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Comparison of quantification methods and subsequent characterization of polyhydroxybutyrate film sample utilizing pretreated cane molasses as carbon source

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Abstract. Polyhydroxybutyrate (PHB) is a biodegradable polyester synthesized as intracellular carbon and energy storage for numerous bacteria and archaea. PHB has been used extensively in biomedical industry. The gas chromatography (GC) method for PHB quantification has some drawbacks, such as large sample size required and involves tedious sample preparation. In this study, reactive pyrolysis-gas chromatography (reactive Py-GC) was applied to determine the PHB content in bacterial biomass. *Bacillus* sp. was cultured in mineral salts medium containing various concentrations of pretreated molasses (10 – 30 g/L) as a sole carbon source. The PHB content obtained by both methods; GC and reactive Py-GC were compared. The obtained PHB was cast into film and the physical properties was determined by DSC and TGA. The biodegradability of PHB film sample was performed by soil burial biodegradation test. Based on the peak intensities of reactive Py-GC analysis, the PHB contents were determined precisely and rapidly without any cumbersome sample pretreatment. Furthermore, the values of the PHB contents coincided overall with those obtained by the conventional GC method with correlation coefficient (R^2) 0.9766. The melting temperature and degree of crystallinity were 172 °C and 61 %, respectively. The PHB film sample used in this study can biodegrade completely within 12 days.

1. Introduction

Polyhydroxybutyrate (PHB) is a type of biodegradable polyester thermoplastics that classified under polyhydroxyalkanoate (PHA) family. This biopolymer is synthesized by various types of bacteria, such as *Cupriavidus necator*, *Bacillus megaterium* and *Aeromonas caviae* under nutrient limiting conditions but in the presence of excess carbon source [1,2,3]. This biopolymer is accumulated in bacteria biomass in the form of cytosolic granule; serves as carbon and energy source for the bacteria during starvation [1,2,3]. The biopolymer can be extracted chemically or biologically from the bacterial biomass [1,3].

Over the past few decades, PHAs have gained many industrial attentions due to their biocompatibility, biodegradability and similar physical property to the commercial polypropylene [1]. Moreover, PHB is known to be degraded by the soil microorganism such as bacteria, archaea and fungi. However, the production cost for PHB is very high compared to petrochemical plastic [1]. Recently, many studies have been conducted to utilize agriculture by-product as the carbon source for the PHA production in order to improve its economic feasibility [2]. Cane molasses is one of the potential carbon sources for PHA production due to its abundance especially in Malaysia and high sugar content [2].

In terms of analysis method for PHB, gas chromatography (GC) is the standard method for PHB quantification; showing high accuracy and reproducibility. However, there are some drawbacks for GC method, for examples: (i) the use of hazardous solvent, (ii) tedious sample pretreatment, and (iii) large sample size required for the analysis. Therefore, it is required to develop a direct and highly sensitive method for PHB quantification [4].

On the other hand, reactive pyrolysis gas chromatography (reactive Py-GC) has been widely used in the forensic and archaeology field due to its rapid and direct method to analyse various polymeric materials, such as oil, polyaromatic compound and plastics [5,6]. The concept behind the reactive Py-GC is to use heat in controllable and reproducible way, and with the aid of strong organic alkali, such as tetramethylammonium hydroxide (TMAH) to breakdown high molecular weight sample selectively into smaller fragments before subjected to the pyrolyzer, attached at the inlet port of the GC for separation and detection. Based on previous studies, the authors have successfully applied the reactive Py-GC method to analyse the poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) content in the bacterial cells directly, without any tedious sample preparation [6].

This study will compare the PHB content obtained by both conventional GC and reactive Py-GC analyses method. Then, the physical properties of PHB film samples produced by *Bacillus* sp. using pretreated molasses as main carbon source were characterized.

2. Materials and method

2.1. Material

The cane molasses used in this study was obtained from Malayan Sugar Manufacturing Company Berhad (MSM), Seberang Perai, Penang, Malaysia. The molasses solution was prepared by dissolving 15 g of molasses into 100 mL of distilled water.

