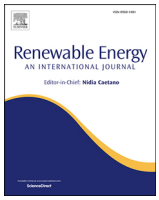
Renewable Energy 263 (2026) 125488

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene



Waste-to-value: Two-Step bioconversion of palm oil mill effluent into biodiesel and polyhydroxybutyrate

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ARTICLE INFO

Keywords:
Bioconversion
Biodiesel
Palm oil mill effluent
Polyhydroxyalkanoate
Polyhydroxybutyrate

ABSTRACT

Converting waste from palm oil mill effluent (POME) into biodiesel and polyhydroxybutyrate (PHB) is a sustainable method for reducing pollution and lowering the cost of these eco-friendly products. This study employed an enzymatic process to convert the oil extracted from POME into biodiesel. The wastewater was then used for PHB synthesis. First, oil was extracted from POME and used as a substrate for biodiesel production. A mixture of immobilized *Candida rugosa* and *Rhizomucor miehei* lipases (1:1, w/w) on PHB was used as a catalyst. Under optimized conditions, the highest biodiesel yield (97.7 ± 0.6%) was achieved within 24 h. The biodiesel produced was characterized and compared with EN 14214 and ASTM D6751 fuel standards. The immobilized lipase maintained its activity over three reuse cycles, indicating its potential for cost-effective application. For PHB production, *Caldibacillus thermoamylovorans* strain PHA005 was used to synthesize PHB from wastewater remaining after oil extraction (WR-POME). Supplementing the effluent with 20 g/L molasses resulted in a maximum PHB production of 59.9 ± 0.5% cell dry mass (CDM) after 96 h, a 1.8-fold increase compared to the non-supplemented effluent. The extracted PHB was confirmed through GC and FTIR analysis. This research presents a sustainable and integrated strategy for POME valorization, producing high-value biodiesel and PHB.

1. Introduction

In tropical regions like Thailand, the palm oil industry has a significant impact on the economy. However, the industry growth produces palm oil mill effluent (POME), a wastewater with high organic content. POME contains substantial organic matter, including carbohydrates (29.6%), proteins (12.8%), lipids (8–35%), oils (0.6–0.7%), and nitrogenous compounds [1]. Untreated POME can cause severe environmental pollution and ecological damage [2]. Consequently, developing innovative and sustainable methods to convert POME from a pollutant into a value-added product is crucial.

Existing research has investigated biodiesel production from POME oil through transesterification, employing chemical methods. Chemical transesterification of POME-derived oil has been widely investigated using alkaline and heterogeneous catalysts [3]. In addition to chemical approaches, enzymatic transesterification has also gained attention due to its mild reaction conditions and environmental benefits. Previous studies have reported biodiesel production from POME via microwave-assisted esterification [4]and lipase-catalyzed transesterification [5].

Although enzymatic transesterification offers benefits such as milder reaction conditions and fewer byproducts, its application to POME valorization is currently limited. The high cost of enzymatic processes is a significant obstacle. Our prior work demonstrated the successful process of producing biodiesel from waste used cooking oil (WCO) using mixed immobilized lipases on polyhydroxybutyrate (PHB), showing an effective biocatalytic system [6]. Utilizing PHB as an enzyme immobilization matrix presents several advantages. As a naturally derived polymer, PHB is biodegradable and biocompatible, which is essential for environmentally friendly applications. Furthermore, PHB immobilization can protect enzymes from harsh conditions, thereby enhancing their stability and extending their activity [6]. PHB provides a biodegradable and stable matrix for enzyme immobilization. Previous studies have demonstrated co-production strategies using renewable or waste-based feedstocks. Integrated bioprocesses for co-producing value-added products from waste biomass have been increasingly reported [7].

