Extraction of Polyhydroxyakanoate (PHA) from Palm Oil Mill Effluent (POME) using Chemical Solvent Extraction

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Abstract. Palm oil mill effluent or POME is the waste water discharge from palm oil milling activities. Polyhydroxyalkanoate or PHA is the linear polyester that produces by bacterial fermentation of sugar or lipids with nature process. The objectives of this research are to extract PHA from POME using chemical solvent and to study the percentage of purity and recovery of PHA. A methodology was developed for PHA extraction from palm oil mill effluent using chemical solvent. PHA from POME can be extracted by using chemical extraction method by using non halogenated solvent that are acetone, methyl isobutyl ketone and ethyl acetate. The result show percentage of mass PHA recovery with acetone has high percentage that is 93% follow by ethyl acetate with 81.2% and lastly with methyl isobutyl ketone with 66%. Meanwhile, the PHA purity percentage by using acetone is 71%, follow by ethyl acetate 58% of purity obtain lastly methyl isobutyl ketone with 49% PHA purity obtain. PHA can be extracted from POME using chemical solvent extraction method.

1. Introduction

Polyhydroxyalkanoate or PHA is linear polyester that produces by bacterial fermentation of sugar or lipids with nature process. PHA is one of the member families of naturally-occurring bio polyesters synthesized by various microorganisms. With extremely different properties of materials, PHA can combine with more than 150 of different monomers. They are biodegradable and biocompatible polyesters intracellular also having similar characteristics with mechanical properties of polypropylene. PHA was collected by many bacteria as energy reserved material and carbon sources. Since PHA was discovered by Lemogine in 1926, it’s had been attracted many research interest because of its biodegradability, biocompatibility, chemical-diversity, and its produce from renewable natural sources. PHA also can be changed by unification, modifying the surface or combining PHA with other polymers, enzymes and inorganic materials. Because of its properties, it is possible to manufactures wider range of application and become the next generation of bio-plastic.

PHA can be classified in three groups depending on the total number of carbon atoms within a PHA monomer. First, short-chain span PHA (scl-PHA;3 to 5 carbon atoms) for example poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV) and their copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate)(PHBV)[1]. Subsequent, medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms) such as poly (3-hydroxyoctanoate) (PHO) and poly (3-hydroxynonanoate)(PHN), which are primarily formed as copolymers with 3-hydroxyhexanoate (HHx), 3-hydroxyheptanoate (HH) and/or 3-hydroxydecanoate (HD) and the third is long-chain length PHA (lcl-PHA; 15 or more carbon atoms)[1]. PHA research has goes through a lot of development and it may become the preferred as the next generation in bio-plastic. PHA is also considered as
pharmaceutically-active composite and been now research as prospective anti-HIV drugs, anti-cancer drugs, antibiotics, and etc.[1] PHA varies considerably in their physical and chemical characteristics. Polymer hydrophobicity, melting point, glass transition temperature and level of crystallinity entirely depend on the composition of the monomer [2]. The family of PHA show evidence of a wide range of mechanical properties from hard crystalline to elastic [3]. Palm oil mill effluent (POME) is a wastewater generated from palm oil milling behavior which requires efficient treatment before release into watercourses due to its exceedingly polluting properties [4]. The untreated waste matter contains 90-95% water and includes the other component such as remaining oil, soil particles and suspended solids. Typical characteristics of POME are shown in Table 1.

Table 1: Typical characteristic of POME [5]

| Parameter                                | Mean  | Range          |
|------------------------------------------|-------|----------------|
| pH                                       | 4.2   | 3.4-5.2        |
| Biological oxygen demand (BOD)           | 25000 | 10250-43750    |
| Chemical oxygen demand (COD)             | 51000 | 15000-100000   |
| Total solids (TS)                        | 40000 | 11500-79000    |
| Suspended solids (SS)                    | 18000 | 5000-54000     |
| Volatile solids (VS)                     | 34000 | 9000-72000     |
| Oil and grease (O and G)                 | 6000  | 130-18000      |
| Ammoniacal nitrogen (NH3-N)              | 35    | 4-80           |
| Total nitrogen (TN)                      | 750   | 180-1400       |

