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Screening of bacteria for PHA production using waste glycerol as carbon source and the ability of new strain to produce P(3HB-*co*-3HV) copolymer

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ABSTRACT

Aims: This study is focused on the isolation, characterization and screening of new bacterial strains isolated from soil and wastewater samples that are able to produce PHA utilizing waste glycerol as sole carbon source in order to create useful products from waste glycerol and at the same time reduce the PHA production cost. A new isolate, *Burkholderia contaminans* Kad1 strain was investigated for its ability to biosynthesize PHA copolymers containing 3HV monomers from waste glycerol and 3-hydroxyvalerate (3HV) precursors.
Methodology and results: PHA producing bacteria were screened using Nile Red and 1% of Nile Blue method. The presence of PHA granules was detected using phase contrast and fluorescence microscopy. *Burkholderia contaminans* Kad1, one out of 23 positive samples, was selected for further study because of its ability to produce high PHA content (47 wt%) and dry cell weight (DCW), (4.2 g/L) when waste glycerol 2% (v/v) was used as the sole carbon source. The 16S rDNA and the PHA synthase gene were sequenced and the PHA produced was confirmed by NMR analysis. A mixture of waste glycerol and sodium valerate fed to the culture gave rise to poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)]. The mole fraction of 3HV monomer in the co-polymer P(3HB-*co*-3HV) sample analyzed using ^{1}H NMR was 23 mol%.
Conclusion, significance and impact of study: This study demonstrated for the first time *B. contaminans* Kad1 was able to use waste glycerol for PHA biosynthesis including the P(3HB-*co*-3HV) copolymer using a mixture of waste glycerol with sodium valerate as the precursor.

Keywords: Burkholderia contaminans, waste glycerol, sodium valerate, P(3HB-*co*-3HV) copolymer

INTRODUCTION

In order for biodegradable plastics to be an alternative for synthetic plastics, it must be economically priced, truly degradable and non-polluting. One such alternative is PHA, which is truly biodegradable and biocompatible. Biodegradable plastics can be completely degraded into CO_2, H_2O, methane and biomass within 1 year. This process is executed by microorganisms. Polyhydroxyalkanoates (PHAs) are linear polyesters of repeating units of 3 hydroxyalkanoic acid monomers which can be different in their carbon chain length. PHAs are produced in nature by bacterial fermentation of lipids or sugars (Kamilah *et al.*, 2013). PHAs are synthesized by bacteria as a reserve source of carbon and energy. The PHA granules are accumulated under limiting nutritional element conditions such as nitrogen, phosphorus, sulfur, oxygen and magnesium in the presence of excess carbon source (Rathi *et al.*, 2013; Sathianachiyar and Devaraj, 2013).

The biodiesel industry generates significant amounts of a by-product called waste glycerol. New utilizations for glycerol have been the subject of much research to extenuate the market glut of this waste and to leverage the economics of biodiesel production. One of the great potential utilization of glycerol is in industrial fermentation where it can be used as a substrate for bacterial growth and the biosynthesis of bacterial products (Chee *et al.*, 2010). An increase in the usage of waste glycerol will reduce its disposal problem and as a bonus, synthesize a value added product. One such usage is in the production of PHA. Annually, it is estimated that approximately 250 million kilograms of waste glycerol byproduct will be produced. In addition to glycerol, the byproduct's composition is different from plant to plant depending on the biodiesel feedstock. Generally, it contains methanol and soap as the two main impurities with different elements such as sodium, phosphorus, potassium, sulfur, magnesium and calcium (Pyle *et al.*, 2008), nitrogen, carbon, glycerides, fatty acid soaps (FFA) and fatty acid methyl ester (FAME) (Ashby *et al.*, 2004; Thompson and He, 2006). The waste vegetable oil (WVO) feedstock that is used in the transesterification process of biodiesel contained 40.5 wt% oleic, 28 wt% linoleic, 18.8 wt%

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palmitic, 6.3 wt% stearic and 1.5 wt% linolenic acids (Thompson and He, 2006). In the glycerol market, the demand for these byproducts was limited by their impurities (Johnson and Taconi, 2007) because the cost of purification is substantial before utilizing them in other industries. The excess amount of generated glycerol may become an environmental problem, since it cannot be disposed of in the environment (da Silva *et al.*, 2009).

