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Bioconversion of novel and renewable agro-industry by-products into a biodegradable poly(3-hydroxybutyrate) by marine *Bacillus megaterium* UMTKB-1 strain

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Abstract

Agro-industry by-products are abundant in various valuable compounds. Some of these raw materials are considered as a cheaper carbon source for polyhydroxyalkanoate (PHA) production when compared with pure substrates. It is however often a costly affair for industries to recover the residual carbon components. In this study, poly(3-hydroxybutyrate) [P(3HB)] was produced using a marine *Bacillus megaterium* UMTKB-1 strain from sweetwater, a by-product from cane sugar refining process. The bioconversion was initiated in shaken-flasks and fermenter experiments. Applications of seawater and sweetwater mixture as well as sweetwater only as PHA culture media were investigated. The produced polymer was characterized using Gas Chromatography (GC) and Gel Permeation Chromatography (GPC). P(3HB) accumulation from tested carbon sources ranged from 3 to 49 wt%. The strain could accumulate P(3HB) in saline conditions when minimal salts medium was replaced with seawater and sweetwater. The P(3HB) content was between 7 and 27 wt%. This strain was also able to grow and accumulate up to 14 wt% P(3HB) when sweetwater was the sole PHA biosynthesis medium. The weights of P(3HB) produced was in the range 3-12 × 10^5 with polydispersity index values ranging from 2.7 to 3.8. The agro-industry by-products have proven to be potential carbon feedstocks for P(3HB) production. The tested strain was able to grow and accumulate P(3HB) in a novel carbon substrate medium, the sweetwater. This by-product could be used as a raw material for P(3HB) production without any pretreatment.

Key words: *Bacillus megaterium*, polyhydroxyalkanoate, poly(3-hydroxybutrate), agro-industry by-product, seawater, sweetwater

Introduction

Sustainability is the key driver of innovation and science. The application of renewable resources as an alternative to fossil resources is done to strike a balance between our environmental, economic, and social interests and to achieve sustainability (Patel et al., 2012). Synthetic plastics have become a major problem in waste management due to their non-biodegradable attributes. Substitution of non-degradable petrochemical-based polymers with bio-based and biodegradable ones is compulsory and is currently being developed in laboratories and commercialized. In search for more environmentally benign materials, polyhydroxyalkanoate (PHA) has drawn increasing attention due to its polymer properties, biodegradability, and the ability to be produced from renewable raw materials. PHA is accumulated intracellularly as carbon and energy storage material in some microorganisms, under the limitation of nutrients and in

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the presence of excess carbon sources (Steinbüchel and Füchtenbusch, 1998). Poly(3-hydroxybutrate) [P(3HB)] is a PHA, the most commonly found in a natural environment (Madison and Huisman, 1999).

The commercialization of PHA is mainly focused on fermentation of Gram-negative bacteria because of their ability to yield higher concentration of PHA compared with Gram-positive bacteria. Nevertheless, PHA produced by Gram-positive bacteria lacks lipopolysaccharide (LPS) endotoxins, and thus provides a wider application in medical such as blood carrier as well as bone plates and pharmaceutical fields such as drug delivery system (Reddy et al., 2003). The Gram-positive *Bacillus* genus is known to produce PHA from different carbon sources such as glycerol, glucose, fructose, and sucrose (Kanjanachumpol et al., 2013; Nuzi et al., 2012). Although *Bacillus* sp. is related to sporulation process, which may reduce the PHA accumulation. PHA produced by this genus shows great advantages in application of PHAs (Reddy et al., 2003).

In addition, some *Bacillus* spp. are known to produce PHA from a combination of industrial wastes or by-products (Kanjanachumpol et al., 2013). At present, commercially available PHA are industrially produced using microbial cultures, and total cost of their production is very high. The commercialization of PHA is also often hindered by its high production cost due to the fermentation set-up cost, especially carbon feed stocks and chemicals used (Lee et al., 2008). The use of renewable resources as carbon sources to replace a pure substrate, especially from industrial and agricultural processes as well as household wastes is an attractive approach in order to the overall PHA production cost.

Sweetwater and cane molasses are by-products of cane sugar refinery process (Albuquerque et al., 2007). Sweetwater is obtained during the process of sugar production by repeated carbonization and filtration of brown sugar, which is known as the residual syrup, whereby no crystalline sucrose can be obtained. The yield of sweetwater is approximately 4.0-4.5% per ton of cane sugar (Wadekar et al., 2012). The cane molasses is obtained from repeated crystallization process of raw sugar and the yield of cane molasses is approximately 3.0% per ton of cane (Parez, 1998). The main component of sugar in sweetwater and cane molasses is sucrose, approximately 17% (w/v) and 30-40% (w/v), respectively (Chen and Chou, 2003). However, glycerol and glycerine pitch are generated as by-products from biodiesel and palm oil-based oleochemical industries. Both glycerine pitch and glycerol have been proven as potential carbon substrates in the P(3HB) biosynthesis (Yazdani et al., 2010).

