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Polyhydroxybutyrate (PHB) production by *Halomonas elongata* BK AG 18 indigenous from salty mud crater at central Java Indonesia

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ABSTRACT

Aims: Bioplastic is a biodegradable polymer produced by particular microorganism as a secondary metabolite. Some halophilic bacteria belonging to *Halomonas* genus have been reported to be a potential of polyhydroxybutyrate (PHB) producer. This study aims to explore the potential of an indigenous halophilic bacterial isolate, *H. elongata* BK-AG18, as bioplastic producer. The indication as bioplastics producer was evaluated by growing in nile red-containing medium and bacterial colonies displayed bright orange fluorescent under ultraviolet light.
Methodology and results: Bioplastic production by *H. elongata* BK-AG 18 was achieved using modified glucose-contained High Medium (HM) after incubated in a rotary shaker for 22 h, 37 °C, 150 rpm. The bioplastic was extracted with chloroform and sodium hypochlorite (1:1) and precipitated in methanol. The highest yield of bioplastic production was 21.36% of the dried bacterial cell weight. The structural characterizations of the bioplastics using Fourier transformed infrared (FTIR), ^{1}H and ^{13}C nuclear magnetic resonances (NMR) spectroscopies showed high similarity to the spectral pattern of polyhydroxybutyrate (PHB). Further characterization using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) revealed that the decomposition and melting temperature at 266 °C and 166.5 °C of the PHB, respectively. The result of PHB has a low degree of crystallinity (9.5%) that close to the rubber-like polymer.
Conclusion, significance and impact of study: This study revealed the high potential of *H. elongata* BK-AG 18 as PHB producer with high mechanical properties.

Keywords: H. elongate BK-AG 18, polyhydroxybutyrate, bioplastic, biodegradable polymer

INTRODUCTION

Plastics are petroleum based polymers produced from 10 of crude oil as raw materials (BP Statistics Review of World Energy/6/2011). Increasing demand for petroleum-based plastics has led to the depletion of oil resources. In addition, plastic is not biologically degradable due to their incompatible structures with most hydrolyzing enzyme excreted by decomposing organisms (Yamada-Onodera *et al*., 2001; Zheng, 2005). Problems related to solid waste of this petrochemical-derived plastic were a serious concern for the global environment. In order to overcome the environmental damage caused by plastic waste, there is considerable interest to develop biodegradable polymers.

One example of the biodegradable polymer materials is polyhydroxyalkanoate (PHA). PHA is a polyester produced by microorganism via fermentation of sugars or lipid (organic compounds) or can be synthesized chemically. PHA is secondary metabolites produced by bacteria under limited nutrients, such as phosphorus, magnesium, and nitrogen and carbon excessive in the growth medium (Madison and Huisman, 1999). That bioplastic was accumulated inside the bacterial cells as discrete granules. This polymer can be degraded and metabolized by bacteria as a source of carbon and energy storage (Byrom, 1994). One of the famous PHA biopolymers is polyhydroxybutyrate (PHB). Besides its biodegradability and biocompatibility, this biopolymer has properties which similar to common plastic, such as propylene. Some bacteria belong to *Halomonas* genus, such as *H. boliviensis* LC1 (Van-Thuoc *et al*., 2007) and *Halomonas* sp. KM-1 (Kawata and Sei-ichi, 2010) have been reported as the potential producer for PHB. In the present study, we explore the potential of *H. elongata* BK-AG 18 obtained from the unique terrestrial hypersaline environment, which was a salty mud crater located at Purwodadi-Grobogan, Central Java, Indonesia (Asy'ari *et al*., 2014). We show here that *H. elongata* BK-AG 18 is also bioplastic producing bacteria that can produce thermally stable PHB.

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MATERIALS AND METHODS

Screening of PHB-producing bacteria

Halomonas elongata BK-AG 18 was grown in modified high medium (HM) containing 20 g/L glucose, 2 g/L yeast extract, 5 g/L NaCl; 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.09 g/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L KCl, 0.25 g/L KH_2PO_4, 2 g/L granulated agar, 0.06 g/L NaBr, and Nile red (dissolved in dimethylsulfoxide) with a final concentration of 0.5 µg dye per mL of medium. The cultures were incubated at 37 °C for 2-15 days. The agar plates were then exposed to ultraviolet light (312 nm) to detect the presence of intracellular bioplastic granules in bacteria. The colonies which appeared bright orange fluorescent under UV light indicated that they were producing bioplastic (Spiekerman *et al.*, 1999).