Since the purified glycerol is of high value and is a chemical with thousands of utilizations, waste glycerol presents great chances for new applications. For that reason, more attention is being paid to the usage of waste glycerol from biodiesel production as biodiesel industrialization has begun on a large scale and at the same time to reduce the production cost of biodiesel by adding value to its waste. Although many investigations have focused on using waste glycerol directly, review papers on waste glycerol usage are rare. Lately, much work has been done utilizing waste glycerol for PHA production by *Cupriavidus necator* DSM 545 (Cavalheiro *et al.*, 2009), *Halomonas* sp. KM-1 (Kawata and Aiba, 2010) and *Pannonibacter phragmitetus* ERC8 (Ray *et al.*, 2016). PHA synthesis by the polymerization of different monomers via a bacterial strain depends on the carbon substrate and the substrate specificity of the PHA synthase. The incorporation of various kinds and ratio of monomers determines the properties of the polymer. Poly(3-hydroxybutyrate), P(3HB) is the most common type of PHA. However, the application of P(3HB) is limited because it is highly crystalline, brittle and stiff in nature (Freier, 2006). The development of polymer blending or copolymer production with other monomers such as 3-hydroxyvalerate (3HV) (Zhu *et al.*, 2012), 3-hydroxyhexanoate (3HHx) (Budde *et al.*, 2011) and 4-hydroxybutyrate (4HB) has widen the applications. The incorporation of 3HV monomer into P(3HB) polymer chains will improve the P(3HB) homopolymer properties. P(3HB-*co*-3HV) has a lower melting temperature and crystallinity, with greater flexibility and toughness (Luzier, 1992). This copolymer is unique among the PHA copolymer family because the structure and size of 3HV and 3HB are similar and this similarity allows both monomers to participate in a co-crystallization process, in which 3HV monomer could be incorporated into 3HB crystal lattice. This phenomenon is called isodimorphism (Kunioka *et al.*, 1989; Zhu *et al.*, 2013). The cost of raw material especially carbon source account for total PHA production cost (Kim and Chang, 1998). Therefore, the recent researches focus on the usage of cheap renewable substrates for production of PHA.

The present study is focused on the isolation, characterization and screening of high PHA producing bacteria from different environmental sources such as soil and waste water using waste glycerol as the sole carbon source to minimize the production cost of bioplastics. Here we report a new isolate, identified to be *B. contaminans* Kad1 by 16S rDNA, PHA synthase gene (*phaC*) and its ability to produce co-polymer P(3HB-*co*-3HV) was investigated.

MATERIALS AND METHODS

Samples collection

Soil and wastewater samples for bacterial isolation were collected from different environmental sources around Malaysia and the details were mentioned in (Table 1).

Isolation of bacteria

Approximately, 0.5 g of soil sample was added to 10 mL of sterile distilled water and mixed for 1 min with a vortex. This mixture is serially diluted and spread on nutrient rich (NR) agar (Hi-Media) containing (per liter) 10 g peptone, 10 g meat extract, 2 g yeast extract and 15 g/L agar powder (pH 7.0) and incubated for 24 h at 37 °C. Selected bacterial colonies were purified on (NR) plates, examined under microscope for further confirmation (Arshad *et al.*, 2007).

Rapid screening of PHA-producing bacteria

Screening with Nile Blue A and Nile Red stains

Single colonies from the spread plate were patched onto new MM agar in duplicates (master and test plates) using sterile toothpicks. The MM medium per liter contained: KH_2PO_4 (2.80 g), Na_2HPO_4 (3.32 g), NH_4Cl (0.50 g), agar powder 15 g, $MgSO_4{\cdot}7H_2O$ (0.25 g) and Trace elements (1.00 mL/L) (Doi *et al.*, 1995). The solution of $MgSO_4{\cdot}7H_2O$ was autoclaved separately and trace elements solution was sterilized by filtration. These solutions were added together with 2% (v/v) autoclaved waste glycerol as the sole carbon source. Waste glycerol in the form of *GLYCERAW*™ containing at least 85% glycerol was a kind gift from Carotech BF Sdn. Bhd located in Chemor, Perak, Malaysia. The trace elements compositions per liter of 0.1 N HCl were as follows (Kahar *et al*., 2004): $CoCl_2{\cdot}6H_2O$ 0.22 g/L, $CaCl_2$ 7.8 g/L, $FeCl_3$ 9.7 g/L, $NiCl_2{\cdot}6H_2O$ 0.12 g/L, $CuSO_4{\cdot}5H_2O$ 0.16 g/L, $CrCl_3{\cdot}6H_2O$ 0.11 g/L (pH 7.0). The plates were incubated at 30 °C for 48 h. The test plate was flooded with 1% Nile Blue A stain (1.0 g of Nile Blue A stain dissolved in 100 mL of 95% ethanol) and observed under UV light (250 nm) to determine the PHA producing bacteria. The bacteria with orange red fluorescence indicated a PHA producing bacteria. A second screening method was also done in which, Nile red stain (0.5 µg/mL) was added to the growth medium (Spiekermann *et al.*, 1999). Bacterial colony with pinkish color when observed under UV light (250 nm) indicated a PHA producer.