In this study, efforts were made to evaluate by-products of agro-based industries such as carbon sources and culture media for PHA biosynthesis. Sweetwater and cane molasses (cane sugar refinery by-product), glycerine pitch (palm oil-based oleochemical industrial by-product) as well as glycerol (biodiesel production by-product) were tested as potential carbon sources for P(3HB) production using a marine bacterium, *Bacillus megaterium* UMTKB-1. Here, we are reporting for the first time the use of seawater and sweetwater mixture as well as only sweetwater as the culture media for P(3HB) biosynthesis. The ability to produce P(3HB) in a medium without the need for mineral salts or additional carbon sources could reduce the overall costs of production. The intracellular content of P(3HB) was determined by GC analysis, whereas its molecular weight was determined using GPC technique.

Materials and methods

Bacterial strain and maintenance

Bacillus megaterium UMTKB-1 strain was used in this study. This strain was isolated from the tissue samples of marine sponge *Callyspongia* sp. collected from waters surrounding Langkawi Island, Malaysia (Nuzi et al., 2012). The bacteria were maintained on nutrient-rich (NR) agar at 4 °C and in 20% (v/v) glycerol stock solution at −20 °C. The partial 16S rRNA gene sequence of the marine strain *B. megaterium* UMTKB-1 has been deposited in the GenBank database at the National Center for Biotechnology Information (NCBI) (Accession No: KF991583).

Media and culture conditions

An NR medium was used for bacteria maintenance and seed culture preparation. NR medium comprised of 10 g/l peptone, 10 g/l lab-lemco powder, and 2 g/l yeast extract. For NR agar preparation, 14 g/l of bacteriological agar (Oxoid) was added. PHA production using a one-stage cultivation method was carried out using minimal salts medium (MSM). MSM consisting of the following components was dissolved in distilled water: 1.5 g/l KH_2PO_4, 3.6 g/l Na_2HPO_4, 0.5 g/l NH_4Cl and

1 ml/l of $MgSO_4 \cdot 7H_2O$ (0.1 M) and was supplemented with 1 ml/l of trace element solution. Trace element solution consisting of the following component, was dissolved in 0.1 M HCl: $FeSO_4 \cdot 7H_2O$ (2.78 g/l), $MnCl_2 \cdot 4H_2O$ (1.98 g/l), $CoSO_4 \cdot 7H_2O$ (2.81 g/l), $CaCl_2 \cdot 2H_2O$ (1.67 g/l), $CuCl_2 \cdot 2H_2O$ (0.17 g/l), and $ZnSO_4 \cdot 7H_2O$ (0.29 g/l). Filtered seawater and sweetwater were used as an alternative PHA production medium in shaken-flask experiments with 1 ml/l of $MgSO_4 \cdot 7H_2O$ (0.1 M) and 1 ml/l of trace element solution. The seawater and sweetwater were filtered using a Whatman® No.1 filter paper to separate any debris. Then, the culture medium was sterilized by autoclaving at 121°C for 15 min under 15 psi pressure. Different carbon sources with carbon to nitrogen (C/N) ratios in the range 25-45 were used.

Carbon sources

The by-products of cane sugar refining process used in this study were obtained from Gula Padang Terap Sdn. Bhd. (14006-V), Kuala Nerang, Kedah, Malaysia. The sweetwater and cane molasses were collected in plastic storage bottles and stored at −20°C for further use. The cane molasses sample was characterized for its components and properties by Technology Park Malaysia (TPM) Biotech Company and ITS Testing Services (M) Sdn. Bhd. Glycerine pitch (Oleochemicals Industry, Penang, Malaysia) and glycerol (Biofuel Industry, Penang, Malaysia) by-products were also tested as potential feedstocks for PHA production. The carbon sources were sterilized separately by autoclaving at 121°C for 15 min under 15 psi pressures and then aseptically added prior to inoculation of seed culture at room temperature.

Production of PHA in shaken-flask cultures

Production of PHA by *B. megaterium* UMTKB-1 strain was initiated in shaken-flask cultures using the one-stage cultivation technique. The effect of different carbon sources on PHA production was determined by supplementing MSM with glycerol, cane molasses, sweetwater or glycerine pitch at C/N ratio of 35 with ammonium chloride (NH_4Cl) as a nitrogen source. The effect of different C/N ratios on PHA accumulation and cell biomass was also tested in shaken-flask cultures. The C/N ratios used were in the range of 25-45. The screening of alternative culture media was carried out using filtered seawater and sweetwater. The *B. megaterium* UMTKB-1 seed culture was prepared by growing the strain in NR medium for 14 h and the inoculum size for PHA biosynthesis was fixed at 0.1 g/l.

