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ISOLATION AND CHARACTERIZATION OF POLYHYDROXYALKANOATE (PHA)-PRODUCING, CELLULOLYTIC BACTERIA FROM MUNICIPAL WASTEWATER

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ABSTRACT

Polyhydroxyalkanoates (PHA) are linear bio-esters produce in nature by certain microorganisms particularly when subjected to limited nutrients sources such as nitrogen while carbon sources are at abundance. PHA has attracted tremendous amount of attention from scientists globally due to their biodegradable property as environment-friendly alternative to petrochemical based plastics. However, the biggest drawback in using PHA is their high production cost. The aims of this study are to assess and characterise PHA-producing bacteria from municipal wastewater and compare their efficiencies in PHA production. A total of 34 out of 282 bacterial isolates from wastewater showed the ability to produce PHA using Sudan Black B staining. These isolates were then subjected to cellulase activity screen using carboxymethyl cellulose (CMC) agar. Two out of the 34 isolates were showed promising cellulose degrading capability and the cellulolytic activities were studied at 24, 48 and 72 hours of incubation respectively. The PHA production efficiencies of both isolates UiTM-E1 and UiTM-E2 were then compared using mineral salt media (MSM) supplemented with 1% and 2% glucose at 24, 48 and 72 hours of incubations respectively. Isolate UiTM-E2 showed the highest PHA production at 36.93% of its cell-dry weight (CDW) at 48 hours incubation.

Key words: Polyhydroxyalkanoates, bacteria, wastewater, cellulolytic

INTRODUCTION

Plastics have proven time and again to play an important role in the improvement of our lifestyles in various ways. They are frequently used in numerous sectors such as packaging, construction, medical, automotive, and many more. According to statistics, the global production of plastic is reaching almost 370 million tons in 2020 and is expected to double over the next 20 years (Rouch, 2021). Even though some plastic waste such as those made of PE, PET, and PP has been recycled, the volume of recycled plastics is tremendously low compared to those that were discarded. Based on the statistics, only 10% of the produced plastics are recycled while the majority of the used plastic and plastic waste ends up in landfill causing pollution of the soil and groundwater due to the leaching of toxic waste derivatives (Thunman *et al*., 2019). One of the major sets back of recycling plastics is its high cost. The high cost of recycling plastics is mainly due to the presence of mixed plastic which would require them to be collected and sorted based on the types of plastic, before removal of impurities, shredded into the small piece before finally being processed for recycling (Wierckx *et al*., 2018).

Naturally, biodegradable materials such as polynucleotides, polyanhydrides, polysaccharides, and many others have been identified as potential candidates to be used as a substitution for non-degradable petroleum-based plastics (Steinbüchel, 2001). Polyhydroxyalkanoates (PHAs) from the group of polyoxoesters have caught the attention of many scientists due to their biodegradable thermoplastic properties (Rawte & Mavinkurve, 2001; Mascarenhas & Aruna, 2017).

Polyhydroxyalkanoates (PHA) are microbial polyesters that serve as intracellular carbon storage and energy reserve. These biopolymers are generally produced by many bacteria when excess carbon is present while other nutrients such as nitrogen are limited. The fully biodegradable property of PHA has caught the interest of many scientists, making PHA a leading candidate to be used in the production of biodegradable plastics (El-malek *et al.*, 2020; Bassi

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et al., 2021). Besides biodegradable plastics, PHA has also been targeted as a synthetic polymer for medical applications due to its biocompatible, biodegradable, and non-toxic characteristics. The monomer of PHA (R-3-hydroxybutyric) is a natural constituent found in the blood. Since PHA is non-toxic to humans, it can be utilized as physical support in tissue engineering (Dwivedi *et al.*, 2020).

One of the major challenges in PHA is the high cost of production which is partly due to the cost of carbon sources (Khatami *et al*., 2021). A recognized strategy to overcome this issue is by using renewable resources like agricultural and industrial waste as carbon feed for microbial PHA production (Mohan *et al*., 2021). However, when using cellulosic biomass as a carbon source, it requires acid pre-treatment to partially hydrolyze the cellulose. This additional step would also contribute to the increase in the overall cost of PHA production. Thus, discovering PHA-producing bacteria with the cellulolytic capability to eliminate the acid hydrolysis pre-treatment step when using cellulosic biomass as the carbon source for PHA production would be highly beneficial since it will open up the possibility of producing PHA using agricultural waste as the carbon source directly without the need of pre-treatment step. This would lower the cost of PHA production substantially.