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https://doi.org/10.1016/j.renene.2026.125488
Received 8 April 2025; Received in revised form 22 February 2026; Accepted 22 February 2026
Available online 23 February 2026

PHB is a polyester belonging to the biodegradable thermoplastic polyhydroxyalkanoate (PHA) family. PHB is of great interest and has numerous applications in the plastic and biomedical industry. However, to our knowledge, there is currently no reported study on the co-production of biodiesel and PHB from POME, a rich and underutilized waste stream. The finding presents a unique opportunity to develop an integrated bioprocess for POME valorization that simultaneously addresses waste treatment, energy production, and bioplastic generation. This study investigates the potential of enzymes immobilized on PHB to convert POME residual oil into biodiesel, aiming to develop a sustainable and valuable method for POME utilization. Furthermore, the wastewater remaining after oil extraction (WR-POME) offers an opportunity for bioconversion. Instead of disposal, this nutrient-rich effluent can be used as a carbon source for microbial PHB production. Prior research has demonstrated the feasibility of using POME after sludge removal for PHA production [8]. PHB is a biodegradable thermoplastic with applications in packaging and biomedical materials [9]. This research introduces a novel two-step bioconversion process that integrates biodiesel synthesis with PHB biosynthesis. This approach maximizes resource recovery from POME while minimizing environmental impact. The strategy proposes a closed-loop, environmentally conscious POME management approach, where residual oil is converted into biodiesel, and WR-POME is utilized for PHB production. Combining enzymatic catalysis and microbial bioprocessing enhances biodiesel production efficiency and promotes a bio-circular-green (BCG) economy by creating valuable bioplastics. This integrated bioconversion process intends to advance sustainable waste utilization and connect waste treatment with bioresource innovation. This study presents the initial report on the simultaneous production of biodiesel and PHB using POME as the substrate and mixed enzymes immobilized on PHB matrices.

2. Materials and methods

2.1. Materials and chemicals

Samples of POME were collected from the Univanich Palm Oil Public Company Limited (Krabi, Thailand). The POME samples were aseptically collected in sterile 5-L containers, tightly sealed, and transported to the laboratory at approximately 4 °C using an insulated ice box to preserve their original physicochemical and microbial properties. Upon arrival at the laboratory, the samples were immediately analyzed for key physicochemical parameters, including pH, biological oxygen demand (BOD), chemical oxygen demand (COD), oil and grease (OG) and total suspended solids (TSS). All analyses were performed according to the Standard Methods for the Examining of Water and Wastewater [10]. The initial characteristics of the raw POME were as follows: pH 4.3 ± 0.2, BOD 25,122 ± 18 mg/L, COD 52,031 ± 25 mg/L, OG 6,108 ± 57 mg/L, and TSS 24,781 ± 30 mg/L. These values are consistent with those reported in previous studies and reflect the high concentrations of organic matter and suspended solids typically found in POME [11].

Lipase powders from *Candida rugosa* and *Rhizomucor miehei*, as well as commercial PHB (particle size 300 μm), were purchased from Sigma-Aldrich (USA). Immobilization of each lipase type was performed separately on PHB, following the method described by Binhayeeding et al. [6]. Briefly, PHB (0.5 g) was pretreated with anhydrous ethanol and activated using 0.6 M glutaraldehyde. The activated PHB (500 mg) was then incubated with 2 mg of lipase protein per gram of PHB at 4 °C for 30 min. After immobilization, the beads were washed with phosphate buffer, filtered, and dried. Finally, the immobilized *C. rugosa* and *R. miehei* lipases were mixed at a 1:1 (w/w) ratio to form the "**mixed immobilized lipase**", which was used as the biocatalyst for biodiesel production. All other reagents and solvents used in this study were of analytical grade and obtained from Merck (Germany).

2.2. Two-step process for biodiesel and polyhydroxybutyrate

2.2.1. Separation of oil from palm oil mill effluent

Oil was extracted from POME following the method outlined by Saowakon et al. [12]. Briefly, raw POME was centrifuged at 8,000 rpm for 15 min, and the sediment was collected and subjected to an oil extraction process using a Soxhlet apparatus. The extraction was performed using a mixed organic solvent system comprising hexane: methanol: acetone in a ratio of 6:2:2 (v/v/v). The sediment was mixed with the solvent at a ratio of 1:6 (w/w) and extracted at 100 °C for 1 h. The solvent was subsequently removed by evaporation to recover the oil. The extracted oil (EO) was then used as a substrate for biodiesel production. Using standard analytical methods, the EO was analyzed for its physicochemical properties, including moisture content (MC), saponification value, and free fatty acid (FFA) content. The supernatant obtained after centrifugation, WR-POME, was used as a carbon source for bioplastic production.