2.2. Hydrothermal oven acid pretreatment

The hydrothermal oven acid pretreatment was performed by adding 10 mL of 1.5 N H_2SO_4 into 200 mL of molasses solution. The molasses solution was then transferred into 600 mL stainless steel hydrothermal reactor, preheat in the Venticell LSIS-B2V/VC55 hot air oven (München, Germany) at 105 °C for 1 hour and followed by incubation for another 1 hour. After the pretreatment, the pretreated molasses solution is cooled to room temperature and the pH were adjusted to 7.0. The pretreated

molasses was centrifuge at 4500 rpm for 15 minutes to remove the impurities in the molasses before used for fermentation [2].

2.3. Microorganisms and media

The bacterial strains used in this study were *Bacillus* sp. The composition of the nutrient broth (NB) for inoculation (pH 7.0) was as follows (per L): 10 g meat extract, 10 g peptone, and 5 g sodium chloride. The mineral medium (MS) composition (pH 7.0) for *Bacillus* sp. was as follows (per L): 1 g$(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 4.47 g $Na_2HPO_4{\cdot}2H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$ and 1 mL of trace element solution. The trace element solution was composed of (per L): 10 g $FeSO_4{\cdot}7H_2O$, 2.25 g $ZnSO_4{\cdot}7H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}5H_2O$, 2 g $CaCl_2{\cdot}2H_2O$, 0.23 g $Na_2B_4O_7{\cdot}10H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}$, and 10 mL 35% HCl. Pretreated molasses was used as main carbon source and test at various concentration ranging from 10 to 30 g/L [2].

2.4. Cultivation condition

Microbial culture was carried out at shake flask scale using one-stage cultivation strategy. The inoculation was done by cultivating the *Bacillus* sp. in nutrient broth (NB) medium at 30 °C, 200 rpm for 24 hours before transferring into the MM medium containing various concentration (10 – 30 g/L) of hydrothermal oven acid pretreated molasses. The cultivation conditions for *Bacillus* sp. are at 30 °C, 250 rpm for 2 days. After the fermentation, the cell biomass was harvested [2].

2.5 Extraction of polymer and film casting

The extraction of PHB was based on procedure reported by Murugan et al. [8], lyophilized cells were stirred in chloroform in a ratio of 1 g : 100 mL for 5 days at room temperature. The cell debris was filtered and the solution was concentrated using a Heidolph Laborata 4000 rotary evaporator (Schwabach, Germany) with the water bath set at 60°C. The concentrated solution was then added dropwise into 10 times volume of vigorously stirred cool methanol. The white precipitate, which is the PHB was recovered by filtration and air dried overnight. The solvent film cast method was performed according to Sridewi et al. [9]. The PHB film samples were prepared by dissolving the 0.3 g extracted polymer in 30 mL chloroform with magnetic stirring for 30 min. The mixture was cast into clean glass petri dishes (9 cm in diameter) and left to dry overnight.

2.6. Gas chromatography (GC) and reactive pyrolysis gas chromatography (reactive Py-GC) analysis for PHB determination

The PHB content in the cell biomass was analysed by GC and reactive Py-GC. For GC analysis, the sample was undergone methanolysis before subjected to GC. The sample preparation was conducted according to the procedure reported by Braunegg et al. [10]. Briefly, 2 mL of chloroform and 2 mL of methanol acidified with 15 % H_2SO_4 were added to the screw capped glass tube that contained 10 mg of lyophilized cell biomass. Then, the sample was heated on the dry bath at 100 °C for 3.5 hours. Upon the completion of methanolysis, the mixture was cooled to room temperature and 1 mL of distilled water was added to the mixture. The mixture was vortexed until 2 phases were formed, which are lower organic phase and an upper aqueous phase. The upper aqueous phase was removed, and sodium sulphate was added to remove any remaining water content. The amount of methyl 3-hydroxybutanoate was quantified using Shimadzu GC 2010 plus gas chromatograph (Kyoto, Japan) equipped with a flame ionization detector and a Merck SPB-1 capillary column (30 m length × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas with a flow rate 13.0 mL/min. The temperature program at 70 °C and ramp up to 280 °C at a rate of 5 °C/min [2]. In this procedure, standard sample was used to construct the standard calibration curve.