In this study, we isolated and characterized PHA accumulating bacteria with cellulolytic capability from municipal wastewater. Two out of 34 bacterial isolates that have the capability of producing PHA which possesses these dual capabilities were selected for identification using the Biolog™ GEN III MicroPlates system. The PHA production efficiencies and cellulolytic capabilities of these two isolates were also analyzed.

MATERIALS AND METHODS

Sample collection

Municipal wastewater was collected from Chancellery building waste treatment plant in Universiti Teknologi MARA (UiTM) Shah Alam for the isolation of PHA-producing bacteria. The sample was collected in a 250 mL Schott bottle and was transported on ice to the laboratory before storage in a 4 °C refrigerator until used.

Bacterial isolation and purification

Ten-fold serial dilutions of the municipal wastewater were prepared using sterile phosphate-buffered saline (PBS) with a pH of 7.4. Sample of 100 µL from each dilution was plated on nutrient agar medium containing 0.5% peptone, 0.3% beef extract, 0.5% NaCl, and 1.5% bacteriological agar. Cycloheximide (10 µg/mL) was also supplemented in the isolation culture medium to prevent fungal growth. The sample was evenly spread using a sterile L-shaped glass rod on the surface of the nutrient agar. The inoculated agar was then incubated at 37 °C incubator for 24 h. Well, isolated colonies were sub-cultured into fresh nutrient agar for pure culture preparation. The isolation procedure was done in triplicates for each dilution.

Screening for PHA-accumulating bacteria

The purified bacterial isolates obtained from the isolation procedure were subjected to screening of PHA production. The isolates were first grown in nutrient agar for 16-18 h. Then they were transferred to Mineral Salt Media (MSM) prepared as described by Raj *et al*. (2014) with 1% glucose supplemented as the carbon source and incubated for 72 h. The MSM The ability of the isolates to accumulate PHA was tested using Sudan Black B dye. The plates containing the streaked isolates were flooded with an ethanolic solution of Sudan Black B (0.05%) and left undisturbed for 20 min. Subsequently, the cultures were washed with 95% ethanol to remove access dye on the agar. Cultures that retained the dark color of the Sudan Black B dye were taken as bacterial isolates capable of accumulating PHA. The PHA accumulating isolates were further characterized using Gram staining to determine their Gram identities as well as to observe their morphological characteristics.

Screening for cellulolytic properties.

Bacterial isolates that were found positive for PHA production by Sudan Black B staining were then cultivated in CMC agar containing 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.5% carboxymethylcellulose sodium salt, 0.02% yeast extract and 1.5% agar. The isolates were streaked onto CMC agar and incubated for 72 h at 37 °C. Then, the plates were flooded with Gram's iodine (1.0 g iodine, 2.0 g KI dissolved in 300 mL of distilled water) for 3-5 min as described by Kasana *et al*. (2008). Isolates that produce clear zones around bacterial growth indicate their cellulolytic capabilities.

Identification of cellulolytic PHA-producing bacteria.

The isolates that were found to be positive as PHA producers while showing cellulolytic activity were further subjected to phenotypic profiling and identification using the Biolog™ GEN III MicroPlates system. Colonies were picked up from nutrient agar plates and inoculated into inoculating fluid (IF-B) according to the manufacturer's instructions. The inoculated IF-B was then dispensed (100 µL) into the 96-wells microplate and incubated at 37 °C for 24 h. Subsequently, the reading of the microplate was performed using an automated computerized Microstation™ system (Biolog Inc., USA) and the results were interpreted by the system identification software (Gen III database, version 5.2.1).

Cellulolytic activity analysis

The isolates with cellulolytic capabilities were first cultivated in nutrient broth for 16 h at 37 °C before 5 µL of the cultures were spotted on freshly prepared CMC agar respectively. The plates were then incubated at 37 °C for 24, 48, and 72 h respectively in triplicates. Subsequently, the plates were then flooded with Gram's iodine for 3-5 min to allow the detection of halo zones indicating CMC hydrolysis. The cellulolytic index for each culture was calculated using the following Equation 1 (Bradner *et al*., 1999).