The shaken-flask cultures were incubated in a Certomat® *R & H* incubator shaker (Sartorious Sedim Biotech, Germany) at 200 rpm at 30°C for 48 h. The cell biomass was monitored by measuring the optical density of the culture broth at wavelength of 600 nm. Triplicates were carried out for each set of experiments. After 48 h cultivation, the cell cultures were harvested by centrifugation at 9000 rpm for 10 mins at 4°C using *Biostat D-DCU II* (Sartorius, Germany) floor stand centrifuge. The cell pellet was then stored at −20°C (in a freezer) overnight to freeze the sample before freeze-drying at −45°C using the freeze-dry machine *Labconco* 4.5 L (Labconco, USA) for 72 h.

Production of PHA in a fermenter

The biosynthesis of PHA was also carried out in a fermenter scale experiment using a 2l *Biostat C plus* (Sartorius Stedim, German) fermenter. MSM was replaced with sweetwater or seawater. Culture conditions were adapted from shaken-flask experiments. The production of PHA in seawater was carried out by supplementing glycerol and sweetwater as the carbon sources without the addition of any nitrogen source. However, sweetwater was used as the sole PHA biosynthesis medium without any additional carbon or nitrogen sources. The seed culture (0.1 g/l) was prepared in 250 ml Erlenmeyer flask using the NR medium. The experiments were carried out in a 1.5 l working volume supplemented with 1.5 ml/l of $MgSO_4 \cdot 7H_2O$ (0.1 M) and 1.5 ml/l of the trace element solution. Air was supplied at a flow rate of 1.5 l/min, the stirrer speed was set at 200 rpm, and the temperature was fixed at 30°C. At a maximum of 48 h of cultivation, the cells were harvested by centrifugation at 9000 rpm for 10 mins at 4°C. The cell pellet were then stored at −20°C in a freezer overnight before freeze-drying.

PHA polymers accumulated in the cell were extracted by stirring the freeze-dried cell with chloroform ($CHCl_3$) for 48 h on an *IKA C-MAG HS 7* hot-plate stirrer to disrupt the cells (Amirul et al., 2008). The $CHCl_3$ extract was then filtered with a Whatman® No.1 filter paper to separate the solution from the cell debris. The filtered mixture was concentrated using *Eyela N-1200 B*

Rotary evaporator (Eyela, Japan) at 50°C. The concentrated solution was then added drop wise into rapidly stirred chilled methanol (CH_3OH) to precipitate the dissolved polymer. The precipitated P(3HB) polymer was recovered by filtration using 0.45 μm PTFE membrane (Sartorius Stedim, Germany) and dried overnight at room temperature.

PHA content determination

The PHA content in lyophilized cells was determined using GC 2010 (Shimadzu, Kyoto, Japan) based on a standard method described by Braunegg and co-workers (1978). Approximately 15 mg of freeze-dried cells was subjected to methanolysis in the presence of CH_3OH and sulfuric acid [85:15 (v/v %)]. The reaction mixture was incubated at 100°C for 2 h and 20 min to convert the PHA monomer into its methyl ester forms. Distilled water (1 ml) was added to the cooled mixture and subsequently vortexed using the Velp Scientific vortex to promote phase separation. The lower organic layer containing reaction products was transferred and dried with anhydrous sodium sulfate, and analyzed by GC using a Fused Silica Capillary Column, 30 m × 0.25 mm × 0.25 μm Supelco SPB™- 1 (Sigma-Aldrich, USA).

PHA molecular weight determination

The molecular weight of the PHA samples was obtained using a GPC Shimadzu LC-9A system equipped with a refractive index detector (*RID-10A*) and a PL-gel MIXED-C column (Polymer Laboratories Ltd, UK) at 40°C. A high-performance liquid chromatography (HPLC) grade $CHCl_3$ was used as eluent at a flow rate of 1.0 ml/min and the sample was injected at concentration of 2.0 mg/ml. The sample was analyzed for weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity index (M_w/M_n) for each polymer. A GPC calibration was performed using polystyrene with a low polydispersity as a standard. The M_w and M_n were calculated using the Mark–Houwink equation:

$$[\eta] = KM^{\alpha}$$

where η is the intrinsic viscosity, K and α are Mark-Houwink constants, and M is the molecular mass (g/mol). The Mark–Houwink constants for polystyrene in chloroform were taken as K = 0.011 ml/mg and α = 0.73, where as those for P(3HB) in $CHCl_3$ were taken as K = 0.016 ml/mg and α = 0.76.