The sample preparation of reactive Py-GC was performed according to the procedure reported by Siti et al. [6,7]. The dry powder sample of bacteria colonies (0.1 mg) was weighed in the stainless steel sample cup (2 mm i.d. × 4 mm height) and 2 µL of 25 % tetramethyl ammonium hydroxide (TMAH) solution was added. The sample cup was dropped into the heated centre of Frontier Laboratory PY-2020iD vertical microfurnace pyrolyzer (Koriyama, Japan) that attached to the Shimadzu GC2010 Plus GC chromatography (Kyoto, Japan). The pyrolyzer was maintained at 400 °C under the carrier gas, helium with a flow rate 50 mL/min. A part of the flow (1 mL/min) reduced by the splitter was introduced into the Frontier Laboratory ULTRA ALLOY-5 (MS/HT) metal capillary separation column (30 m length × 0.25 mm i.d., 0.1 µm film thickness) coated with immobilised 5% diphenyl-95% dimethylpolysiloxane. The column temperature was programmed at 35 °C to 400 °C at a rate of 5 °C/min. The standard sample of PHB was used to construct the calibration curve [6,7].

2.7. Physical properties characterization of PHB film samples using differential scanning calorimetry (DSC) and thermalgravimetric analysis (TGA)

The differential scanning calorimetry analysis (DSC) was carried out using a Perkin Elmer DSC 6 instrument (Massachusetts, USA) under nitrogen flow of 20 mL/min. DSC analysis was conducted in triplicate which were heated from room temperature to 195 °C at a heating rate of 10 °C/min. The cooling cycle was performed from 195 to -10 °C. The degree of crystallinity for PHB was determined using Equation (1), considering the area of the melting peaks as the melting enthalpy:

$$X_{\mathrm{c}} = \frac{\Delta H_{\mathrm{m}}}{\Delta H_{0\mathrm{m}}} \times 100\ \% \qquad (1)$$

where ΔH_{m} is the measured value for PHB melting enthalpy, $\Delta H_{0\mathrm{m}}$ is the melting heat associated with pure crystalline PHB (146 J/mol) [11].

Thermal degradation measurement was performed using a Perkin Elmer Pyris 1 thermalgravimetric analysis (TGA) (Massachusetts, USA). Temperature was raised from room temperature up to 800 °C with a heating rate of 10 °C/min. Triplicate analyses were performed for PHB sample (5 mg). All runs were carried out under a nitrogen atmosphere (20 mL/min) in order to prevent any thermo-oxidative reaction.

2.8. Soil burial biodegradation test of PHB film sample

The PHB films were cut into pieces (1 cm × 1 cm) and cover with nylon cloth (3 cm × 3 cm) before buried in the compost soil with soil composition (58 % sand, 28 % silt and 14 % clay) including 13 % organic matter, at 30 °C, pH 7. Relative humidity was adjusted to approximately 70 % by using a soil moisture sensor, and water was added every 12 hours. All film samples were prepared in triplicate and buried in soil 3 cm from the surface until complete degradation was observed. The films were harvested at 2 days interval.

3 Results and discussion

Figure 1 shows the GC chromatogram of trans-methylated product of PHB after solvent extraction. After elution of the solvent peaks, the peaks of methyl 3-hydroxybutanoate which derived from trans-methylation of 3HB monomer was clearly observed on the chromatogram.

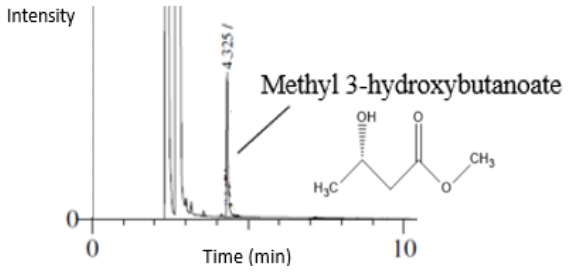

Figure 1. The typical GC chromatograms of *trans*-methylated product of PHB after solvent extraction.