*Units in mg/l except pH

In 2010, palm oil trade in Malaysia produces about 90 million tons of lignocellulose biomass, as well as empty fruit bunches, oil palm trunks, and oil palm fronds, plus POME [6]. POME produced massive amount of methane gas from its anaerobic method and has about 21 times Global Warming Potential (GWP) compared to the other gasses. Therefore, a lot of research been conducted to dropping its risk to the environment. Given that in year 1980-1990s (depending on the country), developments have taken place in the crude palm oil trade to make it environmentally further sustainable, and wastewater treatment systems are now usual in the main areas of manufacture [7]. Wastewater treatment facility is among the most central section in the palm oil mill coordination. This is because the facility is to treat POME, which is being generated in huge amount during the production of crude palm oil (CPO).

Due to the chemical and physical properties of POME, the largest part of the system used in the first phase of the wastewater plant is the anaerobic treatment. The existing systems come across the necessity of the palm oil mill operators to securely discharge the treated POME. Nevertheless, the systems release one of the greenhouse gases (GHG), methane (CH4) into the environment as the by-products of anaerobic digestion of POME.

2. Methodology
The methodology is divided into four parts: sample preparation, methanol pre-treatment, solvent extraction of PHA and PHA purification.

2.1. Sample preparation

All chemicals were obtained from Merck Sdn.Bhd unless otherwise specified. 4L sample of POME was taken from Lepar Hilir Palm Oil Mill. Palm oil mill or POME is waste water generated from palm oil milling activities. Treated POME containing organic acid was filtered to eliminate solid particles and sediments. The light brown fluid of filtered POME obtained was stored at 4°C until further utilize. The filtered POME was evaporated via a rotary evaporator (BuchiRotavapor R-210, 1 L capacity), digital water bath (Buchi Heating Bath B-491) and aspirator pump at appropriate temperatures; 70 °C-95 °C. Since evaporation under vacuum state reduces the boiling point of fluid, the temperature chosen was lower than real boiling point of water, thus reducing power practice.

2.2. Methanol pre-treatment

Ten g samples of dry filtrated POME were mixed with 200 ml of methanol (as a control) at 22±1 °C 5 hours. Biomass was recovered by stirring at 170 rpm for 5 hours in room temperature, washed in distilled water and re-stirring 170 rpm for 30 minutes. PHA substance was determined for all supernatant and PHA molecular weight (MW) determines later. The mixtures were air dried before the next step. The same process was repeated enough for next step with solvent acetone, methyl isobutyl ketone and ethyl acetate extraction.

2.3. Solvent extraction of PHA

After methanol pre-treatment, different amounts of acetone were added to 5 g biomass (mass before methanol wash). Following addition for 24 hours at 170 rpm and 22±1 °C, the PHA solubilised in acetone was divided by filtration and the remaining biomass washed with 20 ml new acetone (solvent). Both volumes of acetone were mixed and rotary evaporated at 50 °C to about 2 ml and the evaporator rinsed with 1 ml new acetone. The collective samples were added drop wise to 10 volumes (20 ml) cold methanol with dynamic stirring. The precipitate was recovered by filtration, air-dried for 4 days, and then weight .The step was repeated using solvent methyl isobutyl ketone and ethyl acetate extraction.

3. Results and Discussion

A lot of non-halogenated solvent that can provide as alternatives to chloroform for PHA recovery have been acknowledged in the academic and copyright journalism [8&9]. In this research, non-halogenated solvent that is acetone, ethyl acetate, and methyl isobutyl ketone were chosen for PHA recovery. The properties and safety characteristic of the solvent in this research would find out how efficient a solvent would be in a recovery procedure. All solvents used in this research have lesser density than water, which it will let uncomplicated decantation of PHA solutions after extraction and centrifugation. Table 2 below the result of PHA produces in every process. Meanwhile figure 1 and figure 2 shows percentage gain and loss during the process.
Table 2: Mass of PHA obtained through all process

| Solvent                         | Acetone | Ethyl Acetate | Methyl Isobutyl Ketone |
|---------------------------------|---------|---------------|------------------------|
| Mass dried sample, g            | 2.000   | 2.000         | 2.000                  |
| Mass after pre-treatment, g     | 1.942   | 1.932         | 1.912                  |
| Mass of POME gained through extraction, g | 1.801   | 1.573         | 1.283                  |
| Mass of POME for purification, g| 0.200   | 0.200         | 0.200                  |
| Mass of POME gained after purification (3 times wash), g | 0.142   | 0.104         | 0.096                  |