2.2.2. Biodiesel production from extracted oil using mixed immobilized lipases on polyhydroxybutyrate

Biodiesel production was carried out in a 250-mL screw-capped flask. The effects of mixed immobilized lipases on PHB for biodiesel production were evaluated under the following conditions: 5% water content, a methanol-to-oil molar ratio of 6:1, enzyme loading of 1% wt, at 45 °C, 250 rpm, for 48 h [6]. In this study, the PHB used as the support for enzyme immobilization was commercially obtained and is distinct from the PHB produced from WR-POME in the subsequent bioconversion process.

Biodiesel production was subjected to a purification step. After 48 h of reaction time, biodiesel was centrifuged and left in a separating funnel for 1-2 h to allow complete separation of the biodiesel into the upper layer. This step helped remove the aqueous and glycerol-rich bottom layer. The washing was repeated until the wash water became clear, confirming the effective removal of glycerol, methanol, and other water-soluble impurities. This procedure, combined with centrifugation and phase separation, ensured the low acid value of the final biodiesel. The biodiesel was then oven-dried at 105 °C for 24 h to eliminate residual moisture. Biodiesel production, specifically through enzymatic transesterification, requires less purification compared to alkali-catalyzed processes. The enzyme reaction with EO results in the utilization of lower-quality feedstocks with higher free fatty acid (FFA) content, which are unsuitable for conventional alkali-catalyzed methods. Therefore, the enzymatic method yields a cleaner biodiesel product with no soap formation and lower acid value. The methyl ester content and biodiesel yield were then determined, and the biodiesel was characterized by ASTM standards. The enzyme was filtered using Whatman No. 1 paper filter to recover the immobilized lipase.

The ability of the immobilized enzyme to catalyze biodiesel was examined under optimal conditions. Each batch consisted of a 5% water content, a methanol-to-oil molar ratio of 6:1, and an enzyme loading of 1% (w/w) at 45 °C and 250 rpm for 48 h. At the end of the reaction, the immobilized enzyme was separated by filtration using Whatman paper (pore size 0.2 μm) and washed three times with 10 mL 0.2 M sodium phosphate buffer (pH 7). The enzyme was then desiccated for 12 h at room temperature. The dried, immobilized lipase was used in a new reaction with a new substrate. Each reaction was initiated by the addition of an immobilized lipase wash in new medium composed of EO, as described above. The repeated reactions were run several times to measure the biodiesel yield. The biodiesel yield was determined after the reaction was complete. Specifically, the immobilized enzyme was considered reusable until the biodiesel yield dropped below 80%. This procedure was repeated for 6 cycles. Each cycle was carried out in triplicate to ensure reproducibility, and the average yield was used for evaluation. The kinetic of immobilized enzyme was already reported by Binhayeeding et al. [6].

2.2.3. Polyhydroxybutyrate production from wastewater remaining after oil extraction using Caldibacillus thermoamylovorans PHA005

C. thermoamylovorans strain PHA005 was used in this current study as a PHB-accumulating bacterium. This strain was isolated from POME, characterized, and identified by 16S rRNA [13]. The strain was collected in laboratory culture collection (Thaksin University, Thailand). The inoculum was prepared using 2 loops of cells in nutrient agar (NA). Cells were transferred into 50 mL nutrient broth (NB) and incubated at 45 °C, 150 rpm. The culture with optical density of 4–4.5 at 600 nm was used as inoculum. The 50-mL NB was inoculated at 3% (v/v) with this culture. The NB medium consisted of 3 g/L of yeast extract and 5 g/L of peptone. NA, the medium was supplemented with 15 g/L of agar to solidify the medium. The pH of both media was adjusted to approximately 7.0 before autoclaving at 121 °C for 15 min. PHB production was carried out via batch fermentation in 500-mL conical flasks, each containing 250 mL of NB and 25 mL of inoculum, achieving an inoculation ratio of 10% (v/v). The fermentation was conducted at 45 °C [13].

PHA production was conducted in 250-mL Erlenmeyer flasks, with WR-POME supplemented with 20 g/L molasses as the sole carbon source. The fermentation was carried out at 45 °C, pH 7.0, and an agitation speed of 150 rpm for 120 h [13]. Samples were collected at 24 h intervals to monitor cell growth and PHA content throughout the cultivation period. This approach, using 20 g/L molasses, was adopted to ensure sufficient carbon availability for efficient PHA production, as molasses serves as an affordable and effective carbon source for microbial growth and polymer synthesis, based on previous studies indicating its effectiveness in enhancing microbial growth and polymer accumulation [14].