Equation 1:

$$Cellulolytic\ index = \frac{(Diameter\ of\ clear\ zone - Diameter\ of\ bacterial\ growth)}{Diameter\ of\ bacterial\ growth}$$

PHA production efficiencies

The PHA production efficiencies were determined by measuring the amount of PHA production by the selected individual isolates using 500 mL of MSM media supplemented with glucose (1% & 2%) as the carbon source for 24 and 48 h respectively. After cultivation, the cells were harvested by centrifugation at 6000 × *g* for 15 min. The cell pellets were respectively washed twice with ice-cold sterile distilled water. The supernatants were carefully removed to minimize the loss of cell mass. The extraction of PHA from the cell pellet was done using the method described by Ratnaningrum *et al*. (2018) with modifications. The cell pellets obtained after centrifugation were dried at 40 ºC until constant weights were achieved, and the weight was recorded as cell dry weight (CDW). The cell pellets were then re-suspended in 15 mL of 2% NaOCl. Then 15 mL of chloroform were added to the suspension respectively, and the mixture was incubated at 24 °C at 150 rpm for 2 h to allow cell lysis. Subsequently, the mixture was centrifuged at 2000 rpm for 10 min to obtain good separation of the chloroform layer. The lower chloroform layer was then collected and allowed to be evaporated. The extracted PHA was then washed twice with ice-cold distilled water and dried until a constant weight is obtained, and the weight is recorded as dry extracted PHA weight. The percentage of PHA accumulation was then estimated using the following Equation 2 (Munir *et al*., 2015):

Equation 2:

$$PHA\ accumulation\ (\%) = \frac{Dry\ weight\ of\ extracted\ PHA\ (\frac{w}{L})}{Dry\ cell\ weight\ (\frac{g}{L})} \times 100$$

Fourier transformed infrared spectroscopy (FTIR) analysis

The FTIR analysis was conducted in the attenuated reflection mode (ATR) by direct analysis of the PHA films with a Thermo Scientific Nicolet 6700 spectrometer equipped with an ATR diamond module. The FTIR-ATR spectra were collected with 32 scans per run at 600 – 4000 cm^{-1} with a resolution on 4 cm^{-1} and an interval of 2 cm^{-1}.

RESULTS AND DISCUSSION

Isolation, characterization, and identification of PHA-producing cellulolytic bacterial isolates

A total of 108, 93, and 113 colonies of bacteria grew on the three 10^{-3} dilution nutrient agar plates respectively. From the serial dilutions of the wastewater sample, 282 well-isolated colonies were randomly picked for screening of PHA producers. Thirty-four bacterial isolated could retain the Sudan Black B dye indicating potential PHA producers (Figure 1). When subjected to cellulolytic analysis, 2 out of the 34 isolates were found positive and were designated as UiTM-E1 and UiTM-E2 as shown in Figure 2. Gram staining analysis revealed both the bacterial strains to be Gram-positive bacilli.

Biolog™ GEN III MicroPlates system which tests for 94 carbon and chemical sources in one microplate has been recognized as a reliable system for identification up to the species level (El-Liethy *et al*., 2018). In the physiological test conducted using the Biolog™ GEN III MicroPlates system, a positive reaction would be indicated by the formation of purple color due to the reduction of the tetrazolium redox dye. A positive reaction would indicate the ability of the isolate to metabolically utilize the specific substrate each well contains. The collective results of these biochemical reactions will then be used to compare to the database of the system thus allowing the identities of the isolates to be determined. Both UiTM-E1 and UiTM-E2 isolates were successfully identified using the GEN III Biolog system as *Bacillus cereus/thuringiensis* with a probability score of 0.910 and 0.851 and similarity index of 0.702 and 0.664 respectively. The only difference between *B. cereus* and *B. thuringiensis* is that the former contains an insecticidal toxin gene, which is usually plasmid-encoded. The loss of this plasmid would render *B. cereus* to be identified as *B. thuringiensis*. Thus, to determine the species of the strains UiTM-E1 and UiTM-E2 respective, further analysis in detecting the presence or absence of the mentioned insecticidal toxin gene is necessary and will be done in near future.

In terms of chemical sensitivity, both *Bacillus cereus/thuringiensis* UiTM-E1 and UiTM-E2 showed similar profiles where they are resistant to NaCl (8%), Rifamycin SV, minocycline, guanidine HCl, lithium chloride, potassium tellurite, aztreonam, sodium butyrate, and sodium bromate. *B. thuringiensis* UiTM-E1 and UiTM-E2 are also sensitive to vancomycin, tetrazolium violet, tetrazolium blue, lincomycin, niaproof 4, and troleandomycin.

