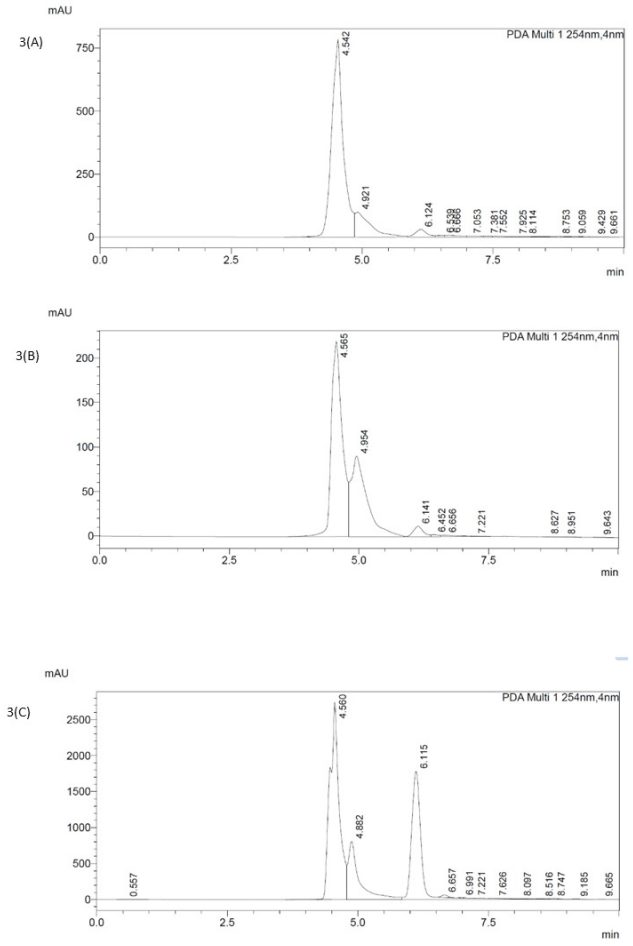

Figure 3 : HPLC analysis of PHA isolated from *B. tequilensis* cells before extraction was carried out at 254 nm: (A) Sample from spent oils (B) Sample from household food waste (C) Sample from spent coffee ground.

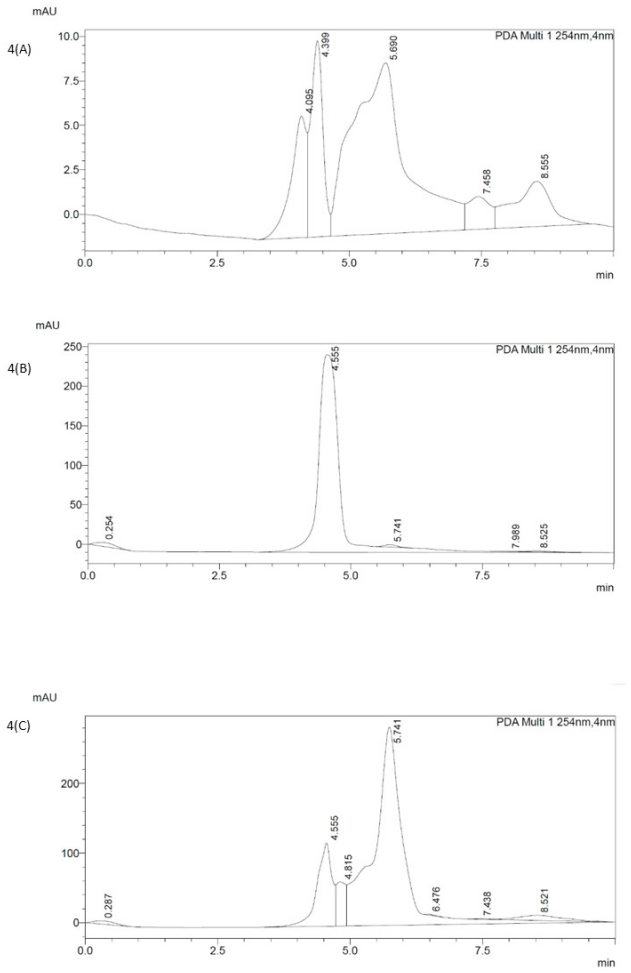

Figure 4 : HPLC analysis of PHA isolated from *B. tequilensis* cells after extraction was carried out at 254 nm: (A) Sample from spent oils (B) Sample from household food waste (C) Sample from spent coffee ground.