2.3. Analytical methods

2.3.1. The determination and characterization of biodiesel

The methyl ester content was determined using Gas chromatography (GC) 6890 (Hewlett Packard) with a PEG-20 M (0.32 mm × 30 m × 0.25 μm) capillary column and a flame ionizing detector (FID, GC-FID). Then, methyl ester content was calculated as described in Binhayeeding et al. [6]. The fuel properties were determined using ASTM and international standards (EN 14214, ASTM D6571), as previously outlined in Binhayeeding et al. [6].

Fourier Transform Infrared (FTIR) spectroscopy (Shimadzu, Japan) was employed to identify the functional groups in the produced biodiesel. The spectra were collected in the range of 4000-400 cm^{-1} [15]. The absorption peaks corresponding to the characteristic functional groups of biodiesels, such as C=O, C–O, and C–H stretching vibrations, were analyzed. The observed peaks were compared with those of standard biodiesel samples, and the spectra were interpreted to confirm biodiesel production.

2.3.2. The determination and characterisation of polyhydroxybutyrate

Total cell concentration was determined by measuring the dry cell mass. Ten mL of culture samples were centrifuged at 13,000 rpm (12,846×*g*) for 15 min at 4 °C. The pellet was resuspended in 10 mL of distilled water and centrifuged again to wash the cells. The washed cells were then dried at 105 °C for 24 h in a hot air oven, followed by cooling in desiccators. This drying process was repeated until a constant weight was achieved [16]. The true cell concentration was obtained by subtracting the PHB concentration from the total cell concentration [17].

One gram of dried biomass was subjected to Soxhlet extraction with chloroform. The chloroform extract containing dissolved polymer was precipitated using methanol as a non-solvent, with slight modifications from previously reported methods [18]. The recovered polymer was dried at 105 °C for 24 h in a hot-air oven, cooled in a desiccator to constant weight, and weighed. The PHB content (%) was calculated based on the dry weight of the extracted polymer using Equation (1).

$$\text{PHB}\ (\%) = [\text{PHB from extraction}\ /\ \text{CDM}]\text{x}\ 100 \quad (1)$$

Where: PHB from extraction is the weight of the PHB polymer obtained after extraction and precipitation. Cell dry mass (CDM) refers to the dry cell weight prior to the extraction process.

FTIR spectroscopy was used to determine the functional groups of PHB. Five mg of isolated PHA was analyzed with a 4000 to 400 cm^{-1} spectral range. The spectra were recorded and identified compared with standard PHB (Sigma-Aldrich, USA). The PHB monomer unit was identified by GC-FID analysis. PHB samples (1-2 mg) were subjected to methanolysis using a chloroform-methanol mixture containing 15% (v/v) sulfuric acid. The resulting methyl esters of hydroxyalkanoic acids were analyzed using a Hewlett Packard GC-6890 system equipped with an HP-INNOWAX capillary column and a flame ionization detector. Compound identification was based on retention times compared to authentic standards [13].

2.4. Statistical analysis

The data were analyzed using one-way ANOVA to assess the differences between treatments. A significance level of $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS software (SPSS Inc.).

3. Results and discussion

3.1. Analysis of extracted oil

Table 1 summarized the characteristics of EO from POME. The oil quality was evaluated based on its moisture content, saponification value, and FFA content. EO exhibited an FFA content of 25.0%, which exceeds the threshold for alkaline-catalyzed transesterification.

FFA levels above 3 wt% have been reported to reduce the effectiveness of base-catalyzed transesterification due to soap formation and catalyst deactivation, leading to lower FAME yields [19]. However, high FFA content does not present an obstacle when using enzymatic catalysts [20]. Previous studies have demonstrated the potential of POME and POME-derived oil (EO) as feedstocks for enzymatic biodiesel production. For example, immobilized lipase systems have achieved ester contents above 97% using POME-based media [21]. In addition, Suwanno et al. [12] utilized crude lipase extracted from oil palm fruit to catalyze biodiesel production from POME-derived residual oil. Furthermore, Matinja et al. [22] investigated the optimization of biodiesel production from POME using lipase immobilized in polyvinyl alcohol (PVA)-alginate-sulfate beads. Moisture content is a key factor in biodiesel production, as it impacts enzyme activity and the transesterification process. High moisture levels can reduce enzyme efficiency, cause soap formation, and lower biodiesel yield. Controlling moisture is essential for optimizing production efficiency and quality [23]. Therefore, water removal from EO by heating is necessary before use to reduce the moisture content to an optimal level, which in this case is 0.22%. This result ensures that the high moisture content does not interfere with enzyme activity during biodiesel production, thereby preventing issues such as soap formation and enhancing biodiesel yield.

