Characterisation of Polyhydroxyalkanoates (PHA) Extracted from Palm Oil Mill Effluent Using Different Solvents

Norazlina Hashim[1]*, Siti Nuratirah Nora[1], Nur Afiqah Zahiah Azamin[1], Lili Shakirah Hassan[1], Suhaila Abdullah[1], Rabiatul Manisah Mohamed[1], Nabihah Abdullah[1], and Abhishek Dutt Tripathi[2]

[1]Faculty of Engineering Technology (Natural Resources and Environment),
University College TATI,
Jalan Panchor, Teluk Kalong, 24000 Kemaman, Terengganu, Malaysia.

[2]Centre of Food Science and Technology, Institute of Agricultural Sciences,
Banaras Hindu University,
Varanasi, Sear Govardhan Dafi, Uttar Pradesh 221005, India.

*Corresponding Author's Email: norazlina@uctati.edu.my

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Abstract

Polyhydroxyalkanoates (PHA) represent a class of biodegradable polymers with immense potential for mitigating environmental issues associated with conventional plastics. Palm Oil Mill Effluent (POME), an abundant byproduct of the palm oil industry, serves as an economically viable source for PHA production. This study focuses on the characterisation of PHA extracted from POME using a variety of solvents and solvent mixtures. The solvents employed in this research include mixtures of acetone (AC) and ethyl acetate (EA) in the ratios of 75:25, 50:50, and 25:75. The choice of solvent during the extraction process significantly influences the yield and properties of the extracted PHA. This research employs a systematic approach to assess the impact of different solvents on PHA yield. The extraction process involves the collection of POME samples from local palm oil mills, followed by the isolation of PHA using solvents of varying polarities. The extracted PHA was characterised using UV-Vis spectroscopy to determine the efficacy and purity of each solvent and solvent mixture in PHA extraction. The UV-Vis spectral analysis revealed distinct absorption peaks corresponding to the presence of PHA. The extraction efficiency and purity of the PHA varied significantly with the choice of solvent and solvent mixture. Acetone exhibited a strong absorption peak, indicating a high efficiency in PHA extraction, while ethyl acetate showed moderate results. Results indicated that the solvent mixture of AC and EA in a 25:75 ratio yielded the highest purity of PHA, 86%, with a loss of 14%. The findings are anticipated to inform the optimisation of solvent selection, furthering the goal of utilising POME as a valuable resource for environmentally friendly PHA production.

Keywords: Palm Oil Mill Effluent (POME), Polyhydroxyalkanoate (PHA), Purity, Solvent Extraction, Yield Optimisation

1.0 Introduction

Bacterial fermentation produces polyhydroxyalkanoates (PHA), a family of biopolyesters made up of 3-hydroxy acid monomers (mostly 3-hydroxybutyric acid copolymerised with longer monomers like 3-hydroxyvaleric acid) that are used as intracellular carbon and energy storage. Although PHA has the potential to replace fossil plastics because of its thermo-physical

characteristics, which are similar to those of petrochemically generated plastics, it is presently more expensive than conventional petrochemical plastics [1]. A combination of upstream and downstream factors causes the current high cost of producing PHA. Much work has been done to lower the upstream costs by examining the suitability of alternative microbial consortia for fermenting unusual feedstock and inexpensive raw materials as feed for PHA-producing bacteria [2],[3]. Various wastewater streams, including sugar cane molasses, wastewater from olive mills, food waste, waste-activated sludge, paper mill wastewater, and cheese whey, have been utilised for this purpose [4]. It has been demonstrated that mixed microbial cultures (MMC) are less expensive to run and have a higher metabolic potential than single strains [5]. However, in terms of economic weight, downstream operations (PHA recovery and purification) are the most significant but also the least studied parts of the entire PHA manufacturing chain [6]. A thorough understanding of the environmental effects of polyhydroxyalkanoates (PHA) in comparison to polymers derived from fossil fuels is provided by life cycle assessment (LCA) research. Compared to traditional fossil plastics like polyethylene terephthalate (PET) and polyurethane (PU), PHA can have a carbon footprint that is 30–60% lower when it is made utilising mixed microbial cultures and low-value or waste substrates, particularly when renewable electricity or environmentally friendly extraction agents are utilised [7].