Table 1 and 2 summarises the carbon and amino acid metabolism obtained through the physiological test conducted using the Biolog™ GEN III MicroPlates

system. Both UiTM-E1 and UiTM-E2 strains showed similar carbon metabolism except for turanose where strain UiTM-E1 showed an inability to hydrolyze the reducing disaccharide. Overall, both the isolates showed diverse carbon metabolism.

Cellulolytic index analysis

The cellulolytic index analysis indicates the capability of the tested bacterial isolates to break down cellulose. The calculated cellulolytic index analysis of *Bacillus cereus/thuringiensis* UiTM-E1 were 3.43 ± 0.74, 3.26 ±0.25, and 1.78 ± 0.80 at 24, 48, and 72 h respectively. The cellulolytic activity of this isolate was highest after 24 h of growth, nevertheless, the reduction of the cellulolytic activity after 48 h was not significant compared to 24 h. However, at 72 h of incubation, the cellulolytic index was significantly lower (Figure 3).

Interestingly, for *Bacillus cereus/thuringiensis* UiTM-E2, the highest cellulolytic activity was seen after 48 h of incubation. The cellulolytic index almost doubled at 48 h compared to 24 h. Additionally, the cellulolytic index of both the isolates was found to be higher than that of the reported cellulolytic index of other reported *Bacillus* species (Apun *et al*., 2000; Hastuti *et al.*, 2014; Nelson *et al.*, 2021). This indicated a high potential for these two isolated to be utilized as PHA producers using cellulosic materials as carbon sources directly, without having a pre-treatment step to break the cellulosic material down to simpler sugars before they can be used to synthesize PHA.

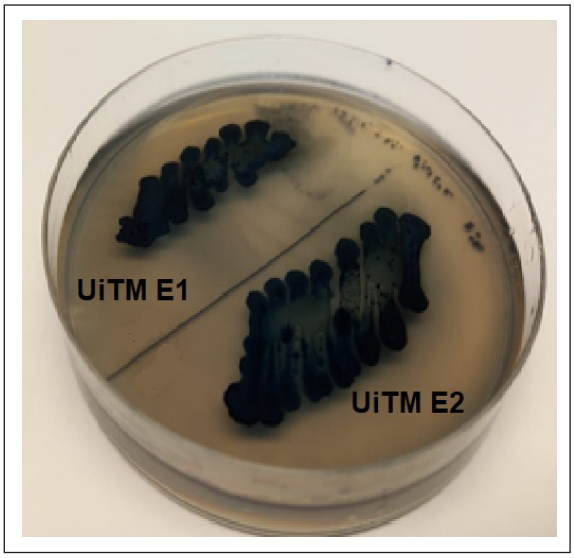

Fig. 1. Sudan Black B staining of *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 after 72 h of incubation on Mineral Salt Media (MSM) supplemented with 1% glucose.

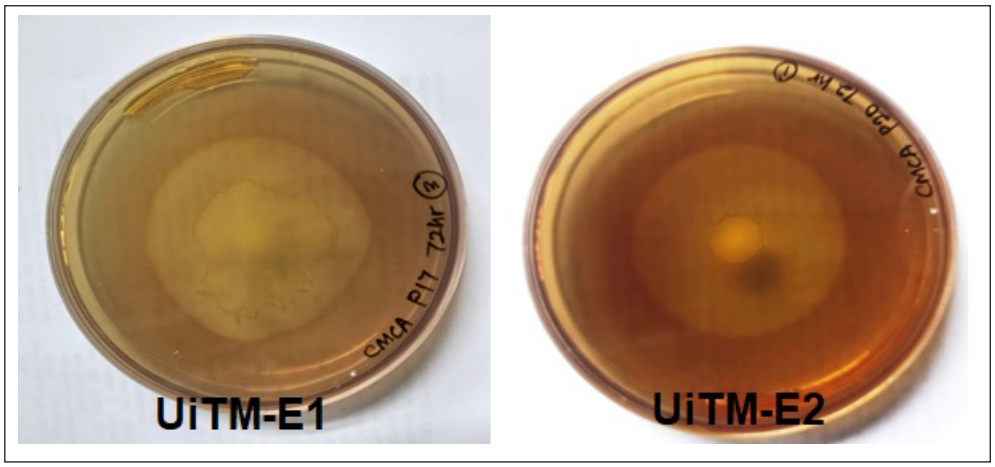

Fig. 2. Cellulolytic activity of *Bacillus cereus/thuringiensis* strain UiTM-E1 and UiTM-E2 on CMC agar after 72 h of incubation.