3.2. Biodiesel production from extracted oil using mixed immobilized lipase on polyhydroxybutyrate

The feasibility of biodiesel production from EO was evaluated. This

Table 1
Characteristics of extracted oil from palm oil mill effluent using solvent extraction.

Characteristics	Unit	Content
Moisture content (MC)	%MC	3.0
Saponification	mg KOH/g of oil	219.3
Free fatty acid (FFA)	%FFA	25.0

study applied mixed immobilized lipases on PHB under reaction conditions previously optimized for WCO by Binhayeeding et al. [6]. Although these conditions were originally established for WCO, they were adopted here to assess whether the enzymatic system could effectively catalyze the transesterification of EO. Given that the fundamental enzymatic reaction mechanism remains the same, these conditions provide a comparative basis for evaluating catalytic efficiency across different feedstocks. The reaction conditions included a temperature of 45 °C, a 5% water content, 1% mixed lipase, and a methanol-to-oil ratio of 6:1, with reaction times of 6, 12, 24, 36, and 48 h.

The results showed that mixed immobilized lipases yielded the highest biodiesel production at 97.7 ± 0.6% after 24 h of reaction time (Fig. 1). This outcome differs slightly from the biodiesel production from WCO using mixed immobilized lipases on PHB [6]. This difference can be attributed to the inherent chemical composition of the oils. EO has higher FFA content, saponification value, and MC compared to WCO, which has a saponification value of 192 mg KOH/g and MC of 0.1%. These characteristics enhance the enzymatic transesterification process, yielding higher biodiesel. Furthermore, EO may have a higher purity than WCO, contributing to enhanced reaction efficiency. Although EO exhibited higher FFA and moisture content than WCO, its composition is more defined and less affected by thermal degradation or food residues typically found in used cooking oil. Additionally, the saponification value of EO was higher than that of WCO, suggesting the presence of shorter-chain esters, which are associated with lower molecular weights and may influence fuel properties [24]. These characteristics, particularly the absence of polymerized fats and contaminants, may contribute to the slightly higher biodiesel yield observed with EO under identical reaction conditions. Although the reaction conditions were optimized for WCO, the differences in feedstock characteristics of EO explain the slightly higher yield observed with EO. Interestingly, the biodiesel yield from EO in this study (97.7%) was higher than that previously reported from POME using crude lipase derived from oil palm fruit (92.07%) [12]. This improvement may be attributed to the use of mixed immobilized lipases, which provide broader substrate specificity and enhanced catalytic stability.

Additionally, biodiesel production from POME using chemical catalysts such as KOH and sulfuric acid (H_2SO_4) has been studied, with varying reported yields. Hayyan et al. [25] demonstrated that using PTSA as a catalyst followed by transesterification yielded biodiesel with a 76.62% yield. In a similar study, Hayyan et al. [26] reported an 83.72% biodiesel yield using sulfuric acid for esterification, followed by transesterification with KOH. Furthermore, Davies et al. [4] achieved an 89% biodiesel yield using a single-step process that involved simultaneous extraction and esterification under microwave irradiation. The lower efficiency of these chemical catalysts can be hindered by factors such as the presence of free fatty acids and water in the feedstock, which can lead to soap formation and lower biodiesel yields. Moreover, chemical catalysts may produce undesired by-products, requiring additional purification steps that can reduce overall yields. In contrast, enzymatic catalysis using lipases provides a more selective, efficient, and environmentally friendly alternative, often yielding higher biodiesel yields with fewer impurities and simpler processing requirements. These findings highlight the potential advantage of the enzymatic approach using mixed immobilized lipases on PHB. Notably, this is the first report on biodiesel production from EO utilizing this enzymatic system. While much research has been conducted on enzymatic transesterification of WCO, studies on biodiesel production from EO using mixed immobilized lipases on PHB remain limited. Therefore, this research offers valuable insights into the potential application of this enzymatic system for use as an EO-based sustainable biodiesel feedstock.