Microscopic observation

Phase-contrast microscopy

A single colony from bacteria was suspended in one drop of water on a slide. Then, the smear was covered with a cover slip and sealed with nail polish. The slide was observed under a phase contrast microscope, *Nikon*

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Table 1: The screening of bacteria producing PHA.

Isolate number	Location	Gram stain reaction	Shape of bacteria in microscope	Plate assay method
1	Penang Botanical Garden	G −ve	Rod	+
2	Penang Botanical Garden	G +ve	Rodococci (Pleomorphic)	+++
3	Penang Botanical Garden	G −ve	Rod	++
3	USM, Penang	G −ve	Cocci	++
5	USM, Penang	G –ve	Cocci	++
6	Cameron Highland, Pahang, Tea farm	G –ve	Cocci	++
7	Cameron Highland, Pahang, Tea farm	G –ve	Cocci	++
8	Gua Kelawar, Langkawi	G +ve	Bacilli	+++
9	Gua Kelawar, Langkawi	G −ve	Cocci	+++
10	Kepala Batas, Penang	G −ve	Cocci	+++
11	Kepala Batas, Penang	G −ve	Cocci	+++
12	USM, Penang	G −ve	Cocci	++++
13	USM, Penang	G −ve	Rod	++++
14	Cameron Highland, Pahang	G −ve	Cocci	+++
15	USM, Penang	G −ve	Rod	++
16	Lata Hijau, Kedah	G −ve	Rod	++
17	USM, Penang	G −ve	Rod	++
18	USM, Penang	G −ve	Cocci	++
19	Lata Hijau, Kedah	G −ve	Cocci	++
20	USM, Penang	G +ve	Rod	++
21	USM, Penang	G −ve	Cocci	+++
22	USM, Penang	G −ve	Cocci	+++
23	USM, Penang	G +ve	Cocci	++

Intensity of Nile Red fluorescence in a plate assay method: +, Poor stained colonies with 1% Nile Blue A; ++, Medium; +++, Good and ++++, Excellent. USM, Universiti Sains Malaysia.

LABOPHOT-2 (Nikon, Japan) at 100× magnification with oil immersion.

Fluorescence microscopy

The method of Nile Blue A staining was used for the observation of PHA granules (Ostle and Holt, 1982). A single colony from bacteria was suspended in a drop of water on a slide and heat-fixed. The smear was flooded with Nile blue A stain and dried at 55 °C for 10 min. After that, the slide was de-stained by utilizing 8% of acetic acid (8.0 mL of acetic acid was added to 92.0 mL distilled water) for 1 min to remove all the unbound stains. Then, the slide washed with water and was allowed to dry. The slide was covered with cover slip. Then, the slide was observed under UV compatible lenses on a UV microscope (Olympus, Japan) using oil immersion 100× magnification and the images were recorded using *Prog Res –C10, Size 5.0* software. The PHA granules inside the bacterial cells showed bright orange fluorescence.

PHA biosynthesis in shake flasks

The purified colonies from NR plates were grown in 5 mL of nutrient broth overnight at 30 °C with shaking at 180 rpm. These cultures were used as inoculums, 3% (v/v) for a 50 mL (MM) broth (MM ingredients without agar powder) with 2% (v/v) waste glycerol. These cultures were used as inoculums, 3% (v/v) for a 50 mL (MM) broth (identical to MM agar but without agar powder) with 2% (v/v) waste glycerol. The inoculated flask was incubated at 30 °C for 48 h with shaking. At the end of cultivation, the cells were harvested by centrifugation (1800 rpm for 10 min, 4 °C). The cell pellet was washed and vortexed with approximately 20 mL of hexane to remove residual oil, then, the pellet was resuspended with 50 mL distilled water to remove the remaining hexane and centrifuged. The washed cells were kept at −20 °C for 24 h before the freeze drying process, and then subjected to gas chromatography (GC) analysis to determine the content and composition of PHA production.