Results and discussion

Chemical constituents and properties of cane molasses

Cane molasses is a by-product of cane sugar refining process. This agro-industrial by-product was analysed for its chemical constituents and properties (Table 1). The cane molasses was dark brown and viscous at room temperature conditions. It exhibits high concentrations of sugars, approximately 41 wt% of sucrose, 6.7 wt% of glucose, and 3.2 wt%. The components of cane molasses recorded in this study corresponded with that reported in a previous study (Chen and Chou, 2003). In general, higher water content recorded in the by-products of cane sugar are essential for the productivity and cell growth of the microorganisms in PHA biosynthesis, whereas, higher glycerol content (32 wt%) was recorded with cane molasses compared with sweetwater as reported by Azemi and coworkers (2016). The cane molasses is acidic; it's pH is 6.07. Meanwhile, the chemical constituents and properties of sweetwater have been identified and reported elsewhere (Azemi et al., 2016). The main sugar components of sweetwater are sucrose (17.4 wt%), glucose (0.6 wt%), and fructose (0.4 wt%). Glycerol content was recorded at a concentration of 10.3 wt%. The pH value of sweetwater is 4.86 (Azemi et al. 2016), which is much more acidic compared with cane molasses reported in this study.

Biosynthesis of PHA using different agro-industry by-products

In general, industrial by-products are evaluated as carbon sources for PHA production because they are abundant in carbon; furthermore, it is expensive for industries to try and recover the residual or remaining carbon components from the by-products itself. The *B. megaterium* UMTKB-1 bacterial strain used in this study has been known to produce P(3HB) from carbon sources such as glucose and glycerol (Nuzi et al. 2012). Initial reports revealed that *B. megaterium* UMTKB-1 strain was able to accumulate 16-30 wt% of P(3HB) content using glycerol as carbon sources (Nuzi et al., 2012).

Figure 1 shows the ability of *B. megaterium* UMTKB-1 strain to use glycerol, glycerin pitch, sweetwater, and cane molasses for P(3HB) production is presented. The highest P(3HB) production (49 wt%) was recorded in MSM medium supplemented with glycerol. This value is higher compared with the initial report by

Table 1. Chemical constituents and properties of cane molasses

Test parameter	Cane molasses
Total sugar [a#]	50.9
Fructose [a#]	3.2
Glucose [a#]	6.7
Sucrose [a#]	41.0
Maltose [a#]	n/d
Appearance [b]	black & viscous
Water content [b#]	12.0
Glycerol content [b#]	32.0
Alkalinity as NaOH [b]	0.91
Specific gravity @ 40 c [b]	1.39
Dynamic viscosity @ 27°C [b] (mPa.s)	3338
Total residue at 160°C [b#]	85.0
Solubility [a]	soluble in water
pH [a]	6.07

n/d – not detected; a – analyzed by TPM Biotech Sdn. Bhd. Malaysia; b – analyzed by ITS Testing Services (M) Sdn. Bhd. Malaysia; # – values shown are in wt%

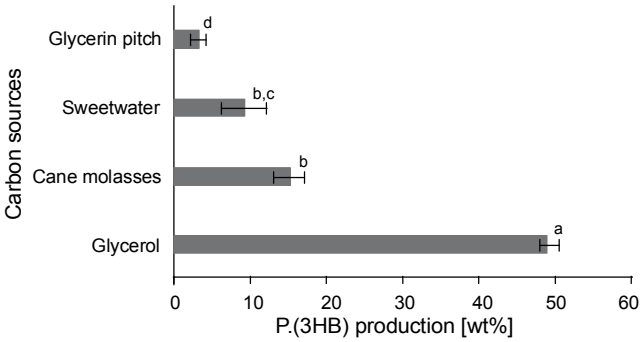

Fig. 1. The ability to synthesise of P(3HB) by *B. megaterium* UMTKB-1 strain using different agro-industry by-products as sole carbon sources. Values are means of three replicates. Mean data accompanied by different alphabet letters indicate significant differences within a group (Tukey's HSD test, $P < 0.05$)

Nuzi and coworkers (2012). In additiona, the bacterial strain could accumulate 9 wt% of P(3HB) production using sweetwater and 15 wt% of P(3HB) was recorded by *B. megaterium* UMTKB-1 strain when cane molasses was used as carbon sources. However, only 3 wt% of P(3HB) was produced using glycerin pitch. Some *Bacillus* spp. are known to grow fast on a great diversity of cheap substrates such as wastes and by-products. *Bacillus megaterium* was able to accumulate high P(3HB) content, approximately 50-70 wt% of P(3HB) content when supplemented with sugar cane molasses and whey (Kulpreecha et al., 2009). Similarity, *B. subtilis* and *B. thuringiensis* were able to produce about 60-65 wt% of P(3HB) from industrial food waste and starch (Gowda and Shivakumar, 2014).