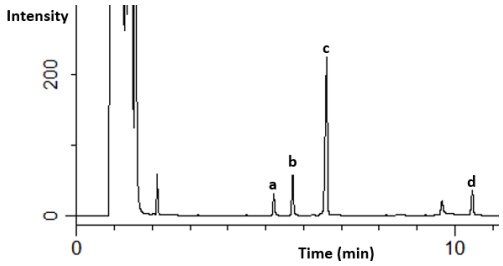

Figure 2 . The reactive pyrolysis gas chromatograms of PHB in the *Bacillus* sp.biomass without any solvent extraction.

Figure 2 shows the reactive pyrolysis gas chromatogram of PHB in the *Bacillus sp.* biomass without any solvent extraction. From the figure 2, four major peaks which attributed to methyl derivatives of 3HB monomer were clearly observed. The similar peak pattern was also reported by Siti et al., 2015 [7]. The assignment of these peaks were summarized in Table 1 along with their effective carbon number (ECN) corresponding to their molar sensitivity to the FID. Peak a-c had been identified as methyl 3-butenoate and its isomers; methyl *cis*-2-butenoate and methyl *trans*-2-butenoate while peak d had been identified as methyl 3-methoxybutanoate [6,7].

Table 1. Identification of the characteristic peaks on chromatogram figure 2

Peak number	Compound name	ECN[a]
a	Methyl 3- butenoate	3.65
b	Methyl *cis*-2-butenoate	3.65
c	Methyl *trans*-2-butenoate	3.65
d	Methyl 3-methoxybutanoate	3.95

[a] Effective Carbon Number for FID [12]

To calculate the PHB content in *Bacillus sp.* biomass, the following equation were applied, as proposed by Siti et al. [6,7] :

$$Y_{3\mathrm{HB}} = \sum_{i=1-3} \frac{\mathrm{I}_i}{\mathrm{ECN}_i} \qquad (2)$$

where I_i is the area of peak *i* in Table 1, and ECN_i is the ECN corresponding to the peak *i*.

Figure 3 shows the comparison of PHB content accumulated in *Bacillus* sp. biomass using conventional GC and reactive Py-GC. The PHB content in *Bacillus sp*. biomass obtained from both methods show increasing trend that correlating to the increase of the concentration of pretreated molasses. To evaluate the accuracy of the reactive pyrolysis method, the PHB content of *Bacillus sp.* biomass obtained from GC and reactive Py-GC were compared. The PHB content obtained by reactive Py-GC is higher compare to GC method due to higher reaction efficiency of 3HB with TMAH, a similar result was also reported by Siti et al. [13]. This results indicate that the PHB content obtained by reactive Py-GC were strongly correlated with those by conventional GC. The correlation coefficient (R^2) between conventional GC and reactive Py-GC data was 0.9766, which indicate that the reactive Py-GC method can be serve as practical tool for direct and rapid PHB quantification in bacterial cell biomass.