Identification of the isolates

The identification was performed based on 16S rDNA analysis. The genomic DNA was extracted utilizing G-spin™ Genomic DNA Extraction Kit (for bacteria) (iNtRON Biotechnology, Inc., South Korea). The 16S rDNA fragment was amplified by PCR utilizing universal primers F (5′-TNA NAC ATG CAA GTC GAG CG- 3′) and R (5′-ACG GGC GGT GTG TAC-3′) (Wise *et al.*, 1997). The PCR parameters were as follows: 94 °C for 2 min, followed by 30 thermal cycles consisting of 94 °C for 45 sec, 58 °C for 30 sec and 72 °C for 2 min, and a final step of 72 °C for 2 min for extension. The identity and similarity of the sequence were compared to other sequences in Gen Bank using BLAST at the National Center for Biotechnology Information website (NCBI).

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PHA synthase gene for identification

The G-spin™ Genomic DNA Extraction Kit (for bacteria) (iNtRON Biotechnology, Inc., South Korea) was used for extraction of genomic DNA. Amplification of the *phaC* was done by PCR utilizing primers G-D (5′-GTGCCGCC(GC)(CT)(AG)(GC)ATCAACAAGT- 3′) and G-IR(5′-GTT CCAG(AT) ACAG(GC)A(GT)(AG) TCGAA-3′) (Romo *et al*., 2007). The parameters of PCR were as follows: 94 °C for 2 min, 30 thermal cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 40 sec, and a final step of 72 °C for 5 min for extension. The identity and similarity of the sequence was compared to other sequences using BLAST at NCBI.

Biosynthesis of P(3HB-*co*-3HV) copolymer from mixtures of waste glycerol and sodium valerate

P(3HB-*co*-3HV) copolymer having 3HV fraction was produced from the mixture of waste glycerol and 3HV precursors like sodium valerate. The total amount from waste glycerol and 3HV precursor was fixed at 2%, [1% (v/v) waste glycerol with 1% (v/v) sodium valerate]. Two separate experiments were conducted, where the precursor was added at 0 h and after 24 h of cultivation in MM. The precursors were added to promote the synthesis of the copolymer. The cells were harvested at 72 h and the copolymer accumulated was analyzed.

Analytical methods

Gas chromatography (GC) analysis

PHA quantification was done by GC analysis utilizing caprylic methyl ester (CME) as the internal standard for determination of PHA content and monomer composition. Around 25 mg of lyophilized cells were weighed into screw-cap test tubes and was subjected for methanolysis (140 min in 100 °C) in presence of 2 mL of 85% (v/v) methanol and 2 mL of 15% (v/v) sulphuric acid (Braunegg *et al*., 1978). The resulting methyl esters were analyzed via GC (*Shimadzu GC-2010 AF 230LV*) equipped with a capillary column SPB-1 (30 m length, 0.25 mm internal diameter, and 0.25 mm film thickness; Supelco, Bellefonte, PA, USA) connected to a flame ionization detector. Nitrogen gas was utilized as the carrier gas (1 mL/min), and the chloroform-dissolved sample (2 mL) was injected utilizing an auto injector (*Shimadzu AOC-20i*). The injector and detector temperatures were set at 270 and 280 °C, respectively. The column temperature was ramped from 70 to 280 °C at 10 °C/min.

Polymer extraction

PHA was extracted from lyophilized cells by stirring around 1 g of cells in 100 mL of chloroform at room temperature for 5 days. The mixture was filtered utilizing *Whatman 3* filter paper, concentrated in a rotary evaporator to around 15 mL, and precipitated out in 100 mL of rapidly stirred chilled methanol. Then, the purified PHA was air-dried and used for nuclear magnetic resonance (NMR) analysis.

NMR analysis

Polymer samples (25 mg) were dissolved in 1 mL deuterated chloroform ($CDCl_3$) and analyzed using *Bruker AVANCE 400 FT*, operating at 400 MHz for ^1H NMR analysis. The internal chemical shifts were reported in ppm relative to signals of tetramethylsilane $Si(CH_3)_4$.