Glycerol is a preferred carbon source for PHA production by *B. megaterium* (Gómez Cardozo et al., 2016). Based on Gómez Cardozo and co-workers (2016), *B. megaterium* grown on a medium supplemented with glycerol can produce 2.87 g/l of P(3HB). The use of glycerol for P(3HB) production by *B. megaterium* has also been reported by Naranjo and co-workers (2013); approximately 4.8 g/l of P(3HB) was produced when growth medium was supplemented with 2% (w/v) of glycerol. Meanwhile, Shahid and coworkers (2013), investigated the effect of combination of carbon sources, glycerol, and succinic acid, for P(3HB) by *B. megaterium* DSM 509 strain, and the bacteria were able to synthesize short-chain-length-medium-chain-length-PHA (scl- and mcl-PHA, respectively) in the absence of nitrogen. Glycerol and glycerin pitch as sole carbon sources were considered as relatively cheap and abundant substrates for industrial microbiology. The production of biodiesel in2016 reached 37 billion gallons with an annual growth of 42%, producing 4 billion gallons of crude glycerol as

Table 2. Effect of different C/N ratios of glycerol on the P(3HB) production*

Carbon sources	C/N ratio	CDW [g/l]	P(3HB) content [wt %] [e]	P(3HB) concentration [g/l] [f]	Residual biomass [g/l] [g]
Glycerol	25	1.01 ± 0.04 [b]	21 ± 3 [c]	0.21 ± 0.01 [d]	0.80 ± 0.03 [a]
	30	1.44 ± 0.03 [a]	31 ± 1 [b]	0.48 ± 0.01 [c]	1.00 ± 0.02 [a]
	35	2.01 ± 0.13 [a]	49 ± 3 [a]	0.77 ± 0.07 [a]	0.80 ± 0.07 [a]
	40	1.35 ± 0.27 [a,b]	47 ± 3 [a]	0.64 ± 0.13 [b]	0.72 ± 0.14 [b]
	45	1.01 ± 0.11 [b]	18 ± 1 [d]	0.18 ± 0.02 [d]	0.83 ± 0.09 [b]
Sweet water	25	0.29 ± 0.07 [b]	3 ± 1 [c]	0.01 ± 0.01 [c]	0.28 ± 0.07 [b]
	30	0.41 ± 0.19 [a,b]	9 ± 3 [a]	0.04 ± 0.01 [a]	0.36 ± 0.19 [a,b]
	35	0.55 ± 0.06 [a,b]	7 ± 1 [a,b]	0.03 ± 0.01 [a,b]	0.51 ± 0.06 [a,b]
	40	0.60 ± 0.07 [a]	6 ± 1 [b]	0.03 ± 0.01 [b]	0.57 ± 0.07 [a]
	45	0.63 ± 0.07 [a]	6 ± 1 [b]	0.03 ± 0.01 [a,b]	0.60 ± 0.07 [a]
Cane molasses	25	1.40 ± 0.20 [b]	3 ± 1 [c]	0.04 ± 0.01 [c]	1.37 ± 0.19 [b]
	30	1.81 ± 0.02 [a]	15 ± 3 [a]	0.27 ± 0.01 [a]	1.54 ± 0.01 [a,b]
	35	1.74 ± 0.06 [a]	12 ± 1 [b]	0.21 ± 0.01 [b]	1.53 ± 0.05 [a,b]
	40	1.82 ± 0.12 [a]	15 ± 2 [a]	0.27 ± 0.02 [a]	1.54 ± 0.10 [a,b]
	45	1.96 ± 0.09 [a]	15 ± 1 [a]	0.29 ± 0.01 [a]	1.66 ± 0.07 [a]

* cultures were harvested after 48 h of incubation in shaken-flasks at 200 rpm at 30 °C; a-d – the data show the mean ± standard deviation of triplicates (means with different superscripts within the same column are significantly different at $P \leq 0.05$ level – Tukey test); e – calculated from GC analysis; f – calculated based on CDW and P(3HB) contents; g – calculated by subtracting P(3HB) concentration from CDW

a by-product and 10 kg of glycerol from a total of 100 kg of biodiesel (Du et al., 2012).

Here, we are reporting for the first time the use of sweetwater as a carbon source for PHA production. Sweetwater is considered to be a potential carbon feedstock, which can be used for biomaterial production (Wadekar et al., 2012; Azemi et al., 2016). Previously, sweetwater was used as carbon source for rhamnolipid production by *Pseudomonas aeruginosa* UMTKB-5 strain (Azemi et al., 2016). The ability of *B. megaterium* UMTKB-1 to use sweetwater for P(3HB) production suggests that it could be developed as promising carbon feedstocks. Glycerin pitch was not selected for further experiments due to low P(3HB) accumulation.