The composition of the monomers has a significant impact on the chemical and physical characteristics of PHA. PHA does not dissolve in water. The PHA family has a broad range of mechanical characteristics, including elastic and hard crystalline [8]. Biodegradability is one of PHA's most unique characteristics. It comes from microorganisms naturally and, if buried in soil, can decompose into water and carbon dioxide (CO_2) in seven months [9]. Many microorganisms in the natural world release PHA-abroad enzymes, which allow the breakdown of PHA in the environment into water-soluble supplements, which are then used as food by the producers. Both equivocal esterase and its subclasses work to break down PHA.

PHAs are formed intracellularly as granules encircled by several proteins. In the industrial setting, bacteria are treated with a variety of enzymes that degrade cell walls and proteins. Microorganisms known as bacteria and archaea generate PHA from a range of carbon sources, including gases, alcohols, alkanes, alkanoic acids and saccharides. Few Gram-positive bacteria accumulate PHA, whereas most are Gram-negative [10]. In archaea, PHA-accumulating organisms are mostly restricted to *Haloarchaeal sp*. One of the most popular methods is to use solvents or compounds composed of oxygen and chlorine to extract PHA as efficiently as possible. Another is to use enzymes that break down the undesirable biological components [11]. PHA extraction involves treating the biomass at the ideal temperature with an anionic detergent and a certain catalyst that causes a biochemical reaction. Although Zeneca Bio Products in the UK is now using this method, its sensitivity to the quantity of chemicals and enzymes involved makes it unsuitable for scaling up [12].

PHA extraction entails using an anionic detergent, a specific catalyst that triggers a biological process and the biomass at the optimal temperature [13]. POME displays various properties as a result of a combination of wastes from three main sources: the hydrocyclone separation of the cracked shell and kernel mixture, the clarifying of extracted crude palm oil and the sterilisation of fresh fruit bunch (FFB) [14]. The manufacture of refined bleach deodorised (RBD) palm oil is depicted in Figure 1 [15].

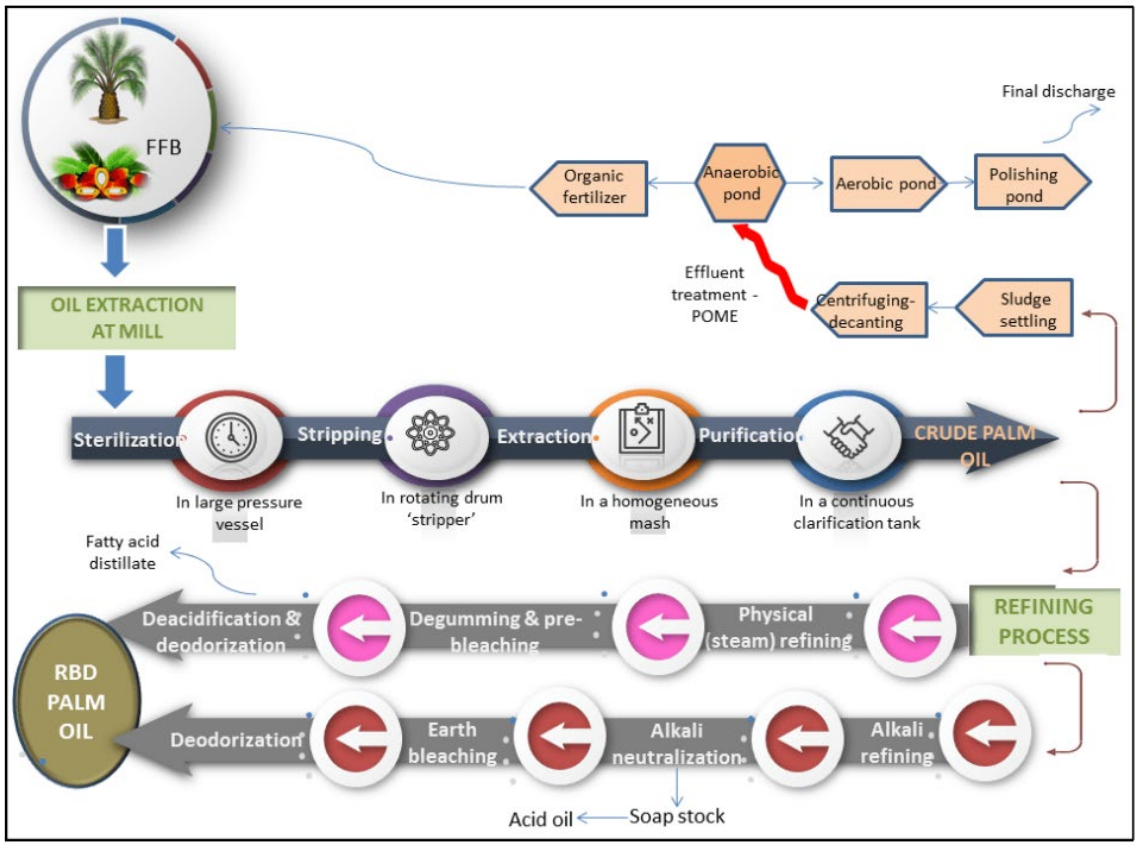

Figure 1: The flow of the palm oil process from the beginning to the final product [15]