Figure 1: Percentage of PHA gain from all process

Figure 2: Percentage of PHA loss in every process
3.1. Pre-treatment

Pre-treatment of POME is needed to formulate the POME further open to solvent extraction and also to get rid of contaminants before they can extracted into the solvent with the PHA. Weighing the dry POME before and after pre-treatment is important to show that contact to remove the largest amount of cellular material and produce of PHA content in the POME. Percentage of PHA recovered and loss after treatment can be calculated by Equation (1) and Equation (2)

Percentage PHA recovered

\[
\text{PHA} \% = \frac{\text{Mass after pre-treatment} - \text{Mass dried sample}}{\text{Mass dried sample}} \times 100\% \quad (1)
\]

Percentage of PHA loss after pre-treatment

\[
\text{PHA} \% = \frac{\text{Mass dried sample} - \text{Mass after pre-treatment}}{\text{Mass dried sample}} \times 100\% \quad (2)
\]

There is not much loss of PHA during pre-treatment. From the result shows that percentage of PHA loss not more than 4 %. The loss of PHA might be happen when filtration process after pre-treatment with methanol. The temperature also will affect the production of PHA. The optimum temperature for pre-treatment process is 22 ± °C. The treatment process will induces cell in POME to a raise in viscosity and loss of PHA. The methanol treatment was applied in this process to coagulate the macromolecules responsible for the increase of produced PHA in the terms of recovery and the loss of PHA because of the handling care or the temperature changes during the process. By using methanol in the washing process, the result demonstrates very small loss of PHA from biomass. Different with other method, pre-treatment with methanol is efficiently dries the biomass prior to extraction. This is because methanol pre-treatment eliminated low molecular weight PHA molecules (probably oligomers), but it does not affect the molecular weight of PHA. Overall, using methanol is suitable for pre-treatment of POME and highly beneficial during treatment.

3.2. Recovery of PHA

From the result, percentage of mass PHA recovery with acetone has high percentage that is 93 % follow by ethyl acetate with 81.2 % and lastly with methyl isobutyl ketone with 66 %. A PHA balance of recovery process in which from the methanol process pre-treatment was subsequently direct solvent extraction under ambient condition then precipitate in cold methanol was conducted to determine where PHA was lost during the process. Percentage of PHA recovered and loss after extraction can be calculated by Equation (3) and Equation (4).

Percentage recovery after extraction calculation:

\[
\text{PHA} \% = \frac{\text{Mass of pome obtain through extraction} (g)}{\text{Mass after pre-treatment} (g)} \times 100\% \quad (3)
\]
Percentage of PHA loss after extraction

\[
\text{PHA} \% = \frac{\text{Mass after pre-treatment} - \text{Mass of pome obtain through extraction} [g]}{\text{Mass after pre-treatment} [g]} \times 100\% \quad (4)
\]

For acetone, the overall sum of PHA loss detected from the recovery process was about 6.3 % during this process. By using ethyl acetate as solvent, the result shows that the percentage of loss during recovery process was 18.8 %. The loss is higher than using acetone. And lastly, by using methyl isobutyl ketone, the result show 33.9 % loss of PHA. This loss is higher than acetone and ethyl acetate. Extraction was performed at optimal temperature 50 °C to recover almost all PHA in the POME. The extraction step process of produces PHA involves different unit operations that ensure the proper removal of the biopolymer from the interior of the cells. In this process, it is necessary to consider the aspect reduction of products losses in the different stages of the process, obtaining a product of high purity and using low toxicity chemicals that is not harmful to the environment. From the result, the mass recovery of PHA that obtains using non-halogenated solvent is high (more than 50 %).