This study assessed the reusability of mixed immobilized lipase for biodiesel production. Our results showed a decline in biodiesel production efficiency from 97.7 ± 0.6% to 83.8 ± 0.4% after more than three cycles (Fig. 2). This decrease may be attributed to lipase leakage during recovery and washing [27], or potential conformational changes induced by methanol. Moreover, methanol might inhibit lipase activity, deactivating enzymes after multiple cycles [6]. In a previous study, we demonstrated that immobilized *C. rugosa* and *R. miehei* lipases retained over 80% of their initial activity after 30 days of storage at 4 °C, indicating good long-term enzyme stability. These findings support the reusability of the immobilized lipases used in the present study [6]. For laboratory-scale experiments, the mixed immobilized lipase was evaluated for six cycles to demonstrate operational feasibility and stability rather than to define the economic limit of reuse. Previous studies have reported that immobilized lipases can be reused for multiple cycles, typically in the range of 5–10 cycles depending on the immobilization matrix and reaction conditions [28,29]. In contrast, the present study showed a noticeable decline in biodiesel yield after three cycles, indicating comparatively lower operational stability. This difference may be attributed to enzyme leakage, methanol inhibition, or the properties of the immobilization support used in this study. Although immobilization introduces additional steps and costs, it allows the enzyme to be reused, thereby reducing overall production costs and making the process more economically feasible [30]. The use of immobilized lipase allows enzyme reuse, reducing overall production costs compared with soluble enzymes. Moreover, PHB, as a biodegradable support material that can be produced from renewable resources or waste streams, not only enhances enzyme stability and simplifies recovery but also minimizes

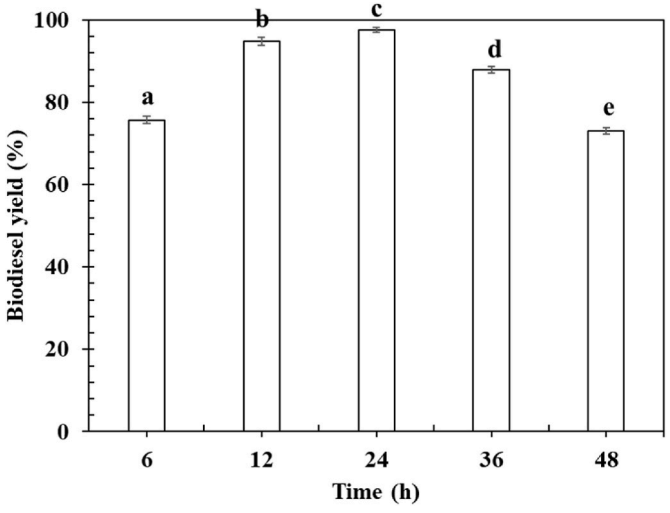

Fig. 1. Biodiesel production from extracted oil using mixed immobilized lipase on polyhydroxybutyrate. Different letters indicate significance ($p < 0.05$). Bars represent the standard deviation from triplicate determinations.

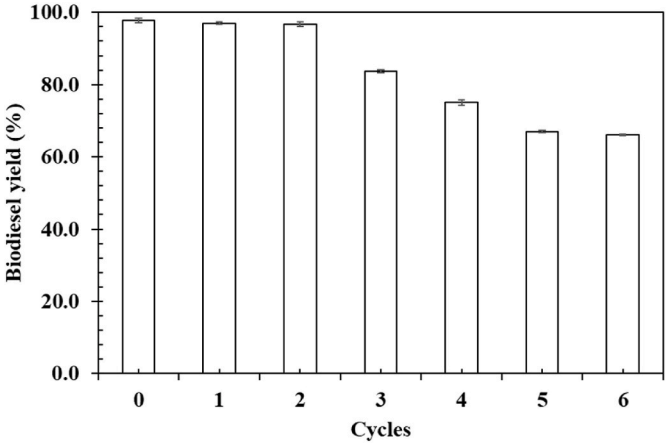

Fig. 2. Reusability of immobilized lipase on biodiesel production.

environmental impact and residual accumulation, improving both the cost-effectiveness and sustainability of the process [31].