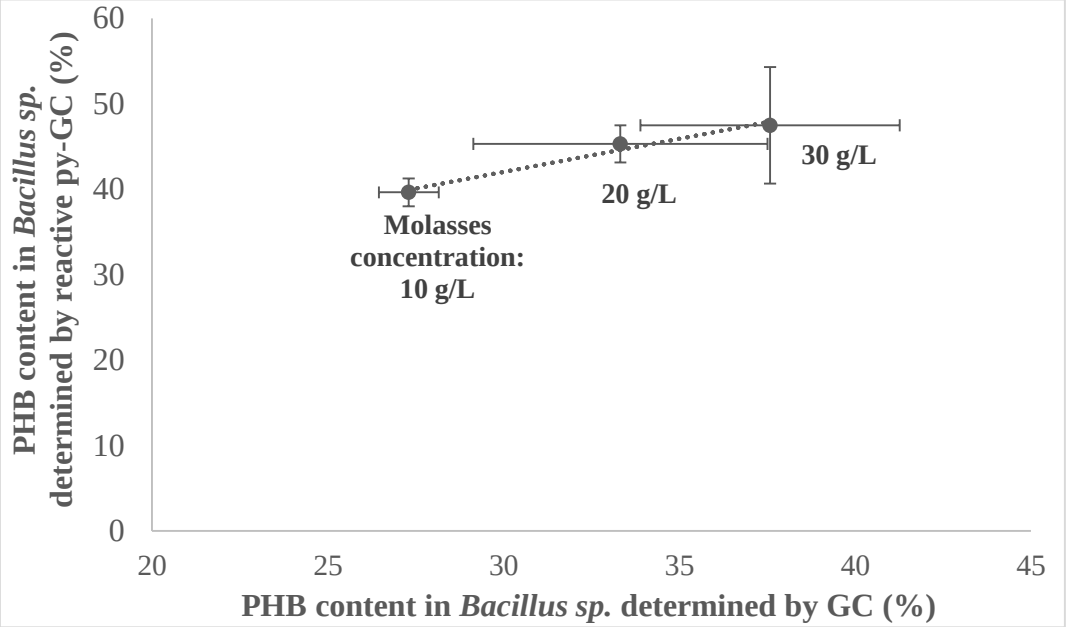

Figure 3. Comparison of PHB content accumulated in *Bacillus* sp. biomass using conventional GC and reactive Py-GC.

Figure 4 and Figure 5 shows the thermogram for both DSC and TGA for PHB films samples. The interpretation of physical properties for the PHB film samples are listed in Table 2. The glass transition temperature (T_g), which is -4.40 °C, is in the mentioned range in a previous study [13]. The T_g of PHB was depended on molecular factor, such as molar mass and degree of crystallinity (X_c), as well as the heating and cooling rate. Previous study reported that the T_g increase with the increase of molar mass of PHB up to 2×10^{-3} g/mol [15]. The crystallization temperature (T_c) obtained in this study is parallel as reported by Karami et al [18], which may due to the T_c was depend on the rate during cooling scan [19]. The melting temperature (T_m) of the PHB using pretreated molasses as the carbon source is 172.14 °C, which is consistent with the publications [16,17,20,22]. The decomposition temperature (T_d) of PHB film sample is 271.35 °C. These values are consistent with the results of PHB produced by *Rhodococcus equi* [17,20], which is mainly associated with the ester cleavage of PHB component by the β-elimination reaction [21]. The X_c and T_m of PHB in the present study are consistent with the results of PHB produced from *C. necator* cells when glucose is used as the carbon source [22]. Figure 4 shows the biodegradation of PHB film samples in term of weight reduction. From this figure, the PHB film samples can completely degrade within 12 days of soil burial biodegradation under the condition set in this experiment. The biodegradation of PHB in term of weight reduction was higher than in previous studies due to its degree of crystallinity and molecular weight of PHB, the soil types, organic content, including the bacteria and fungi consortium exists in the soil [23].