The solvent were chosen in this research, were able effectively dissolve these polymers. The decision to focus on acetate, ethyl acetate and methyl isobutyl ketone is because due to their properties that suitable in separation of the organic phase from aqueous phase during PHA recovery from cell of the POME. Referring Figure 1 and 2, by using acetone, PHA recover from the cell is 93 % recovery and only 6.3 % PHA loss. Using ethyl acetate, the recovery obtain is 81.2 % and the loss of PHA 18.8 %. For recovery of PHA using methyl isobutyl ketone, the result show 66 % recovery and 33.9 % loss of PHA. The probability presence of water can reduce the solvating power of methyl isobutyl ketone. The loss of PHA during the recovery process also might be happen at evaporating stage. Evaporation can be problematic in the process because the polymer tends to coat at the vessel after the solvent remove. The loss also can be happen while drying the PHA, recovery from sample will take time in drying the PHA after process.

Riedel et al., 2012 tested different non-halogenated solvents for extraction of poly (hydroxybutyrate-co-hydroxyhexanoate) [P (HB-co-HHx)] from Ralstonia eutropha [10]. The solvents studied were a methyl isobutyl ketone and ethyl acetate and isoamyl alcohol. From the result the highest polymers recovery obtained were with the solvents methyl isobutyl ketone and ethyl acetate, of 84 % and 93 %, respectively. In this research, the recovery PHA extraction from Palm Oil Mill Effluent, the results shows for the solvent ethyl acetate is slightly lower, 81.2 %. For methyl isobutyl ketone, recovery result shows the about 66 % by the research.

Xuan Jiang et al.,2005 test acetone extraction of PHA from Pseudomonas putida KT2440 [11]. By using acetone as the solvent, the recovery of PHA obtain from this research is 66 %. In this research, PHA was extracted from POME show the recovery obtain is 93 %.

3.3. Purity of PHA

From the purity of PHA result, it shows the percentage of purity decrease after extraction procedure. The PHA purity percentage by using acetone is 71 % with the PHA loss about 29 %. Follow by ethyl acetate 58 % of purity obtain and 48 % loss of PHA and lastly methyl isobutyl ketone 49 % PHA obtain with 55 % of PHA loss. Percentage of PHA recovered and loss after extraction can be calculated by Equation (5) and Equation (6).
Percentage recovery after purification calculation:

\[ \text{PHA} \% = \frac{\text{Mass of pome get after purification (3 times wash)}}{\text{Mass of pome for purification (g)}} \times 100\% \]  

(5)

Percentage of PHA loss after purification calculation:

\[ \text{PHA} \% = \frac{\text{Mass of pome for purification (g)} - \text{Mass of pome get after purification}}{\text{Mass of pome for purification (g)}} \times 100\% \]  

(6)

The loss of PHA during purity process is due effect of the methanol use during washed the PHA and the amount of solvent necessary since it eradicate contaminants that can be co extracted with the PHA. The purity of the PHA three times washed cycle show there were PHA loss in every cycle. For acetone, average of PHA loss in every cycle is about 10 %. For ethyl acetate is 16 % and lastly, for methyl isobutyl ketone is 18.3 %. As the purity of PHA increased with each consecutive dissolution-precipitation, the peak produce is around 238 nm and 280 nm.

Riedel et al. 2012 tested different non-halogenated solvents for extraction of poly (hydroxybutyrate-co-hydroxyhexanoate) \([P \text{ (HB-co-HHx)}]\) from Ralstonia eutropha. The solvents studied were a methyl isobutyl ketone and ethyl acetate and isoamyl alcohol. From the result the highest polymers purity obtained were with the solvents methyl isobutyl ketone and ethyl acetate is 93 % and 94 % respectively. In this research, the recovery PHA extraction from Palm Oil Mill Effluent, the results shows for the solvent ethyl acetate is slightly lower, 58 %. For methyl isobutyl ketone, recovery result shows the about 49 % by the research. The PHA loss in every washing step with methanol is factor of lower result obtained.

Xuan Jiang et al. 2005 test acetone extraction of PHA from Pseudomonas putida KT2440. By using acetone as the solvent, the purity of PHA obtain from this research is 95 %. In this research, PHA was extracted from POME show the recovery obtain is 71%.