Table 1. Carbon utilisation profiles of *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 using the Biolog™ GEN III MicroPlates system

	Test substrate	*Bacillus cereus/thuringiensis* strain UiTM-E1	*Bacillus cereus/thuringiensis* strain UiTM-E2
.Sugars	D-maltose	+	+
	D-mannose	-	-
	D-galactose	-	-
	D-trehalose	+	+
	Sucrose	+	+
	Dextrin	+	+
	α-D-lactose	-	-
	Turanose	-	±
	D-cellobiose	+	+
	β-gentiobiose	+	+
	D-melibiose	±	±
	Stachyose	-	-
	D-raffinose	-	-
	α-D-glucose	+	±
	D-froctose	+	+
	L-fucose	±	±
	D-rhamnose	-	-

(+) reaction is indicated by colour change, (-) reaction is indicated by no change in colour, while (±) reaction is indicated by weak colour change

Table 2. Amino acid metabolism profiles of *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 using the Biolog™ GEN III MicroPlates system

	Test Substrate	*Bacillus cereus/thuringiensis* strain UiTM-E1	*Bacillus cereus/thuringiensis* strain UiTM-E2
Amino acids	D-serine	+	+
	L-arginine	+	+
	Gly-Pro	+	+
	L-alanine	+	+
	L-aspartic acid	-	-
	L-histidine	+	+
	L-glutamic acid	+	+
	L-pyroglutamic acid	±	+
	L-Serine	+	+

(+) reaction is indicated by colour change, (-) reaction is indicated by no change in colour, while (±) reaction is indicated by weak colour change.

PHA production efficiency analysis

The capacity of *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 of producing PHA was determined and compared using MSM media prepared as described by Teeka *et al*. (2010) supplemented with various concentrations of glucose as the sole carbon source were studied at different incubation period and the results of the study were presented in a graph shown in Figure 4. Both *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 showed higher production of PHA using 2% glucose. The highest percentage of PHA production was 36.93 ± 1.17% by *Bacillus cereus/thuringiensis* strain UiTM-E2 in MSM media supplemented with 2% glucose after 72 hr of incubation. The highest amount of PHA produced by *Bacillus cereus/ thuringiensis* strain UiTM-E1 was 33.55 ± 0.81% in MSM supplemented with 2% glucose after 72 h of incubation. It is highly possible to improve on this high amount of PHA production with optimization as shown by Evangeline and Sridharan (2019) with their *Bacillus cereus* VIT-SSR1 where the PHA production using molasses as a carbon source could reach 40.3 ± 0.77% after optimization.

In comparison, stain UiTM-E2 was able to produce a higher amount of PHA than strain UiTM-E1 even though they were identified as the same genus and possibly the same species. Similarly, the dry cellular biomass of strain UiTM-E2 was also found generally higher than that of strain UiTM-E1. Evidently, as the CDW of strain UiTM-E1 increases, the percentage of PHA production also increases over time. Nevertheless, in the case of strain UiTM-E2, the CDW after 48 h of incubation showed a significant decrease, but interestingly, even with the decrease in CDW, the amount of PHA production continued to increase.

Fourier transformed infrared spectroscopy (FTIR) analysis

FTIR analysis was done to identify the side chains and functional groups of the extracted PHAs from *B. cereus/thuringiensis* strains UiTM-E1 and UiTM-E2. The peaks observed in the region from 2930 cm^{-1} to 2975 cm^{-1} indicate the symmetrical and asymmetrical stretching of the methyl groups. The peaks in the spectra region of 1720 cm^{-1} confirm the presence of carbonyl group (C=O) which strongly suggests medium length chain (MCL) PHA molecule (Lopez-Cuellar *et al*., 2011). The intermittent vibration bands in the spectra between 1455 cm^{-1} and 1000 cm^{-1} are assigned to the stretching vibrations of C-O groups.