In Figure 2(C), PHA from the selected spent coffee ground samples isolates from *B. tequilensis* showed C=O band near 1739 cm^{-1}. The extracted polymer displayed a C-H bond stretch in the range of 2974 cm^{-1} -2851 cm^{-1}. Compared to PHA standard by Yasin and Al-Mayaly (11), the peaks of C-H in the methylene group O-H were between 2962 – 2877 cm^{-1}. As a result of the disturbance in the structure of the polymer, this difference could be attributed to the extraction process. In this experiment, peaks were compared between the PHA standard and the extracted polymer. It was found that this polymer might not be as pure as the PHA standard. Figure 2(C) have similar highest peaks which were at 1008 cm^{-1} that represented the C-O-C chemical groups of the polymer. The peaks between 1636 cm^{-1} and 1240 cm^{-1} were due to the presence of the C=O and C-O compared to the FTIR analysis PHA standard from Yasin and Al-Mayaly (11).

HPLC Analysis

The HPLC method was used to analyze all three samples, including those before extraction. This is shown in Figure 3. HPLC analysis obtained from this experiment was being compared to the standard HPLC analysis research by Sayyed et al. (17). Figure 3(A), 3(B) and 3(C) showed one large peak at 4.5 min which corresponded to the retention time from previous studies by Watanabe et al. (18) and Mozes-Koch et al. (19) and might potentially be identified as PHA or poly(3-hydroxybutyrate), PHB. There is a similar retention time for all three samples when compared to the PHB standard, indicating that monomers are formed during the reaction. However, there is a tendency for some HPLC methods to require a longer processing time to analyze PHA, but by using a shorter column and acetonitrile as a mobile phase can significantly shorten the analysis to as little as 10 minutes (18). As a result of the modification of this HPLC method, a high-throughput analysis of PHA composition using HPLC analysis has been made possible to reduce the analysis time. The highest peaks for Figure 4(A) and 4(B) were at 4.5 min but for Figure 4(C) was at 5.7 min. A single peak that appeared at the same retention time was an

indication that the PHA produced in this case was the same as that produced in the previous case.

DISCUSSION

Factors affecting the PHA production from *Bacillus tequilensis*

Using all these three types of carbon source as a low-cost carbon and nitrogen substrates like peptone and yeast extract and isolates using *B. tequilensis* able to produce PHA with up to 14 g on cell dry weight basis. PHA is produced by most microbes at a pH of around 7. Compared with acidic and alkaline pH levels, neutral pH produces greater dehydrogenase enzyme activity and substrate degradation in microaerophilic environments. As a result, PHA production increased (8). Incubation time also plays an important role for PHA production. Based on previous research, incubation time at 24 h, 48 h, 72 h and 96 h were the most studied. A 72 h incubation time was optimized as it proved to produce PHA using *Bacillus sp.* the highest at 63% (9, 11). The amount of PHA accumulated in the media studies was similar. These accumulations peaked after 72 hours of cultivation. A PHA content of 7 % to 8 % was achieved using food waste as a carbon source during cultivation of *Bacillus sp*, which was comparable to the result (8 % to 9 %) obtained from previous research using volatile fatty acids from food waste (9). It is known that as the temperature increases above 40 °C, biopolymers production tends to decrease due to denaturation of the polymers (16). There was found to be an optimal temperature of 30 °C for producing 10 % of PHA from 0.96 g/l DCW and similar results were obtained from other *Bacillus sp.* which proved that 30 °C was the optimum temperature for producing PHA (7). Above all, in this study, there were two types of condition that showed no significant influenced of the percentage of PHA production, which were liquid substrate and solid substrate. The first was medium containing liquid spent oils or household food waste while the second was, solid substrate for spent ground coffee. Based on previous study, both types of substrates have their advantages and were able to produce a high percentage of PHA up to 70 %. Respectively, in this study both types of fermentation were able have the same percentage of PHA production which were 7% - 8%.

CONCLUSION

In this study, all food waste was determined to be an excellent source for isolating PHA-producing bacteria. In addition, *B. tequilensis* has also been confirmed as a potentially useful biopolymer producer with the ability to accumulate PHA by using cheap, readily renewable carbon sources, namely spent oils, spent coffee grounds and household food waste. In order to successfully produce biodegradable polymers, it is extremely crucial to select bacteria that produce PHA in an efficient manner and to work towards optimizing conditions that are most conducive to PHA synthesis.

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