Effect of different C/N ratios on P(3HB) production

The C/N ratio is a very important variable to be considered during PHA production. The preferable C/N ratios are in the range between 20 and 50 (Amirul et al., 2008). *Cupriavidus* sp. USMAA2-4 strain grown on a medium supplemented with oleic acid as a carbon source at a C/N ratio of 20 resulted in maximum PHA production (54.8 wt%) and cell growth (8.9 g/l) (Aziz et al., 2012). Based on a research performed by Kulpreecha and co-workers (2009), the C/N ratio of 25 resulted in a maximum cell growth (8.24 g/l) and P(3HB) accumulation of 50% of cell dry weight (CDW) by batch bacterial cultures. In addition, the *Azospirillum* and *Azotobacter,* as well as other rhizosphere bacteria, were only able to produce PHA under a C/N ratio of 20 (Itzigsohn et al., 1995). The *B. megaterium* UMTKB-1 strain was tested with different C/N ratios ranging from 5 to 25 and using different carbon sources (Nuzi et al., 2012). However, the cell biomass and P(3HB) production recorded by Nuzi and co-workers (2012) was lower as compared with other *Bacillus* spp. used in other studies (Kulpreecha et al., 2009; Kanjanachumpol et al. 2013). Therefore, in this study, higher C/N ratios were tested using *B. megaterium* UMTKB-1 strain to observe their effects on cell growth and P(3HB) accumulation. As shown in Table 2, the highest P(3HB) content (49 wt%) and CDW (2.01 g/l) were obtained from glycerol at C/N ratio of 35. Cer-

tainly, in this study a higher P(3HB) accumulation and cell growth were observed than that in a study by Nuzi and co-workers (2012).

However, when different C/N ratios were tested in sweetwater the P(3HB) content was in the range 3-9 wt%. For cane molasses, we recorded similar P(3HB) content between 3 and 15 wt%. In addition, the cell biomass grown on sweetwater and cane molasses was in the range 0.29-0.63 g/l and 1.40-1.96 g/l, respectively. Based on statistical analysis, the use of different C/N ratios with sweetwater did not exhibit significant changes in the overall P(3HB) concentration. Nevertheless, with cane molasses, significant increase in P(3HB) concentration was observed when C/N ratio was increased from 25 to 30, but remained almost unchanged with C/N ratio above 35. Almost 7-fold increment in P(3HB) concentration was observed when cane molasses was used as a growth medium, compared with that when sweetwater was used.

Cane molasses has been widely used as a renewable low cost feedstock in an industrial-scale fermentation of PHA by *Bacillus* sp. (Kanjanachumpol et al., 2013; Kulpreecha et al., 2009). A high sugar content in cane molasses supports cell growth as well as PHA accumulation (Du et al., 2012). Chaijamrus & Udpuay (2008) reported when *B. megaterium* 6748 strain were grown in cane molasses as carbon sources and corn steep liquor as nitrogen sources, an about 35 wt% P(3HB) production were produced. Cane molasses is proven a suitable carbon source for *B. megaterium* in PHA production due to the presence of a high-level sugar content.

Biosynthesis of P(3HB) in seawater as culture medium

Seawater was tested as an alternative culture medium to the commonly used MSM, in shaken-flasks and fermenter experiments. As the *B. megaterium* UMTKB-1 strain is a marine isolate, it can withstand saline conditions (Rodríguez-Contreras et al., 2016). Substitution of MSM with seawater is a positive approach in lowering PHA production costs. *Bacillus megaterium* S29 was able to grow optimally at sodium chloride (NaCl) concentration of 1-10% (w/v) (Rodríguez-Contreras et al., 2016). No additional nitrogen source was added into the culture. *Bacillus megaterium* can adapt to a wide variation in the osmotic pressure and has a wide tolerance range of pH level, from acidic to alkaline conditions. Based on research conducted by Lee and co-workers (2015), the optimum pH for *Bacillus* spp. was determined to be in the range 5-9.

Among the three carbon sources tested, glycerol was also found to be the most suitable carbon source for P(3HB) production in seawater. The strain was able to accumulate 27 wt% of P(3HB) when seawater was supplemented with glycerol, followed by 18 wt% with supplementation with sweetwater and 7 wt% with cane molasses. The comparison between P(3HB) accumulation using glycerol and sweetwater as a carbon sources were two-fold different. In a similar study reported by Pandian and coworkers (2010), in order to promote P(3HB) production in fed-batch production by *B. megaterium* SRKP-3 seawater was used as a nutrient source and rice bran as a carbon source. Maximum production of 11.32 g/l of P(3HB) content was observed (Pandian et al., 2010). In a separate study, seawater was evaluated as a culture medium for PHA production (Yue et al., 2014). The wild type *Halomonas campaniensis* LS21 strain produced approximately 26% of P(3HB) with cellulose and starch as carbon sources in seawater used as a growth medium (Yue et al., 2014). The major advantages of bacterial fermentation in saline conditions are minimal contamination by bacteria (which lack saltwater resistance) and the ability to use filtered seawater as a culture medium.