3.4. Impurities in PHA recovered

PHA sample were recovered after direct solvent extraction and after one to three cycle of dissolution in solvent followed by precipitation in methanol. Then the sample was tested using UV spectroscopy.
From the result above shows in figure 3, the sample dissolved in chloroform produce peak at 239.2 nm and 277.8 nm. Nucleic acid and aromatic amino acids are absorbed around 277 nm. For the wavelength 500 nm the absorbance is 0.0014 A. But further identification of the contaminant was not pursued.

From the result above shows in figure 4, the sample dissolved in chloroform produce peak at 240.2 nm and 273.8 nm. Nucleic acid and aromatic amino acids are absorbed around 273 nm. Absorbance value for 500 nm of wavelength is 0.013 nm.
From the result above shows in figure 6, the sample dissolved in chloroform produce peak at 238.8 nm and 275.2 nm. Nucleic acid and aromatic amino acids are absorbed around 275 nm. Absorbance value for 500 nm of wavelength is 0.016 nm.

3.5. Absorbance and transmittance
The table 3 below shows the result of absorbance and percentage of transmittance between non treatment of PHA and after treatment of PHA.

Table 3 Absorbance and transmittance value of non-treatment and treatment of PHA

| Solvent            | Non treatment | Treatment  |   |   |
|--------------------|--------------|------------|---|---|
|                    | Absorbance,Au| Transmittance% | Absorbance,Au | Transmittance% |
| Acetone            | 0.079        | 83.4       | 0.014        | 96.83         |
| Ethyl acetate      | 0.370        | 42.7       | 0.013        | 97.05         |
| Methyl isobutyl ketone | 0.187    | 65.0       | 0.016        | 96.38         |

For acetone, the absorbance value for non-treatment of PHA is 0.079 Au and the transmittance of the sample is 83.4 %. After the treatment, the absorbance value for sample acetone solvent is 0.014 Au and the transmittance values 96.83 %. For ethyl acetate, the absorbance value for non-treatment of PHA is 0.370 Au and the transmittance of the sample is 42.7 %. After the treatment, the absorbance value for sample acetone solvent is 0.013 Au and the transmittance value is 97.05 %. Lastly, for methyl isobutyl ketone, the absorbance value for non-treatment of PHA is 0.187 Au and the transmittance of the sample is 65.0 %. After the treatment, the absorbance value for sample acetone solvent is 0.016 Au and the transmittance value is 96.83 %. The result shows the difference of the value absorbance and transmittance of non-treatment and after treatment of purity PHA. The higher the percentage obtained the more purity of the sample. In order to treat the sample, the sample must be washed with methanol for about three times. This is to ensure that the sample will achieve the maximum of its purity. UV test
was done on non-treatment sample and after treatment to show the change of purity level of the sample. From the result, the percentage of acetone was increase about 14.43%. The result for ethyl acetate show the percentage of transmittance increase about 54.35% and for methyl isobutyl ketone also show the increasing about 31.38% of transmittance.

4. Conclusions
The extraction of PHA from palm oil mill effluent (POME) using chemical solvent gives the percentage of recovery as high 90% and purity 71%. By using non halogenated solvent, it gives difference percentage of recovery and purity according to its chemical properties in separated the PHA cell in the sample. Extraction of PHA by using halogenated solvent can lead the percentage of recovery and purity about 96% but, it is highly toxic solvent with low boiling temperature. Percentage of mass PHA recovery with acetone has high percentage that is 93% follow by ethyl acetate with 81.2% and lastly with methyl isobutyl ketone with 66%. Meanwhile, The PHA purity percentage by using acetone is 71%, follow by ethyl acetate 58% of purity obtain lastly methyl isobutyl ketone with 49% PHA purity obtain. PHAs have big potential substitutes for petrochemical plastic, because they have similar properties to thermoplastic materials, are biodegradable and can be produced from renewable raw materials, with their applicability directly linked to the production and extraction process. Because of this, the extraction step should reduce product losses, obtain product of high purity and with its preserved physical and thermal characteristic, less toxicity and can be develop into industrial scale.

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