Production of bioplastic

In order to cultivate the highest yield of PHB, the growth curve *H. elongata* BK-AG 12 was needed to determine first. *Halomonas elongata* BK-AG 18 was previously cultivated in Luria-Bertani medium (20 g/L glucose, 2 g/L yeast extract, 5 g/L NaCl) overnight. Then, it was transferred into liquid HM medium and incubated in the rotary shaker at 37 °C, 150 rpm. One milliliter bacterial culture was sampled aseptically every two hours and cell density (OD) was measured by UV/Vis spectrophotometer at 600 nm until the absorbance value of bacterial density attained stationary phase. The results were then plotted as OD_{600} toward inoculation time in order to obtain the growth profile of the bacteria in HM medium.

In the production phase, the bacteria were grown in HM medium by varying the production times, NaCl concentrations, and amount and type of carbon sources. The bacterial optimum condition obtained from this experiment was then used for the production of bioplastic in this study.

Polymer extraction

Bacteria cells were harvested by centrifugation at 8000 rpm. The cell pellet was dispersed with chloroform and sodium hypochlorite (1:1). Then the dispersion solution was centrifuged at 5800 rpm for 10 min at room temperature. Chloroform phase in the lowest layer was collected. The chloroform phase was added by 4× volume by methanol in order to precipitate the polymer. The precipitated polymer was washed with acetone and dried for further analysis (Rohini *et al*., 2006). The yield of bioplastic production by *H. elongata* BK-AB8 bacteria was obtained by comparing the dry weight of bioplastic to the weight of dry cell, expressed in percent.

Polymer structure analysis: Fourier Transformed Infrared spectroscopy (FTIR)

FTIR analysis of PHB was performed using FTIR Prestige 21 instruments, Shidmazu, Japan. Two mg of a polymer sample was mixed with potassium bromide (10× polymer volume) to make a solid pellet. Fifteen mg of this pellet was used for FTIR analysis.

Polymer structure analysis: Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance of ^{1}H was recorded at 500 MHz, whereas the NMR of ^{13}C was recorded at 125 MHz by using Agilent 500 MHz NMR in deuterated chloroform at room temperature.

Thermal property study with Differential Scanning Calorimeter (DSC) and Thermogravimetry Analysis (TGA)

DSC-TGA analysis of PHB was performed using Linseis STA Platinum Series (Linsesis Application Laboratory Thermal Analysis). The polymer sample was 10-15 mg taken and inserted into the aluminum pan and then heated under nitrogen exposure at 30 °C to 600 °C. The temperature and flow rate was increased to 10 °C/min and 15 mL/min, respectively.

The degree of crystallinity (%) of PHB can be calculated based on the enthalpy measurement from the thermogram of DSC using the following equation:

$$X_C = \frac{\Delta H_f^m}{\Delta H_f^{100\%}} \times 100\%$$

Where X_C is the % crystallinity, ΔH_f^m is the measured heat fusion (J/g), and $\Delta H_f^{100\%}$ is enthalpy of fusion of fully crystalline PHB, which is equal to 146 J/g (Barham et al., 1984).

RESULT AND DISCUSSION

Nile red test for bioplastic producing bacteria

Some bioplastics producing bacteria, such as *H. boliviensis* LC1 (Van-Thuoc *et al*., 2007) and *Halomonas* sp. KM-1 (Kawata and Sei-ichi, 2010) were screened by nile red test developed by Spiekerman *et al*. (1999). The bright orange fluorescence displayed by the bacterial colonies under the exposure of UV light indicated that those bacteria produced bioplastic. In the present work, we applied this method to evaluate the potential of *H. elongata* BK-AG 18 as bioplastic producer by growing them in the medium containing Nile red. Bright orange fluorescence was showed by the bacterial colonies (Figure 1) indicating that these bacteria confirmed to be bioplastic producing bacteria.

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Figure 1: Nile red test to reveal the potential of *H. elongata* BK-AG 18 as a bioplastic producing bacteria. Bright orange fluoresence of bacterial colonies under UV light indicated that bioplastics were produced by the bacteria.