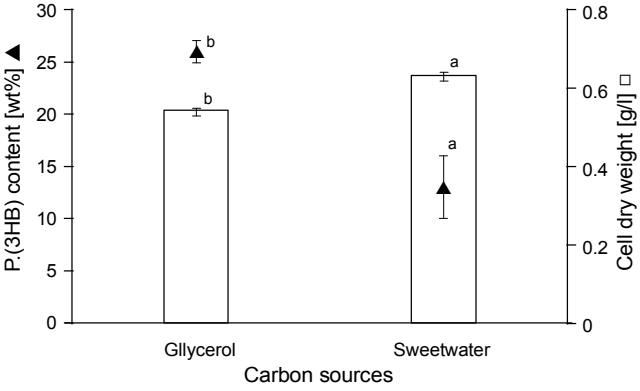

Fig. 2. The biosynthesis of P(3HB) by *B. megaterium* UMTKB-1 strain in 2l bioreactor using seawater as culture medium. Values are means of three replicates. Mean data accompanied by different alphabet letters indicate significant difference within a group (Tukey's HSD test, $P < 0.05$)

The production of P(3HB) by *B. megaterium* UMTKB-1 was up-scaled to a 2l bioreactor based on the results from shaken-flask cultures. In this batch culture experiment, two different carbon sources, glycerol, and

Table 3. Biosynthesis of P(3HB) by *B. megaterium* UMTKB-1 in seawater medium via shaken-flask cultures through one-stage cultivation*

Carbon sources	CDW [g/l]	P(3HB) content [wt%] [d]	P(3HB) concentration [g/l] [e]	Residual biomass [g/l] [f]
Glycerol	0.69 ± 0.04 [b]	27 ± 1 [a]	0.17 ± 0.01 [a]	0.52 ± 0.04 [b]
Sweetwater	0.95 ± 0.18 [a]	18 ± 2 [b]	0.15 ± 0.05 [a]	0.80 ± 0.13 [a]
Cane molasses	0.57 ± 0.03 [b]	7 ± 1 [c]	0.04 ± 0.01 [b]	0.53 ± 0.02 [b]

* the cells cultures were harvested after 48 h of incubation in shaken-flasks at 200 rpm at 30 °C; a-c – the data show the mean ± standard deviation of triplicates (means with different superscripts within the same column are significantly different at $P \leq 0.05$ level – Tukey test); d – calculated from the GC analysis; e – calculated based on CDW and P(3HB) contents; f – calculated by subtracting P(3HB) concentration from the CDW value

Table 4. The biosynthesis of P(3HB) by *B. megaterium* UMTKB-1 strain in sweetwater medium via shaken-flask and fermenter cultures in a one-stage cultivation [a]

Culture mode	CDW [g/l]	P(3HB) content [wt%] [a]	P(3HB) concentration [g/l] [b]	Residual biomass [g/l] [c]
Shaken-flask	0.82 ± 0.01	14 ± 3	0.12 ± 0.02	0.66 ± 0.01
2l bioreactor	1.32 ± 0.01	50 ± 2	0.66 ± 0.01	0.66 ± 0.01

* the cell cultures were harvested after 48 h of incubation in a 2l bioreactor and shaken-flask cultures at 200 rpm at 30 °C (data show the mean ± standard deviation of triplicates); a – calculated from the GC analysis; c – calculated based on CDW and P(3HB) contents; c – calculated by subtracting P(3HB) concentration from CDW

sweetwater were tested with seawater as the culture medium (Fig. 2). P(3HB) accumulation of 26 wt% was recorded using glycerol compared with 13 wt% with sweetwater, meanwhile, the P(3HB) concentrations were recorded as 0.14 g/l and 0.08 g/l, respectively. This finding highlights the reproducibility of results obtained in shaken-flask cultures as similar results were observed in the fermenter scale experiment. Nevertheless, higher yield is normally expected in fermenter-scale experiments, which should be possible by further optimizing the culture conditions. For example, an effective transfer rate for *B. megaterium* DSM 32 strain was obtained under volumetric oxygen transfer rate ($k_L a$) of 0.006 s^{-1} and when P(3HB) concentration of 3.3 g/l was recorded (Faccin et al., 2013).

Biosynthesis of P(3HB) in sweetwater medium

Several bacteria are known to grow and produce PHA from agro-industrial residues such as wheat bran and corn-starch (Sharmala et al., 2012). In this experiment, sweetwater was used as the sole growth and P(3HB) production medium. An effort was made to test the possibility of a complete elimination of MSM, carbon, or nitrogen sources. Experiments were carried out using both shaken-flasks and a fermenter. The strain was able to grow and accumulate up to 14 wt% of P(3HB) in shaken-flask cultures (Table 4). Nevertheless, better accumulation was observed in a fermenter-scale experiment. P(3HB) content of 50 wt% with 1.32 g/l of CDW was recorded. This amount to an about 5-fold higher P(3HB) concentration when compared with shaken-flask cultures. Wastes are often rich in organic contents and less rich in nutrient contents and such a state of unbalanced nutrients may support the growth of some microorganisms and PHA production. Based on research conducted by Solaiman and co-workers (2006), the culture medium for PHA production is not only limited to standard MSM, but the media from inexpensive materials such as waste from agro industry has sufficient amount of nutrients needed for cell growth and maintaining metabolic pathways.