RESULTS AND DISCUSSION

Screening of PHA producers

PHA producers were screened by plate method utilizing Nile Blue A and Nile Red staining. The bacterial strains when stained with Nile Blue A showed bright orange fluorescence and pinkish colony with Nile Red, both viewed under UV light. These isolates were suspected as positive strains that can produce PHA (Figure 1) and merits further investigation for formation because other lipids fluoresced similarly (Spiekermann *et al.*, 1999; Teeka *et al.*, 2010).

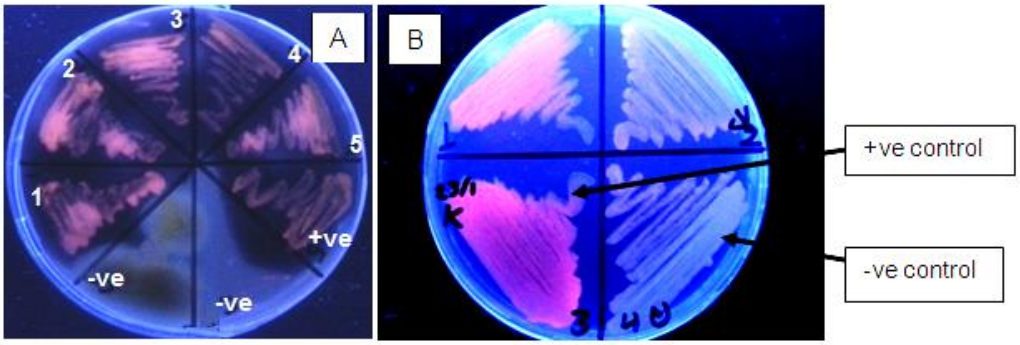

Figure 1: (A) Polyhydroxyalkanoate (PHA) producing bacteria detected on agar plate stained with Nile Blue A. (B) Bacterial cells producing PHA fluorescence when grown on agar medium containing Nile Red. (A and B) observed under UV transillumination after 24 h of incubation at 30 °C.

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A total of 23 bacterial isolates with different colony morphologies were isolated from various environmental sources. All were selected after screening with Nile Red and Nile Blue A staining. Nineteen isolates were Gram negative, while only four isolates were Gram positive (Table 1). All isolates were tested for PHA production using viable colony staining method based on the intensity of staining. Out of 23 isolates, only 2, showed excellent staining, 8 good, 12 medium and 1 poor. The PHA production of isolate 13 indicated that this strain could utilize waste glycerol efficiently and accumulated PHA inside the cell higher (47 wt%) than the others (Figure 2). For this reason isolate 13 was selected for further study because of its high PHA production.

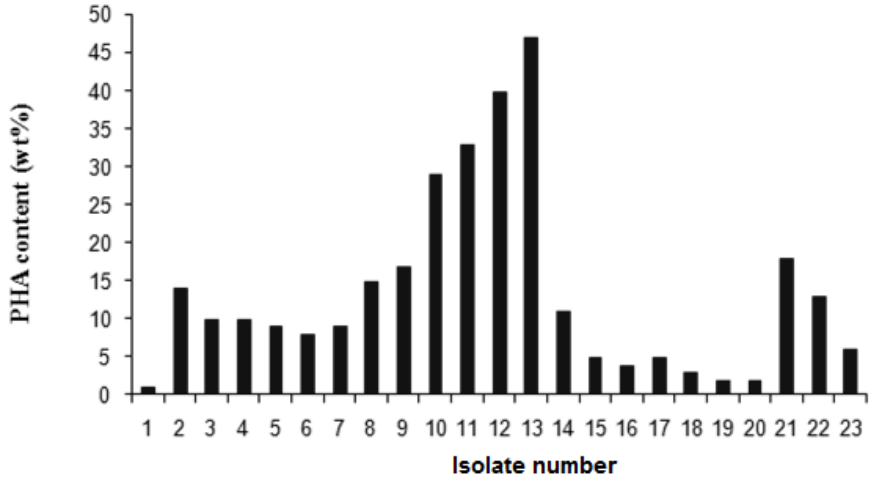

Figure 2: PHA production by 23 bacterial isolates using waste glycerol as the sole carbon source. Isolate 13 is the highest PHA production (47 wt%).