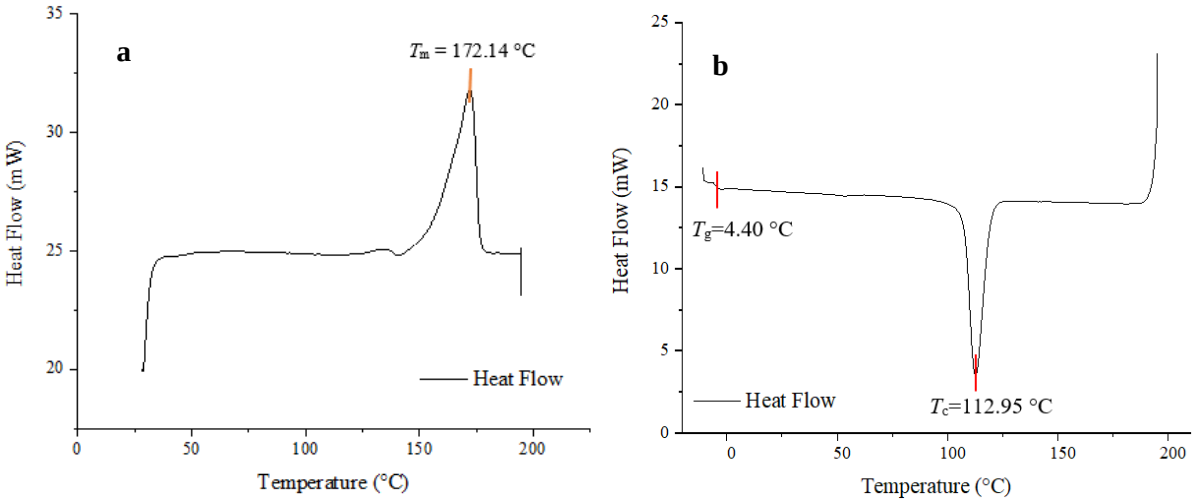

Figure 4. DSC thermograms of PHB film sample a) at the first heating, b) during the cooling scan.

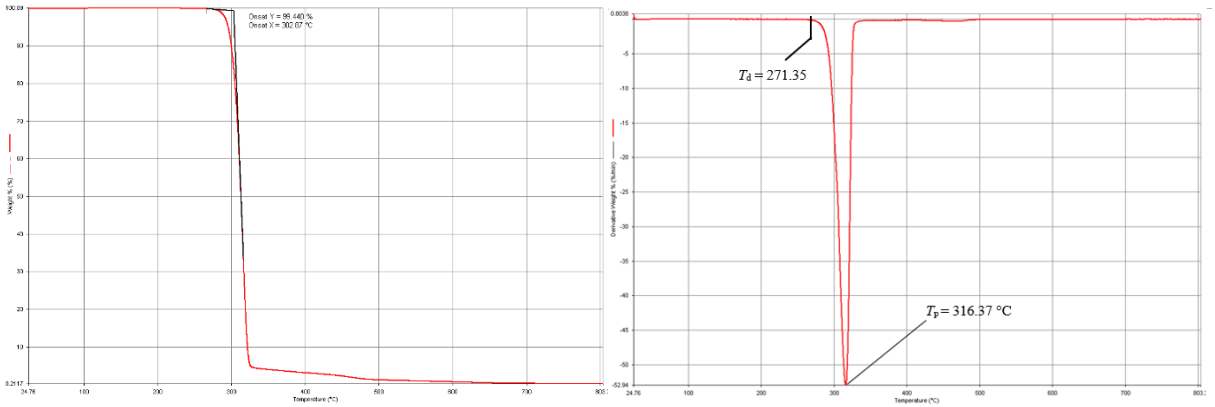

Figure 5. TGA thermogram of PHB film samples.

Table 2. Physical properties of PHB film sample

Physical Properties	This study	Altaee et al. [16]	Wang et al. [21]
Glass transition temperature, T_g (°C)	-2.42	2.79	-
Crystallization temperature, T_c (°C)	112.95	-	-
Melting temperature, T_m (°C)	172.14	173.00	172.05
Enthalpy of melting, ΔH_m (J/g)	90.50	-	89.70
Degree of crystallinity, X_c (%)	61.64	-	61.44
Degradation temperature T_d (°C)	271.35	276.00	249.60
Extrapolated onset temperature, T_o (°C)	302.87	-	-
Maximum weight loss temperature, T_p (°C)	316.37	-	269.80

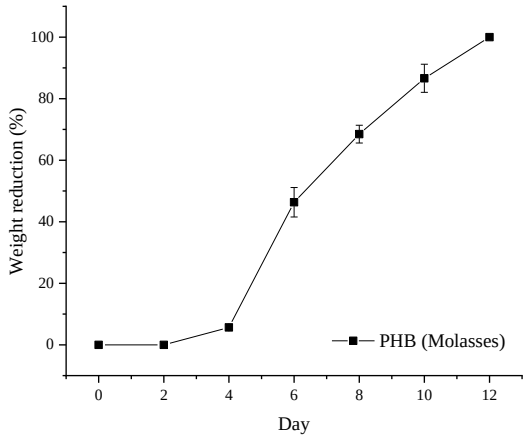

Figure 6. The biodegradation of PHB film sample in term of weight reduction.