Nowadays, palm oil is acknowledged as a premium oil that is widely utilised, mostly for cooking in developing nations. As a result, plantations of oil palm trees are expanding throughout Latin America, Africa, and Asia [16]. The production of palm oil poses significant issues in terms of land and water contamination. POME is rich in chemical oxygen demand (COD) and biochemical oxygen demand (BOD) and can be found in high solid, oil and grease forms [17]. Given their detrimental effects on a wide variety of life forms, these environmental contaminants are of great concern. The preferred method for treating POME is the anaerobic-aerobic bioreactor (IAAB), which may lower COD and BOD in 115 days [15,16]. Many strategies have been investigated recently to create more affordable and efficient treatment methods. Numerous unconventional, low-cost approaches, such as using microorganisms, were put forth as POME treatment strategies [18]. The

efficiency of the mill's operation and process control has a significant impact on POME's properties. Table 1 shows the characteristics of each raw POME.

Table 1: The general characteristics of each raw POME [18]

Parameter	Concentration Range	Current Discharge Limit
Chemical oxygen demand (COD)	15000-100000	-
Biochemical oxygen demand (BOD)	10250-43750	100
Total suspended solids (TSS)	5000-54000	400
Oil and grease	130-18000	50
Temperature	80-90	45
pH	3.4-5.2	5-9

The raw POME is a thick brownish colour in the viscous and colloidal suspension, which contains high water contents (95–96%), suspended solids (2–4%), and oils (0.6–0.7%). POME is an unpleasant-smelling, acidic material with high COD and BOD levels that can also be harmful to the environment [20]. Numerous researchers have reported on the minerals, trace elements, heavy metals and nutrients found in POME. The XRF analysis of POME, sampled in Penang, Malaysia was obtained as below; Carbon 51.0%, Oxygen 35.3%, Sodium 0.0632%, Magnesium 1.09%, Aluminium 0.215%, Silicon 0.552% Phosphorus 0.429%, Sulfur 0.553%, Chlorine 2.75%, Potassium 6.77%, Calcium 1.09%, Manganese 0.0243%, Iron 0.141%, and Rubidium 0.0286% [21]. Numerous elements, such as the degree of FFB maturity, various treatment systems, different batches of FFB and industrial systems, all had an impact on the effluent's quality. Therefore, the Malaysian Department of Environment (DOE) passed the Environmental Quality Act (EQA) 1974 and established standard limitations for the release of POME into the environment to regulate and reduce the pollution impact on the environment [22].

The wet palm oil milling process is the most commonly used technique in Malaysia for removing palm oil from FFB. Sterilisers, strippers, digesters, press machines, clarifiers, separators, extractors and purification systems are the unit operations utilised for extractions. It is possible to maximise efficiency and lessen adverse environmental effects by comprehending the function of each unit in the extraction process. Sterilisation of FFB, stripping, digesting, pressing, clarification and kernel oil recovery are the usual procedures in a wet palm oil mill (POM) [23]. Sterilisation, which involves heating the FFB, is the initial stage in the extraction of crude palm oil. Pressurised steam is used to sterilise the FFB for 60 to 90 minutes at a temperature of 140°C and a pressure of 3×10^5 Pa. The kernel will be forced to separate from the shell wall by the steriliser's hot, pressurised steam [24]. Moreover, it stops the quick production of free fatty acids and deactivates hydrolytic enzymes. Continuous sterilisers and moving conveyors with steam injection at atmospheric pressure have been more popular in recent years

[23]. This sterilisation procedure generates condensate, which is one of the primary sources of POME. The steriliser condensate has a substantially greater total dissolved solids content than the effluent from the hydro cyclone wastewater.

The current study used the POME as the zero-cost feedstock to produce the PHA. The main objective was to examine the yield of PHA obtained from the POME synthesis. Different solvent ratios were used to evaluate which solvent gave the maximum yield of POME.

2.0 Methodology

The methodology of this research is meticulously divided into four critical stages: 1) Sample preparation of POME; 2) Ethanol pre-treatment; 3) Solvent extraction of polyhydroxyalkanoates (PHA); and 4) PHA purification. Each of these stages is vital to ensure the successful isolation and characterisation of PHA from POME.