Microscopic observation

The bacteria that produce PHA have granules, which appeared as light refractive inclusion bodies in cell's cytoplasm when used phase contrast microscope. These isolates indicate the existence of PHA granules. Also the presence of PHA granules inside the bacterial cells can be detected by using Nile Red stain because it can diffuse into the cytoplasm of cells and then bind onto the PHA granules which fluoresce under UV light. The observation of cells under UV can visually discriminate between positive and negative PHA-producing strains.

Identification by 16S rDNA analysis

A partial sequence of 16S rDNA of 1400 bp was obtained by PCR. The BLASTn analysis revealed 99% identical to a partial sequence of 16S rRNA gene of *B. contaminans* (accession number NR 104978.1). This result indicates that Isolate 13 is also a *B. contaminans.* Therefore, Isolate 13 will be henceforth named *B. contaminans* Kad1.

Identification by presence of *phaC* synthase gene

The deposited sequence of *phaC* gene in NCBI for *Burkholderia* genus was estimated to be around 1.5-1.8 kb and varied to different species. A set of universal primers were used to amplify a conserved region for *phaC* in order to screen and identify the presence of the gene. In this study, the partial sequence of the *B. contaminans* Kad1, *phaC* was amplified by PCR. A 500 bp fragment was obtained (Figure 3), and its sequence indicated that this gene belongs to *B. contaminans* (98% features polyhydroxyalkanoic acid synthase, class I) when analyzed using BLAST at the NCBI. The similarity to polyhydroxyalkanoic acid synthase, class I indicate the synthase is having substrate specificity to short chain 3HA-CoA, 3HA-CoA, 4HA-CoA, 5HA-CoA and 3MA-CoA that contain to 3 to 5 carbons (Rehm, 2007).

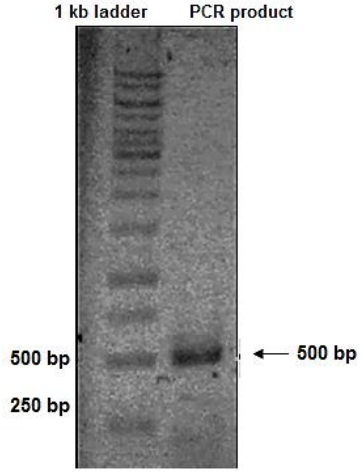

Figure 3: PCR amplification of PHA synthase gene of *B. contaminans* Kad 1.

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PHA production by bacterial isolates

Burkholderia contaminans Kad1, when grown in MM with waste glycerol 2% (v/v) as sole carbon source yielded 47 wt% of PHB in 72 h cultivation. This value is higher than those reported for an unidentified strain AlK7 which yielded 35 wt% PHB in 72 h when cultivated in waste glycerol containing free fatty acid whereas in pure glycerol cultivation the highest PHB content was 33 wt% in 120 h (Teeka *et al.*, 2010). In another report, *C. necator* fed on waste glycerol and pure glycerol in a two-stage fermentation, produced 0.34 and 0.36 g PHB/g glycerol from waste and pure glycerol, respectively (Cavalheiro *et al.*, 2009).

Several researchers have reported that the growth and PHA accumulation in bacteria that had been cultured in biodiesel-derived waste glycerol were inhibited due to sodium salt and other impurities in waste glycerol (Mothes *et al.*, 2007; Cavalheiro *et al.*, 2009). Mothes *et al.* (2007) reported that the ICP-AEC analysis showed that MM that supplemented with unpurified waste glycerol contained 1.384 g/L salt. It was shown that the concentration of salt which caused the reduction in PHA production was 5 g/L.

A similar study of PHA production from soy-based biodiesel co-product stream (CSBP) found that *P. oleovorans* produced 1.3 g/L bacterial (DCW) containing up to 27% PHB when the concentration of CSBP was varied from 1 – 5%. *Pseudomanas corrugata* on the other hand could produce 2.1 g/L dry cell weight containing 42% mcl-PHA. They explained that glycerol could be utilized as a substrate for PHB while the FFA (free fatty acid) and FAME (fatty acid methyl esters) that were also present in CSBP, could be metabolized in the β-oxidation pathway to form the precursors for mcl-PHA production (Ashby *et al.*, 2004).

Many factors affecting on PHA production cost include carbon substrate cost, PHA yield and the method of PHA recovery (Choi and Lee, 1999) and the substrate's cost accounts for approximately 40% of the total PHA production cost (Choi and Lee, 1997). However, the use of *B. contaminans* Kad1 to convert abundant waste glycerol into higher value products could help to reduce the cost of carbon source as well the need to discharge it to the environment.