Sweetwater, which is mainly composed of water (79.9 wt%), can substitute distilled water that is normally used when preparing salts medium. Besides, sugars

(18.5 wt%) as well as glycerol (10.3 wt%) present in sweetwater may serve as carbon sources for growth and PHA accumulation. Trace elements and magnesium sulfate ($MgSO_4 \cdot 7H_2O$) supplements in the culture medium will acts as an additional nutrient that is need for cell growth of the bacterial strain. However, further analysis is required to study the nitrogen concentration in sweetwater. Sweetwater is slightly acidic and perhaps the pH has to be adjusted when other pH sensitive strains are to be grown, unlike *Bacillus* sp. that tolerates pH in the range between 5 and 9. Our findings indicate that sweetwater can be used as an alternative culture medium for PHA production. The yield may be improved by further optimizing culture conditions.

Molecular weights of P(3HB) produced by B. megaterium UMTKB-1 using different carbon sources

The weight-average molecular weights (M_w), number-average molecular weight (M_n), and polydispersity indices (M_w/M_n) of P(3HB) synthesized from different carbon sources were obtained using GPC analysis. M_w/M_n provides an estimate of the size distribution of fragments with different degrees of polymerization in the homopolymer produced. In general, the M_w of P(3HB) produced by wild-type bacteria was in the range 88-133 × 10^3 kDa with polydispersity index of 1.32-2.2 (Tsuge, 2002). The general range of P(3HB) produced by *Bacillus* spp. was in the range 55-100 kDa molecular weight (Singh et al., 2009).

In the present study, P(3HB) produced from glycerol in shaken-flask culture recorded the highest M_w of 5.51 × 10^5 Da followed by cane molasses at 3.42 × 10^5 Da. Conversely, the M_n recorded with glycerol was 1.43 × 10^5 Da with polydispersity index of 3.8 whereas cane molasses was 0.93 × 10^5 Da with polydispersity index of 3.7. The *B. thuringiensis* grown on a medium supplemented with glycerol was found to have M_w of 3.85 × 10^5 Da with a polydispersity index of 2.1 (Kumar et al., 2015). The polymer produced by *Cupriavidus necator* DSM 545 strain had lower molecular weight when the medium was supplemented with glycerol at M_w 3.04 × 10^5 Da (Cavalheiro et al., 2009). The presence of fatty acid in crude glycerol has been shown to affect the molecular weight of the polymeric chains in *C. necator* (Cavalheiro et al., 2009). However, the M_w obtained in this study is higher compared with the reports by Kumar et al. (2015) and Cavalheiro et al. (2009) when glycerol was used in the P(3HB) production. Besided the substrate Besides the substrate, M_w of P(3HB) produced was also affected by culture conditions as well as by the P(3HB) recovery method (Chen and Page, 1994). This may explain the differences in M_w of P(3HB) when the same substrate is used.

However, for P(3HB) produced from sweetwater as sole growth and production medium and a source of minerals, the highest M_w of 7.01 × 10^5 Da and M_n with 2.50 × 10^5 Da were recorded, followed by a polydispersity index with 2.8. The M_w of PHA is dependent on the frequency of the chain transfer reaction, in which the PHA polymer chain is transferred from the PHA synthase after polymerization to a chain transfer reagent such as water or alcohol (Tomizawa et al., 2010; Higuchi-Takeuchi et al., 2016). The presence of mixed components of sugars, glycerol, and water in sweetwater could have contributed to the lower chain transfer reaction frequency, thus resulting in high M_w. This is the first report of *Bacillus* sp. being able to produce high M_w P(3HB). P(3HB) polymer with a high M_w is considered as a high-quality material with a wide range of applications.

Conclusions

The marine isolate *B. megaterium* UMTKB-1 could utilize agro-industry by-products for P(3HB) production. Glycerol was the most preferred carbon sources. The strain also demonstrated growth and P(3HB) accumulation in a mixture of seawater and carbon sources. This is the first report on PHA production using sweetwater. This novel carbon substrate could be successfully used for PHA production in a bacterial fermentation process. This substrate is also able to act as the sole growth and PHA production medium. This work clearly demonstrates the possibility to use by-products as raw materials without the requirement of any pretreatment for P(3HB) using a *B. megaterium* UMTKB-1 strain.

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