2.1 Sample Preparation

The preparation of the sample started with the filtration process of POME to remove solid and particle sediments. Then, the filtrated liquid particle was dried using an oven to remove the remaining water at a temperature of 50°C.

2.2 Methanol Pre-Treatment of POME

At 22±1°C, 10 g samples of dry filtrated POME were mixed with methanol (as a control). After 5 hours of stirring at 170 rpm at room temperature, the biomass was recovered by washing in distilled water and swirling again for 30 minutes at 170 rpm. The mixes were allowed to air dry.

The percentage of PHA recovered and lost after treatment was calculated by using Equations (1) and (2).

Percentage of PHA recovered:

$$PHA\ \% = \frac{Mass\ after\ pre-treatment\ (g)}{Mass\ dried\ sample\ (g)}\ x\ 100\% \quad (1)$$

Percentage of PHA loss after pre-treatment:

$$PHA\ \% = \frac{Mass\ dried\ sample\ (g) - mass\ after\ pre-treatment\ (g)}{Mass\ dried\ sample\ (g)}\ x\ 100\% \quad (2)$$

2.3 Methanol Pre-treatment of POME

The solvents used in the extraction were acetone (AC) and ethyl acetate (EA). After methanol pre-treatment, AC:EA (75:25) was added to the 5g biomass. Following addition for 24 hours at 170 rpm and 22±1°C, the PHAs solubilised in AC:EA were divided by filtration, and the remaining biomass was washed with new AC:EA solvent. Both volumes of AC:EA were mixed and rotary

evaporated at 50°C to about 2 mL

The mixed samples were added dropwise to ten volumes (20 mL) of cold methanol while being stirred dynamically. The precipitate was filtered, then left to air dry for four days before being weighed.

The yield of PHAs obtained was calculated. The experiments were repeated using solvent mixtures of AC:EA with different ratios of (50:50) and (25:75). The comparison in terms of PHAs yield was made with PHAs obtained from pure acetone solvent extraction from a previous study [25]. The percentage of PHA recovered and lost after extraction was calculated by using Equations (3) and (4).

Percentage recovery after extraction calculation:

$$PHA\ \% = \frac{Mass\ of\ POME\ obtained\ after\ extraction\ (g)}{Mass\ of\ POME\ obtained\ after\ pre-treatment\ (g)}\ x\ 100\% \quad (3)$$

Percentage of PHA loss after extraction:

$$PHA\ \% = \frac{Mass\ after\ pre-treatment\ (g) -\ Mass\ after\ extraction(g)}{Mass\ after\ pre-treatment\ (g)}\ x\ 100\% \quad (4)$$

2.4 Purification of PHA

200 g of PHAs were reprecipitated in ice-cold methanol after being dissolved in an AC:EA solvent (75:25). Three iterations of the dissolution–precipitation cycle were conducted. Following the dissolution of a dried PHA sample in AC:EA, a UV-visible spectrophotometer was used to perform a spectral scan using AC:EA as the blank. At least two duplicate samples from separate experiments were used for each analysis. After being filtered, the precipitate was allowed to air dry for four days before being weighed. Other ratios of AC:EA solvent mixes were used to repeat the procedure.

The percentage of PHA recovered and loss after extraction can be calculated by using Equations (5) and (6).

Percentage recovery after purification:

$$PHA\ \% = \frac{Mass\ of\ POME\ after\ purification\ (g)}{Mass\ of\ POME\ before\ purification\ (g)}\ x\ 100\% \quad (5)$$

Percentage of PHA loss after purification:

$$PHA\ \% = \frac{Mass\ before\ purification - mass\ after\ purification\ (g)}{Mass\ before\ purification\ (g)}\ x\ 100\% \quad (6)$$

3.0 Results and Discussion

The PHA production process must meet several criteria to be both economically viable and sustainable. High-purity polymers must also be able to be consistently isolated using an effective recovery procedure. This polymer was successfully dissolved by the following non-halogenated solvents: acetone, ethyl acetate and solvent mixtures of acetone and ethyl acetate in varying ratios.