Bacteria growth profiles

The growth profile of *H. elongata* BK-AG18 was indicated by the value of OD_{600} and monitored based on its cell density. The results showed that the highest OD_{600} value for *H. elongata* BK-AG18 was at 22nd h (OD=1.16) and the bacteria could survive more than 150 h prior to entering the death phase (Figure 2). Bacteria produce bioplastic as a secondary metabolite at the stationary phase, because the bacteria have well adapted to the environment (growth medium) and can fully produce their secondary metabolite. *Halomonas elongata* BK-AG18 has long stationary phase, therefore, the bioplastic production was estimated to occur within this phase.

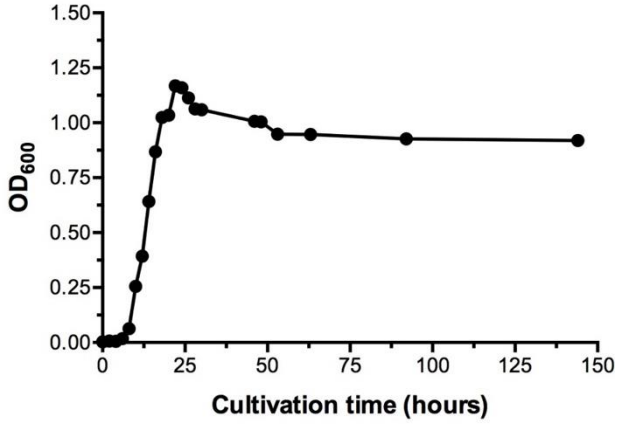

Figure 2: Growth profile of *H. elongate* BK-AG 18 in HM medium at 37 °C.

Bioplastic production optimization

Optimization of bioplastic production for *H. elongata* BK-AG 18 was done by varying NaCl concentration and production time (Figure 3). It shows that the optimum conditions to produce bioplastic were in the medium containing 5% NaCl (Figure 3A) and at 22 h of production time (Figure 3B). The best time for cultivating bioplastic was about 22 h of incubation because the longer the incubation, that would be the less bioplastic obtained. Bacteria were tended to use the bioplastic after a longer period of incubation for their own metabolism and decreased the bioplastic amount. At the optimum condition, the yield of bioplastic production was about 21.36%.

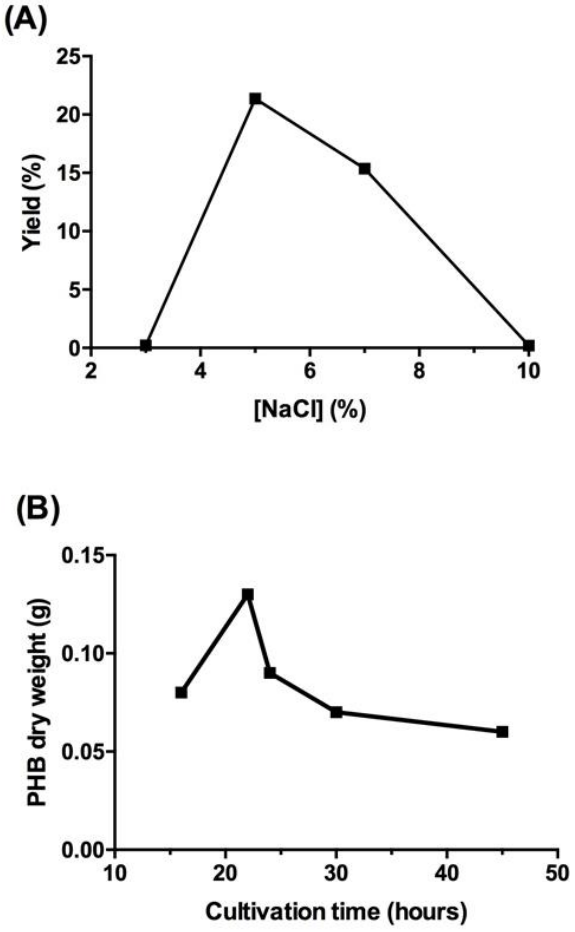

Figure 3: Effect of NaCl concentration (A) and cultivation time at the optimum NaCl concentration (B) in bioplastics production by *H. elongate* BK-AG 18.