4 Conclusion

Reactive Py-GC proves to be direct and highly sensitive method to determine the PHB content in the *Bacillus sp.* cell biomass without the need of tedious sample pretreatment and relatively small sample size (0.1 mg) required. Furthermore, the physical properties of PHB film samples produced by using the pretreated molasses as carbon source is similar to the previous studies.

5 References

[1] Sudesh K 2012 Polyhydroxyalkanoates from palm oil: biodegradable plastics (Heidelberg: Springer) p 1-32
[2] Khok Y S, Hussin M H and Baidurah S 2019 J Biocatal Agric Biotechnol **17** 51-59.
[3] Murugan P, Han L, Gan C Y, Maurer F H, and Sudesh K 2016 J Biotechnol **239** 98-105.
[4] Alves L P S, Almeida A T, Cruz L M, Pedrosa F O, De Souza E M, Chubatsu L S, Müller-Santos M and Valdameri G 2017 Braz J Med Biol Res **50** 1
[5] Wampler T 2014 Pyrolysis-Gas Chromatography *Reference Module In Chemistry, Molecular Sciences And Chemical Engineering* p 1-14
[6] Baidurah S, Murugan P, Joyyi L, Fukuda J, Yamada M, Sudesh K and Ishida Y 2016 *J Chromatogr* **1471** 186-191
[7] Baidurah S, Kubo Y, Kuno M, Kodera K, Ishida Y, Yamane T and Ohtani H 2015. *Anal Sci* **31** 79-83
[8] Murugan P, Gan C Y and Sudesh K 2017 *Int J Biol Macromol* **102** 1112-1119.
[9] Sridewi N, Bhubalan K and Sudesh K 2006 *Polym Degrade Stabil* **91** 2931–2940
[10] Braunegg G, Sonnleitner B and Lafferty R M 1978 *Eur J Appl Microbiol Biotechnol* **6** 29–37
[11] Seoane I, Manfredi L, Cyras V, Torre L, Fortunati E and Puglia D 2017 *Polymers*, **9** 561
[12] Jorgenson A D, Picel K C and Stamoudius V C 1990 *J Anal Chem* **62** 683–689
[13] Baidurah S, Kubo, Y, Ishida Y and Yamane T. 2018 *Pure Appl Chem,* **90** 1011-1017
[14] Srubar W V III, Wright C Z, Tsui A, and Frank C W 2012 *Polym Degrad Stab* **97** 1922–1929
[15] Androsch R, Radusch H J and Funari S S 2007 *Eur Polym J* **43** 4961–4974
[16] Pachekoski W M, Agnelli J A M and Belem L P 2009 *Mater Res* **12** 159-164.

[17] Altaee N, Fahdil A, Yousif E and Sudesh K 2016 *J Taibah Univ Sci* 10 543-550.
[18] Karami S, Ahmadi Z, Nazockdast H, Rabolt J F, Noda I and Chase B D 2018 *Mater Res Express* **5** 015316.
[19] Wellen R M R, Rabello M S, Araujo Júnior I C, Fechine G J M and Canedo E L 2015 *Polímeros* **25** 296-304.
[20] Zhang M and Thomas N L 2011 *Adv Polym Tech* **30** 67-79
[21] Choi J Y, Lee J K, You Y and Park W H 2003 *Fiber Polym* **4** 195-198.
[22] Wang Y, Chen R, Cai J, Liu Z, Zheng Y, Wang H, Li Q and He N 2013 *PLoS One* **8** e60318.
[23] Kijchavengkul T and Auras R 2008 *Polym Int* **57** 793–804

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