3.1 Methanol Pre-Treatment of POME

Cells in POME will become more viscous and lose PHA as a result of the therapy procedure. Methanol treatment was utilised in order to coagulate the macromolecules that led to the increase in PHA production. The results indicate that very little PHA from biomass is lost during the washing process when methanol is used. Methanol pre-treatment efficiently dries the biomass before extraction. The reason for this is that methanol pre-treatment destroys low molecular weight PHA molecules (probably oligomers) but does not change the molecular weight of PHA. Low molecular weight PHA fractions can be effectively removed from biomass by methanol washing, which aids in purifying the finished product without breaking down the primary polymer chains [26]. All things considered, methanol can be used to pre-treat POME and is quite helpful during treatment. The percentage of POME and POME loss during the pre-treatment phase is displayed in Figure 2.

POME is not significantly lost during pretreatment. The table indicates that over 10% of POME loss has occurred. POME may be lost during the filtration procedure following methanol pretreatment. The production of PHA will also be impacted by temperature. 22±°C is the ideal temperature for a pre-treatment procedure. The proportion of POME and the percentage of POME loss during the pre-treatment phase are displayed in the graph below.

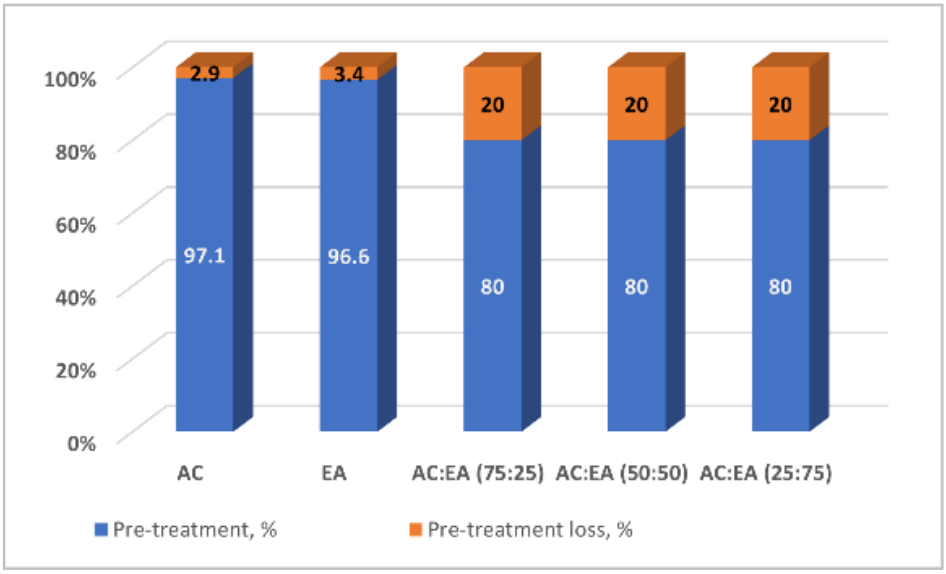

Figure 2: Percentage of POME and POME loss during the pre-treatment process

3.2 Recovery of PHA

Several unit activities are used in the extraction step procedure to produce PHA, which guarantees that the biopolymer is appropriately removed from the inside of the cells. Reducing product losses at various stages of the process, producing a highly pure product and utilising environmentally friendly, low-toxicity chemicals are all important considerations in this process. According to the results, a non-halogenated solvent yielded a significant bulk recovery of PHA (more than 50%). The percentages of PHA loss and recovery throughout the extraction procedure are displayed in Figure 3.

The PHA recovery for a solvent mixture of 75:25 AC:EA is 33.1 % followed by 53.1 % for a 50:50 AC:EA and 60.0 % for 25:75 AC:EA. Pretreatment of the methanol, direct solvent extraction in ambient settings, and precipitation in cold methanol were all steps in a PHA balance of recovery approach that was utilised to determine where PHA was lost during the process. The loss of PHA by using a solvent mixture of 75:25 AC:EA is 66.9 %, followed by 46.9 % loss from 50:50 AC:EA, and 40.0 % loss by using the 25:75 AC:EA solvent mixture. PHA may also be lost during the evaporation phase of the healing process. In the procedure, evaporation can be difficult since the polymer tends to cover the vessel once the solvent is removed. It will take time to dry the PHA, and loss might also happen while the sample is drying.

In 2020, Shakirah and co-workers achieved a high percentage of mass PHA recovery using acetone (93.0 %), followed by ethyl acetate (81.2 %) [25]. The overall PHA loss for acetone that was found during the recovery phase was roughly 7.3 %. The percentage of loss throughout the recovery process was 18.6 %, according to the results obtained using ethyl acetate as a solvent. Compared to utilising acetone, the loss is greater.