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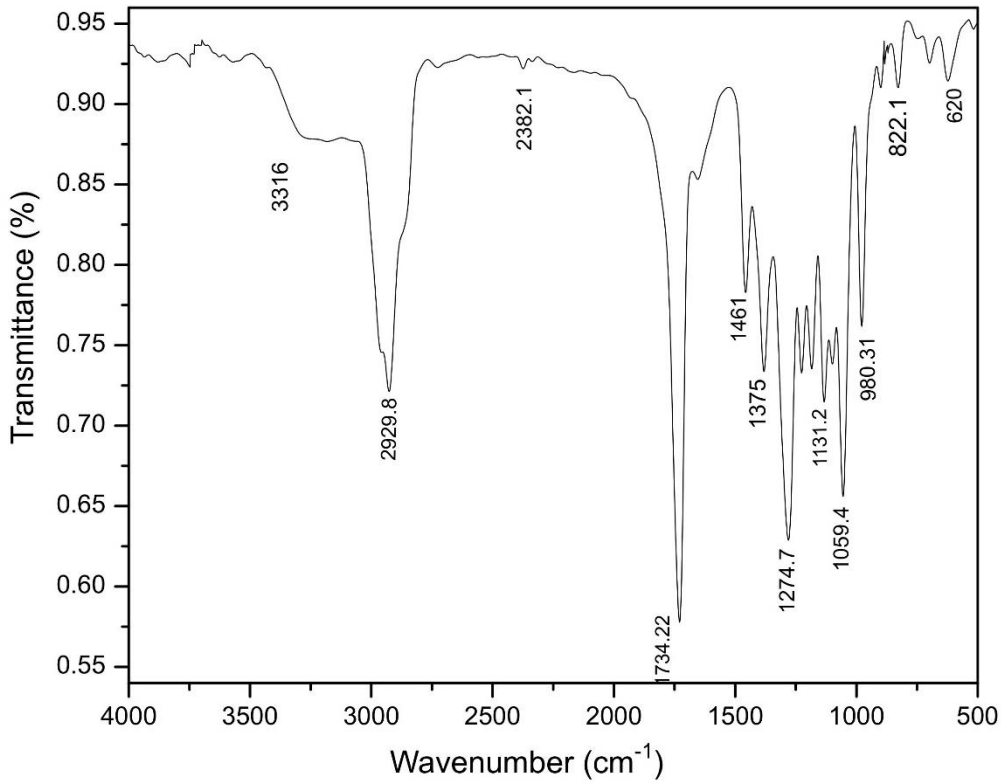

Figure 4: FTIR spectrum of bioplastic produced by *H. elongate* BK-AG 18.

Bioplastic extraction and purification

Bioplastic extraction was carried out using a mixture of sodium hypochlorite-chloroform (1:1). Sodium hypochlorite worked as a detergent that disturbs the structure of cell wall and releases bioplastic to dissolve in chloroform (Kumaravel *et al*., 2010). In the extraction process, these two solvents, sodium hypochlorite and chloroform formed two phases i.e. water and an organic phase. Organic phase that contains bioplastic were collected for further process.

Bioplastic deposition process was carried out by the addition of methanol into the organic phase. Polarity difference between methanol and bioplastic will reduce bioplastic solubility. After bioplastic was precipitated, they were washed with acetone to remove the remaining of organic compounds and water. This purified bioplastic was then used for further characterization.

Structural analysis of bioplastic

The purified bioplastic was first characterized its structure by Fourier transformed infrared (FTIR) spectroscopy. There were several major functional group in the FTIR spectrum of bioplastic i.e. C-H (alkane) vibration at wave numbers of 2900 cm^{-1}, C=O (carbonyl) at wave numbers of 1700 cm^{-1}, C-O-C (ester) at wave numbers of 1280 cm^{-1} and C-O (ester) at wave numbers of 1050 cm^{-1} (Figure 4). These all four major peaks were similar to the characteristics of polyhydroxybutyrate (PHB) (Shah, 2012).