Biosynthesis of P(3HB-*co*-3HV) copolymer from mixture of waste glycerol and sodium valerate

In this study, the biosynthesis of P(3HB-*co*-3HV) copolymer from mixtures of waste glycerol and sodium valerate was evaluated. The effect of adding the precursor at different time (0 h and 24 h of cultivation) was studied. At 0 h, the precursor was added at the beginning of the culture. In the second experiment, the cells were grown for 24 h before adding the precursor. This way, adequate cell density can be achieved before encountering the toxic effect of the precursor when fed after 24 h. The results showed improvement in bacterial (DCW), PHA content and incorporation of the second carbon source when the addition of sodium valerate was done at 24 h of cultivation (Table 2). The 3HV incorporation was only 8 mol% at 0 h compared to 24 mol% when the precursor was added after 24 h of cultivation.

Table 2: Biosynthesis of P(3HB-*co*-3HV) copolymer by *B. contaminans* Kad1 from mixture of waste glycerol and sodium valerate[a].

Precursor addition time (h)	Dry cell weight[b] (g/L)	PHA content[c] (wt%)	Total (3HB)[d] (g/L)	PHA composition (mol%)	
				3HB	3HV
Sodium valerate					
0	4.2±0.1	26±1	1.09	92	8
24	5.6±0.1	35±1	1.96	76	24

[a]Cells were cultivated in MM containing 1% (v/v) of waste glycerol with 1% (v/v) Na- valerate incubated for 72 h at 37 °C with the agitation speed 180 rpm.
[b]Dry cell weight after lyophilization.
[c]PHA content in lyophilized cells was determined by using GC analysis.
[d]Total P(3HB) = DCW × P(3HB) content.
P(3HB) content and DCW was determined in triplicate; the mean and standard deviations are provided.

The P(3HB-*co*-3HV) concentration was higher in the 24 h culture (1.96 g/L) compared to the 0 h culture (1.09 g/L). Likewise, the cell biomass increased from 4.2 g/L to 5.6 g/L and PHA production from 26 wt% to 35 wt% when sodium valerate was added at 24 h. The sodium valerate had been shown to be metabolized through the β-oxidation cycle producing 3-hydroxyvaleryl-CoA which was directly incorporated to form P(3HB-*co*-3HV) without catabolism (Doi *et al.*, 1988). The low conversion percentage of short-chain fatty acids such as sodium valerate is common. This is thought to be due to accumulation of undissociated fatty acids which inhibits substrate utilization in the cell (Wang *et al.*, 2010).

NMR analysis to identify the homopolymer and copolymer

The ^{1}H NMR spectra of the PHA extracted from *B. contaminans* Kad1 (Figure 4A) showed corresponding peaks assigned to methyl (-CH3), methylene (-CH2) and methine (-CH) proton groups. The ^{1}H NMR spectra of the sample and the structure of the standard polymer are

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identical; confirming that extracted intracellular compound is polyhydroxybutyrate (PHB). The presence of 3HB and 3HV is confirmed in the ^{1}H NMR spectrum (Figure 4B). Methyl proton resonance V5 of 3HV monomer can be assigned to a terminal (-CH3) group at 0.9 ppm and the methyl proton resonance B4 of 3HB monomer at 1.3 ppm, the methylene (-CH2) group V4 of 3HV monomer at 1.7 ppm indicating the presence of 3HV in the copolymer chain. The mole fraction of copolymer P(3HB-*co*-3HV) was calculated from the ratio of the V5 of 3HV monomer and B4 of 3HB monomer. The mole fraction of 3HV monomer in the copolymer from waste glycerol and 3HV precursors using ^{1}H NMR was 23 mol%, which was not much different from the 24 mol% obtained by GC.