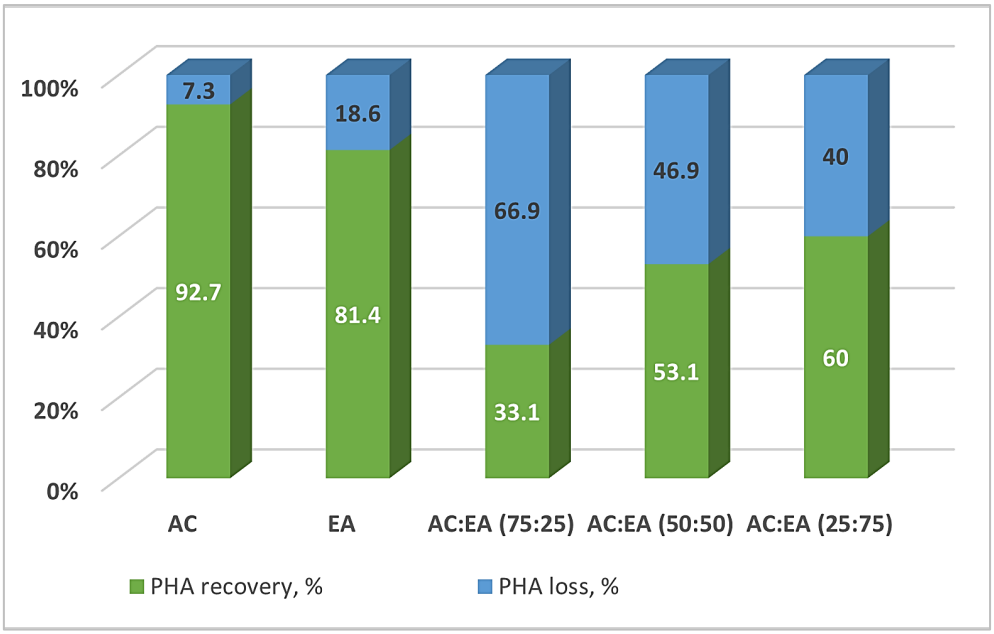

Figure 3: Percentage of PHA recovery and PHA loss during the extraction process

3.3 Yield of Pure PHA

PHA is lost throughout the purification process because of the impact of the methanol used to wash the PHA and the quantity of solvent required, which removes impurities that may co-extract the PHA. PHA loss occurred in each of the three cycles of washing with methanol and 1 ml of solvent, according to the purity of the PHA. The purification process's PHA loss and purity percentages are displayed in Figure 4.

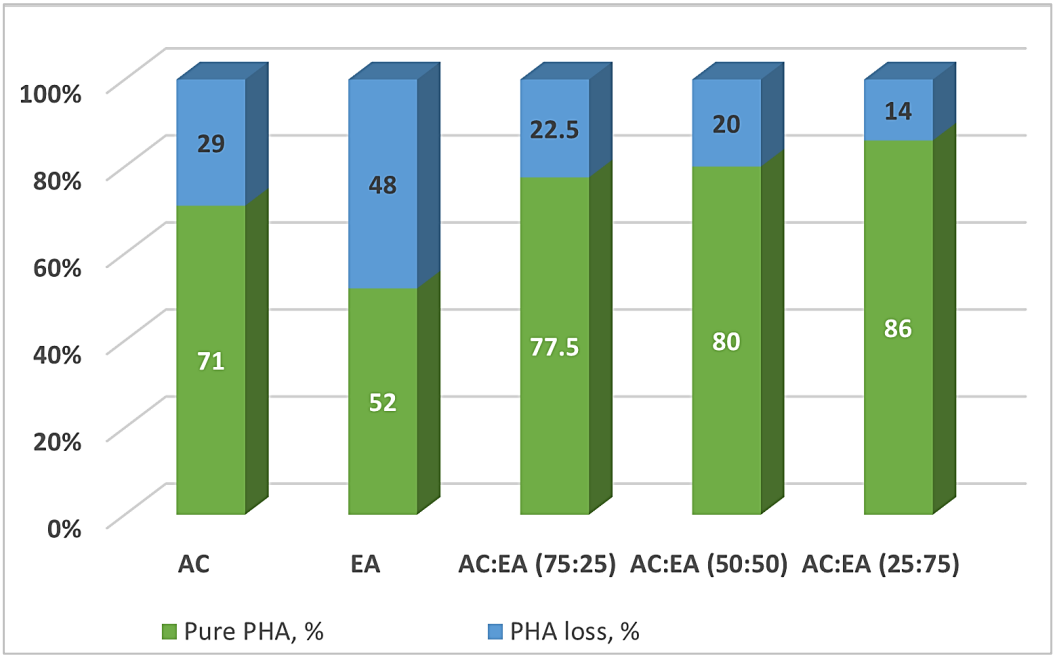

Figure 4: The percentage of pure PHA and PHA loss during the purification process