Further structural analysis of the bioplastic sample was carried out by using nuclear magnetic resonance (NMR) spectroscopy measurements. The ^{1}H NMR spectrum of bioplastic sample (Figure 5A) showed a doublet proton signal of methyl group at chemical shift of 1.26 ppm; a double triplet proton signal of methine group (-CH-) coupled to a proton of methylene group and three protons of methyl group at chemical shift of 2.58 ppm; and multiple proton signals of methyl group at 5.25 ppm. The impurities signal also appeared at a chemical shift of 2.16 ppm as a singlet proton signal corresponded to the proton of methyl groups of methanol and a singlet proton signal at a chemical shift of 3.47 ppm corresponded to the proton of –OH group of methanol. The ^{13}C NMR spectrum of bioplastic sample (Figure 5B) showed the similarity in its chemical shifts of carbon signals to the commercial PHB (Table 1). There were four major carbon signal

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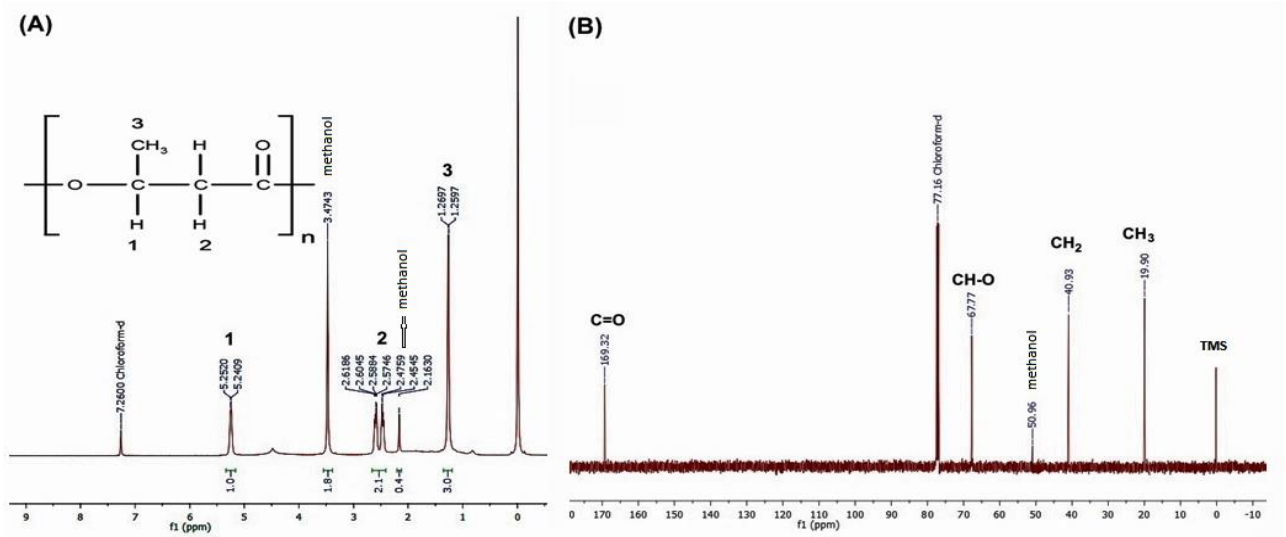

Figure 5: NMR spectra of bioplastic produced by *H. elongate* BK-AG 18: (A) Spectrum of ^{1}H-NMR and (B) spectrum of ^{13}C-NMR.

appeared at chemical shifts that corresponded to carbon atoms in four different chemical environment within the structure of PHB, [-O-CH-(CH_3)-CH_2-(C=O)-]$_n$. However, there was an additional carbon signal at a chemical shift of 50.96 ppm, which was lower than other carbon peaks. This signal, as also shown in its ^{1}H NMR spectrum, was likely corresponded to methanol solvent that was trapped in the polymer chain and formed hydrogen bond with the polymer. This methanol signal indicated that methanol used in the bioplastic production could not be completely removed during the separation process.

Table 1: The chemical shift of ^{13}C NMR spectra of PHB produced by *H. elongate* BK-AG 18 compared to the commercial PHB (Chaijamrus and Udpuay, 2008).