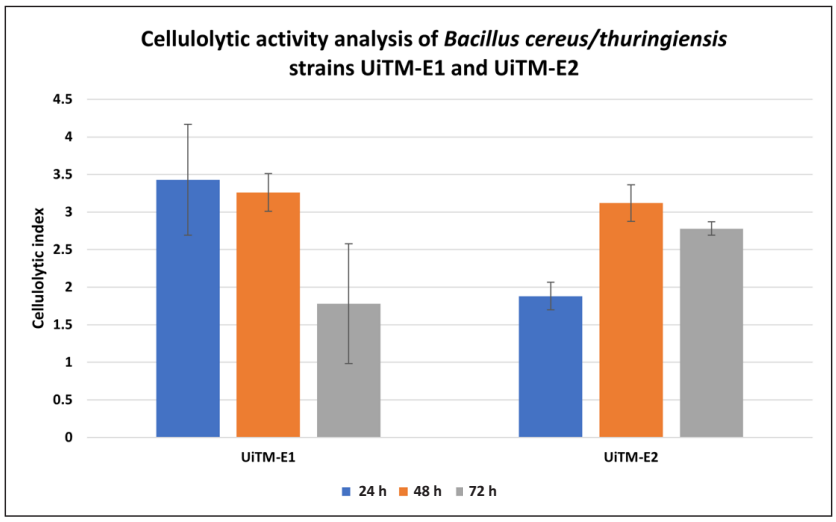

Fig. 3. Cellulolytic activity of *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 at 24, 48, and 72 h of incubation (mean of triplicates ± standard deviation) through the cellulolytic index analysis performed on carboxymethyl cellulose agar.

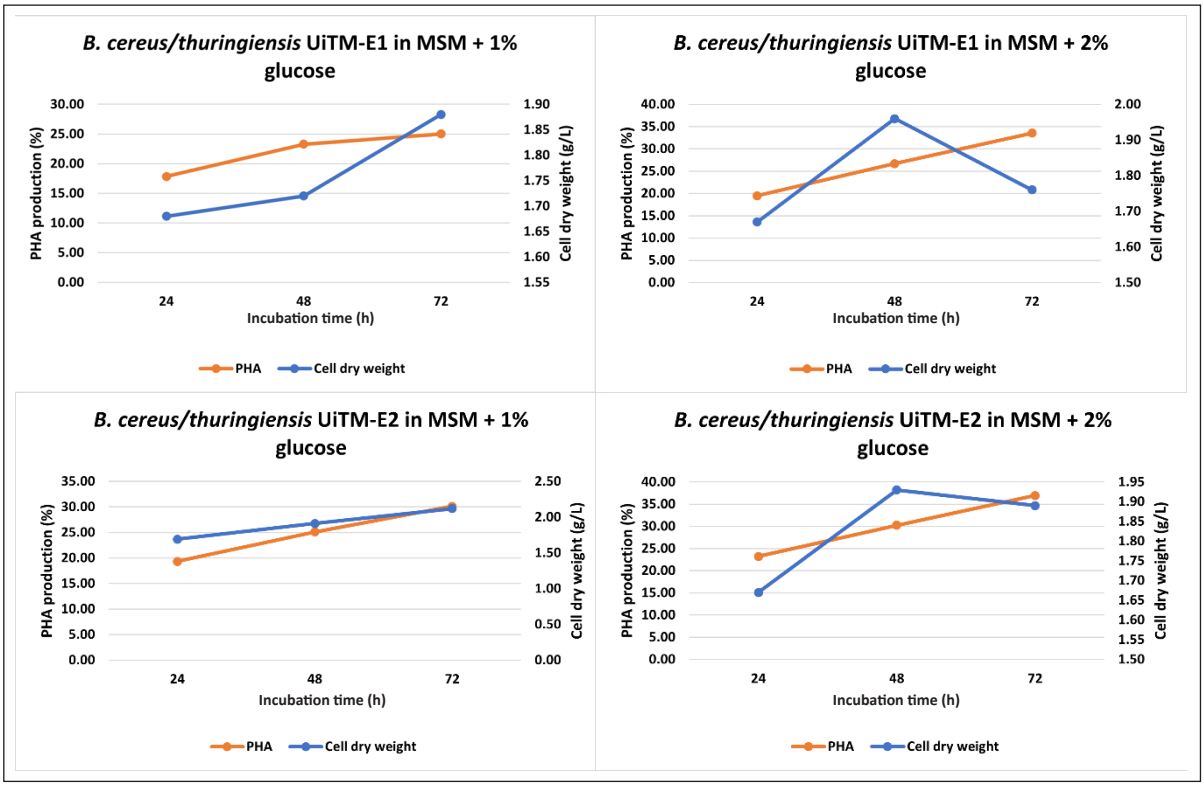

Fig. 4. Dry cell weight (g/L) and PHA production (%) of *Bacillus cereus/thuringiensis* strains UiTM-E1 and UiTM-E2 in Mineral salt media (MSM) supplemented with 1% and 2% glucose at different growth incubation time (24, 48 & 72 h) respectively. The values reported are means from triplicate ± Standard Deviations (SDs).