From the result in Figure 4, the solvent mixture of acetone and ethyl acetate with a ratio of 75:25, the purity obtained is 77.5 % with a 22.5 % loss of PHA. The purity obtained from the solvent mixture of 50:50 AC:EA is 80.0 % with a percentage PHA loss is 20.0 %. Lastly, the solvent ratio of 25:75 gave 86.0 % with a 14.0 % loss of PHA during the purification process as a result of the methanol used in the PHA and the quantity of solvent needed, as it removes impurities that may co-extract the PHA.

Methanol washing is a critical step in the purification process, aimed at removing residual contaminants. The effectiveness of this step depends on the amount and concentration of methanol used. In the case of the 25:75 solvent mixture, the conditions for methanol washing appear optimal. Methanol effectively eliminates impurities without causing significant PHA loss. This optimal washing condition is essential for maintaining high purity and recovery rates of PHA. If the washing step is not adequately optimised, it could result in either insufficient removal of contaminants or excessive loss of PHA. Shakirah et al. developed a lower level of purity after the extraction procedure [25]. The PHA purity by using acetone was 71.0 % with the PHA loss about 29.0 % followed by 52.0 % purity and 48.0 % loss of PHA by using ethyl acetate solvent.

Overall, the initial extraction phase is crucial, as it determines the raw yield of PHA from the sample. The graph indicates that acetone is the most effective solvent for this purpose, producing the highest mass of PHA compared to other solvents tested. This suggests that acetone is particularly efficient at solubilising or separating PHA from the biomass or other components present in the sample. Ethyl acetate follows Acetone in terms of extraction efficiency, although it yields a lower mass of PHA. This difference may be due to the solvents' varying abilities to interact with the PHA, possibly influenced by factors such as polarity, solubility parameters or the presence of other organic materials in the sample.

Following extraction, the PHA undergoes a preliminary purification step, during which the mass of PHA decreases across all solvents and mixtures. This reduction likely reflects the removal of non-PHA materials, such as cell debris, residual biomass, or solvent impurities. The decrease in mass during this stage is expected, as purification typically involves steps that selectively retain PHA while eliminating unwanted components. Interestingly, acetone continues to outperform the other solvents during this purification stage, maintaining a higher mass of PHA than either ethyl acetate or the solvent mixtures. The final purification step involves washing the PHA three times to ensure that impurities are thoroughly removed. At this stage, the mass of PHA decreases significantly for all solvents and mixtures. The graph shows that the lines representing different solvents converge, indicating that despite the initial differences in extraction efficiency, the final purified yield of PHA is relatively similar across all conditions.

3.4 Characterisation of PHA

The three figures below exhibit the peaks of PHA obtained from the UV-Vis characterisation. Figure 5 shows the UV-Vis spectrum of PHA for solvent mixture AC:EA (75:25) treatment. The result shows peaks at 293.4 nm and 226 nm. For the wavelength 500 nm, the absorbance is 0.765 A. The 293.4 nm peak wavelength corresponds to a specific electronic transition where the sample absorbs light most intensely. The absorbance peak at 293.4 nm indicates that this particular wavelength is sensitive to the electronic structure of the sample in chloroform. The higher the absorbance at this peak, the greater the absorption of light by the sample at this wavelength. The peak at 226 nm signifies another electronic transition or possibly a different chromophore in the sample molecule that absorbs light at this specific wavelength.

Figure 6 shows the UV-Vis spectrum of PHA for solvent mixture acetone and ethyl acetate (50:50) treatment. Based on the results, the sample produced peaks at 294.4 nm and 235.4 nm. The absorbance value for 500 nm of wavelength is -0.016A. Peaks at 294.4 nm and 235.4 nm. These peaks represent the wavelengths where the sample absorbs light most strongly. When molecules in the sample absorb photons at these wavelengths, electronic transitions from the ground state to excited states result in maximum absorbance. The negative absorbance value (-0.016A) at a wavelength of 500 nm indicates that the sample does not significantly absorb

light at this wavelength. Instead, light may pass through or not be absorbed, resulting in the low absorbance value.

Figure 7 shows the UV-Vis spectrum of PHA for solvent mixture acetone and ethyl acetate (25:75) treatment. The result displays the peaks at 350.2 nm and 223.8 nm. The absorbance value for 500 nm of wavelength is -2.037A. This peak indicates absorption of light at a wavelength of 350.2 nm. In UV-Vis spectroscopy, such absorption peaks often correspond to electronic transitions within molecules. This wavelength suggests that the sample contains compounds that absorb light in the UV range, possibly due to conjugated double bonds or aromatic systems.