C atoms	Chemical shift (ppm)	
	PHB sample	Commercial PHB
CH_3	19.90	19.81
CH_2	40.93	40.72
CH	67.77	67.34
C=O	169.32	169.48

Thermal property analysis by DSC and TGA

The thermal properties of PHB samples were investigated by DSC-TGA based on the peaks (endothermic curve) or valleys (exothermic curve) shown in the thermogram. The standard PHB has a melting point peak at 172.1 °C (Chaijamrus and Udpuay, 2008). DSC thermogram of bioplastic sample produced by *H. elongata* BK-AG18 showed three valleys (Figure 6A). The first two valleys at 57.7 °C and 166.5 °C were the melting point of the sample while the other valleys at 287.5 °C and 314.6 °C were its decomposition points. The last two valleys were confirmed by thermal gravimetric analysis (TGA) that the PHB sample was decomposed at the temperature about 266 °C (Figure 6B).

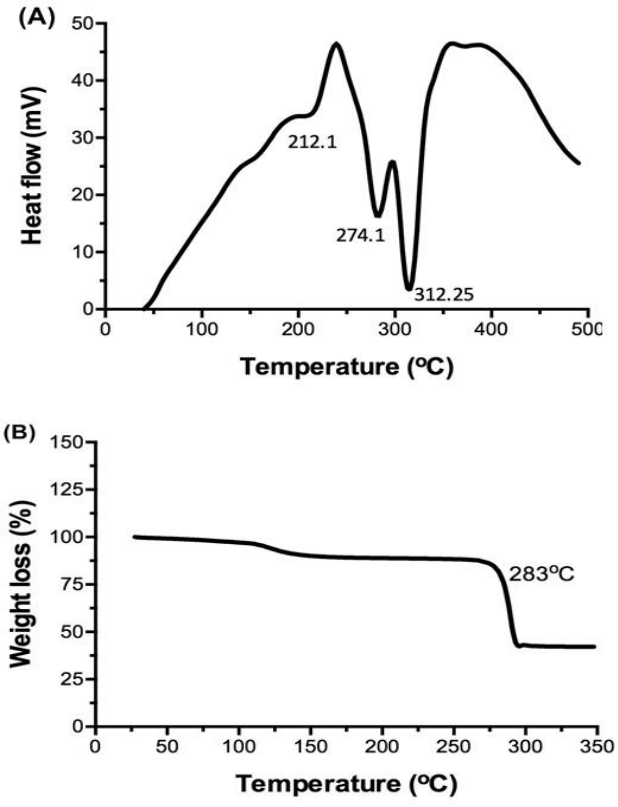

Figure 6: Thermal property of PHB produced by *H. elongate* BK-AG 18 studied by (A) DSC and (B) TGA.

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The thermal properties of PHB samples were investigated by DSC-TGA based on the peaks (endothermic curve) or valleys (exothermic curve) shown in the thermogram. The standard PHB has a melting point peak at 172.4 °C (Marjadi and Dharaiya, 2014) but in another study, it was 168.05 °C (Güngörmedi *et al*., 2014). Therefore, referring to the melting point of the commercial PHB, an endothermic peak at 166.5 °C is more likely the melting point of PHB. From DSC study, we also calculated the degree of crystallinity by using equation 1 (Table 2). The estimated degree of crystallinity of our PHB sample was about 9.5%, which is significantly smaller than the commercial one i.e. 45% and PHB isolated from *Staphylococcus epidermidis* i.e. 49.2% (Marijadi and Dharaiya, 2014). The other study conducted by Chaijamrus and Udpuay (2008) found PHB isolated from *Bacillus megaterium* ATCC 6748 has a degree of crystallinity about 60%. The higher of the degree of crystallinity, the stiffer is the polymer. High crystalline polymers are usually having poor mechanical properties with low extension at break (Savenkova *et al*., 2000). *Halomonas elongata* BK-AG 18 thus produces PHB that closes to a rubber-like polymer with the high mechanical property.

Table 2: Thermal characterization of PHB by DSC.

Sample	T_m	ΔH_f^m (J/g)	X_C (%)
PHB_{sample}	166.5	13.88	9.5
PHB (Marijadi and Dharaiya, 2014)	173.4	71.9	49.2
Commercial PHB (Marijadi and Dharaiya, 2014)	172.4	65.7	45

CONCLUSION

Halomonas elongata BK-AG 18 was able to produce bioplastic in the modified HM medium containing 5% NaCl with the yield about 21%. The structural analysis using FTIR and NMR to the bioplastic sample exhibited high similarity to the structure of (PHB). The bioplastic obtained by *H*. *elongata* had high mechanical property as concluded from the result of DSC analysis.

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