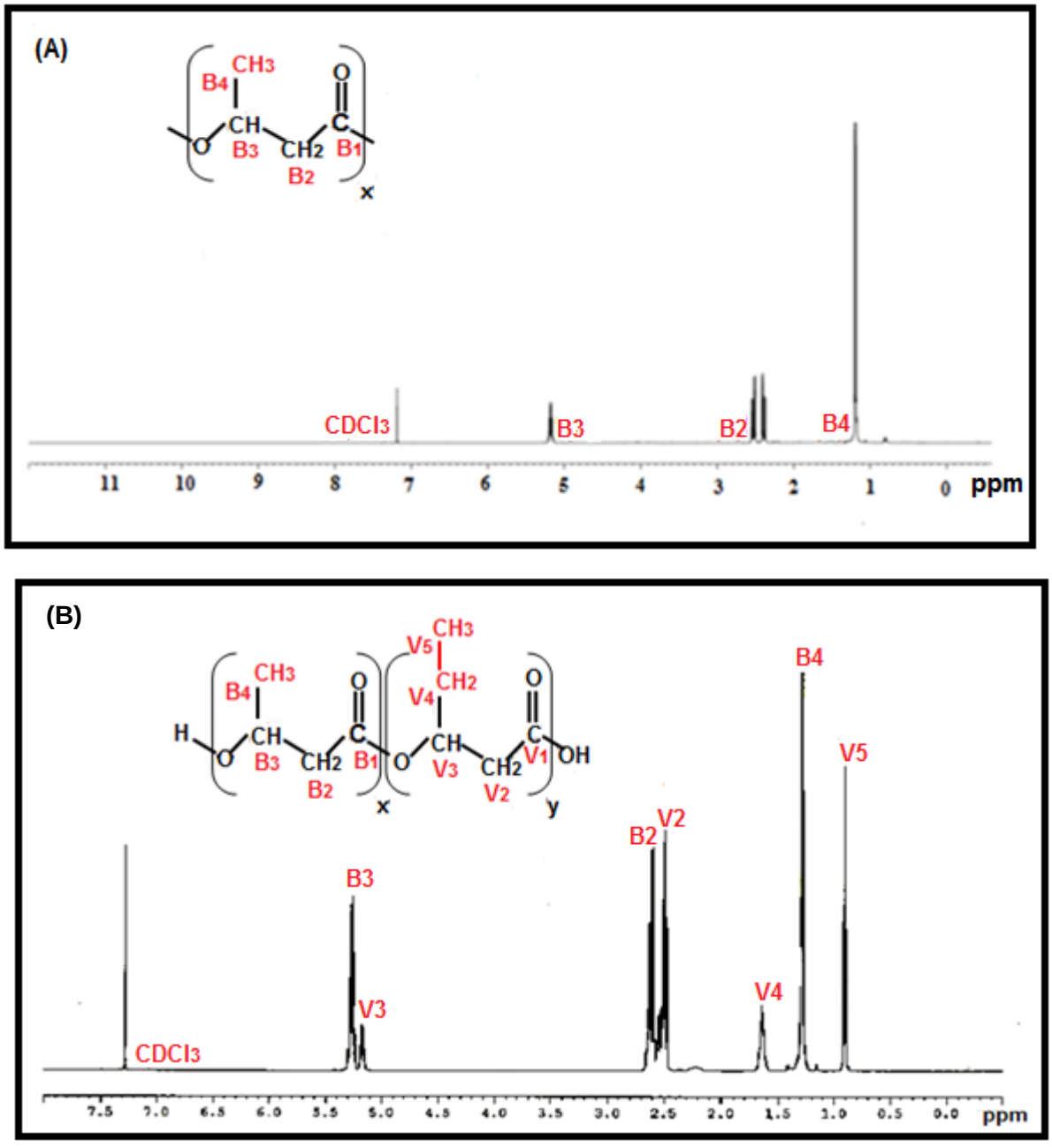

Figure 4: (A) ^{1}H NMR spectrum confirms the presence of P(3HB) polymer produced by *B. contaminans* Kad1. (B) ^{1}H NMR (400 MHz) spectrum of P(3HB-*co*-3HV) in deuterated chloroform using waste glycerol with sodium valerate as precursors.

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CONCLUSION

In this study, various bacterial strains were isolated from different environmental sources and screened for PHA production using waste glycerol as the sole carbon source. *Burkholderia contaminans* Kad1 was selected as a potential strain for the conversion of waste glycerol into PHB. This strain has the ability to produce a copolymer using waste glycerol with sodium valerate as the precursor. The mole fraction of 3HV monomer in the co-polymer P(3HB-*co*-3HV) sample analyzed using ^{1}H NMR was 23 mol%. The usage of waste glycerol as a carbon and energy source for bacterial fermentation decreases the cost of PHA production and as well as adding value to waste by-product of biodiesel. Our results indicate waste glycerol is a good carbon and energy source for PHA production.

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