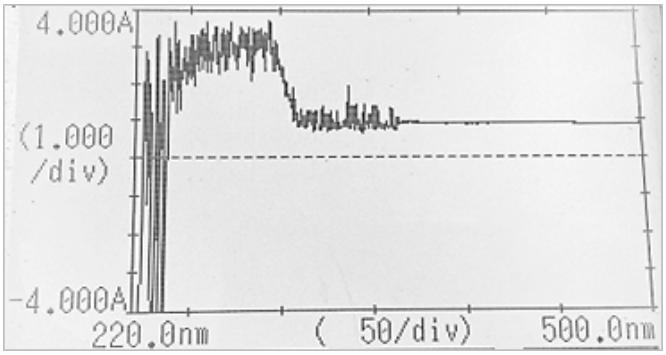

Figure 5: UV-Vis spectrum of PHA for solvent mixture of acetone and ethyl acetate (75:25) treatment

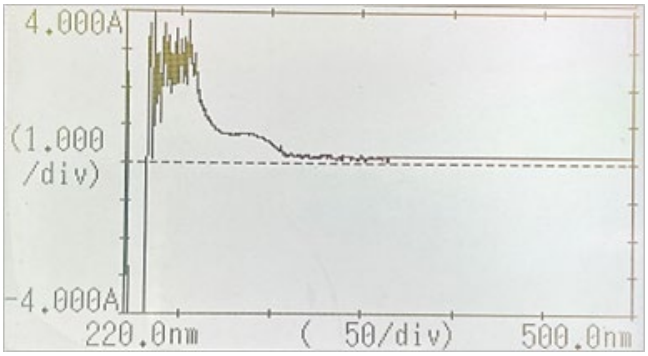

Figure 6: UV-Vis spectrum of PHA for solvent mixture of acetone and ethyl acetate (50:50) treatment

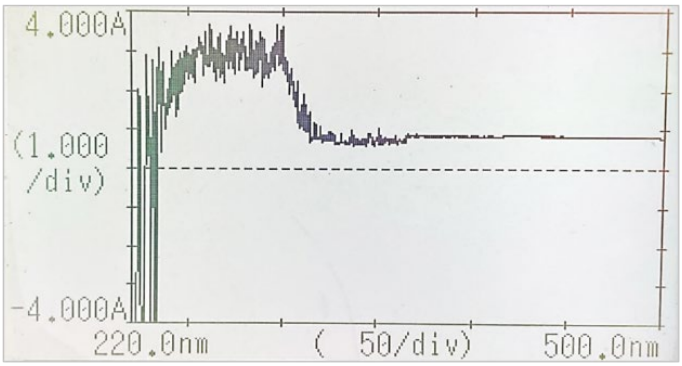

Fig. 7: UV-Vis spectrum of PHA for solvent mixture of acetone and ethyl acetate (50:50) treatment

4.0 Conclusions

In conclusion, POME is wastewater generated from palm oil milling that can be extracted and treated to synthesise the PHA using different solvents. The non-toxic solvent mixture of acetone and ethyl acetate with varying ratios was employed. It yielded varied results, influenced by solvent polarity, selectivity, and efficiency in dissolving PHA. The solvent mixtures presented a high efficiency, with the 25:75 AC:EA ratio showing the highest PHA yield. Acetone demonstrates good extraction efficiency for PHA. The solvent effectively extracts PHA from palm oil effluent, owing to its strong ability to dissolve organic compounds. It ensures high extraction yields and the purity of PHA, making it suitable for industrial-scale applications where purity is critical.

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Author Contributions

Norazlina Hashim: Conceptualisation, Supervision; **Siti Nuratirah Nora**: Data Curation, Writing-Original Draft Preparation; **Nur Afiqah Zahiah Azamin**: Data Validation, Writing-Reviewing and Editing; **Lili Shakirah Hassan**: Co-Supervision, Data Validation; **Suhaila Abdullah**: Methodology; **Rabiatul Manisah Mohamed**: Data Curation; **Nabihah Abdullah**: Methodology; **Abhishek Dutt Tripathi:** Methodology.

Conflicts of Interest

The manuscript has not been published elsewhere and is not under consideration by any other journal. All authors have reviewed and approved the manuscript, consent to its submission, and declare that there are no conflicts of interest.

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