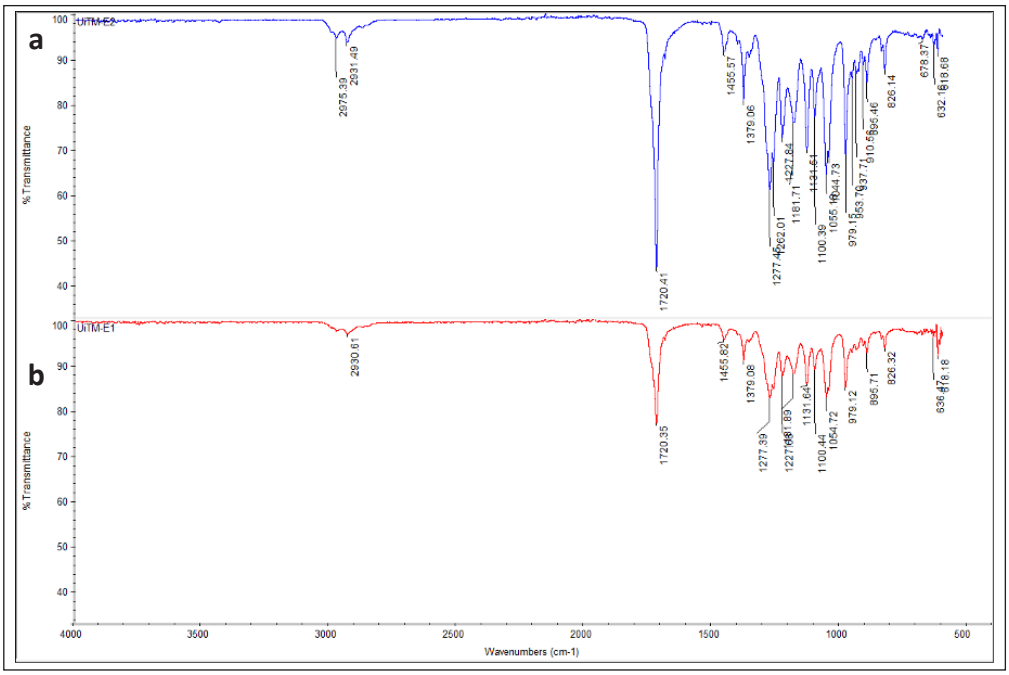

Fig. 5. Fourier transformed infrared spectroscopy (FTIR) analysis of the PHAs produced by *B. cereus/thuringiensis* strains UiTM-E1 (b) and UiTM-E2 (a) in the attenuated reflection (ATR) mode. Spectra were recorded ranging from 4000 cm^{-1} to 600 cm^{-1}.

CONCLUSION

Two bacterial isolates which have been partially identified and designated as *Bacillus cereus/thuringiensis* UiTM-E1 and *Bacillus cereus/thuringiensis* UiTM-E2, isolated from the wastewater of the Chancellery building waste treatment plant in UiTM Shah Alam were found to have both the ability to produce PHA as well as capable of breaking down carboxymethyl cellulose. Thus, these two isolates may be good candidates to be used in the production of PHA from cellulosic waste material allowing the valorizing of agricultural waste by using it as carbon feed for PHA production. This would potentially reduce the cost of PHA production in two ways, first by using cellulosic agricultural waste as a carbon source, which would also help in the management of agricultural waste, and second by eliminating the pre-treatment steps of the cellulosic materials before being used as carbon feed. Using agricultural waste for PHA production would also help in reducing environmental pollution due to waste management processes. Additionally, this would potentially allow a more economical process of PHA production. Further study on efficiencies of PHA production by these two bacterial isolates using various types of cellulosic waste material would need to be conducted and optimized.

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