3.3. The characterization of biodiesel

FTIR analysis confirmed the chemical structure and functional groups in the biodiesel produced from EO using mixed immobilized lipase on PHB. The FTIR spectra revealed key peaks that matched the characteristic functional groups of biodiesels, similar to those of commercial biodiesel. The spectra of commercial biodiesel and the biodiesel produced in this study (Fig. 3) showed similar features, confirming the biodiesel's chemical resemblance to commercial biodiesel, as reported in previous studies [32].

A notable peak at 1745.71 cm^{-1}, corresponding to the aliphatic carbonyl (C=O) stretching vibration, indicates esterification and confirms the conversion of free fatty acids in RO oil to fatty acid methyl esters (FAMEs) [33]. Similar characteristic ester carbonyl bands around 1744–1745 cm^{-1} have been widely reported in FTIR analyses of biodiesel, including enzymatically synthesized systems [34]. These results confirm that the biodiesel produced using mixed immobilized lipase on PHB shares the expected chemical structure with commercial biodiesel, demonstrating the process's efficiency and the viability of RO as a feedstock for biodiesel production.

The properties of biodiesel produced from EO were evaluated and compared with Thailand's ASTM and EN14214 standards to assess its suitability as a fuel (Table 2). The biodiesel exhibited a methyl ester content of 96.5%, meeting the required minimum and confirming the efficiency of the transesterification process.

The biodiesel produced from EO exhibited excellent fuel quality, meeting the key specifications required by both EN 14214 and ASTM standards. The methyl ester content confirmed efficient transesterification, while other important parameters such as acid value, water content, viscosity, and flash point demonstrated good oxidative stability, fuel purity, and combustion performance. Detailed values are provided in Table 2. This result suggests that the biodiesel is well-purified and free from residual methanol. Overall, the biodiesel from EO met most fuel quality standards, demonstrating its potential as a viable alternative fuel. Its high flash point enhances both storage safety and stability.

3.4. Application of wastewater remaining after oil extraction for polyhydroxybutyrate production

PHB is a biodegradable polyester synthesized by microorganisms, with a growing focus on utilizing alternative, renewable, and inexpensive feedstocks for PHB production. *C. thermoamylovorans* PHA005 was isolated for PHA production, and its biochemical characterization revealed its ability to utilize various substrates. The optimal conditions for PHA production by *C. thermoamylovorans* PHA005 were identified as 45 °C, pH 7.0, and agitation at 150 rpm, based on previous studies and preliminary tests [13]. *C. thermoamylovorans* PHA005, a heat-tolerant strain, offers several advantages. Its ability to thrive at high temperatures, such as 45 °C, reduces the risk of contamination from other microorganisms [35], making the fermentation process more efficient and stable. Additionally, higher temperatures can increase the rate of PHA production, potentially improving yields and reducing the overall fermentation time. This strain's resilience to heat also makes it well-suited for large-scale industrial applications where temperature control can be challenging [36], providing a more cost-effective and sustainable solution for PHB production.

This study investigated the production of PHB using wastewater from WR-POME as a substitute for commercial media. Under the optimal conditions, PHB production using WR-POME without additional carbon resulted in a CDM of 2.7 ± 0.06 g/L and a PHB content of 33.0 ± 0.3 % after 120 h (Fig. 4a). These results suggest that *C. thermoamylovorans* PHA005 can utilize residual sugars and fatty acids in WR-POME for growth and PHB biosynthesis [8], though carbon availability limits production. When 20 g/L of molasses was added, CDM (3.2 g/L) and PHB content (59.9 ± 0.5 %) significantly increased after 72 h (Fig. 4b), demonstrating that molasses enhances growth and PHB synthesis.

This finding is consistent with those of Zhao et al. [37], who reported that sugarcane molasses significantly enhanced PHB production by *Paracoccus* sp. P2. Additionally, previous studies support molasses as an effective and affordable carbon source for microbial growth and biopolymer production [38].

Similar trends have been observed in other microorganisms. For instance, *Priestia* sp. YH4 produced 61.7% PHB under optimized conditions with sugarcane molasses [39], and *Enterobacter cloacae* yielded up to 56% PHA with 4% molasses and 60 h of incubation [40]. Likewise, *Cupriavidus necator* produced 35.7% PHB using commercial sugarcane

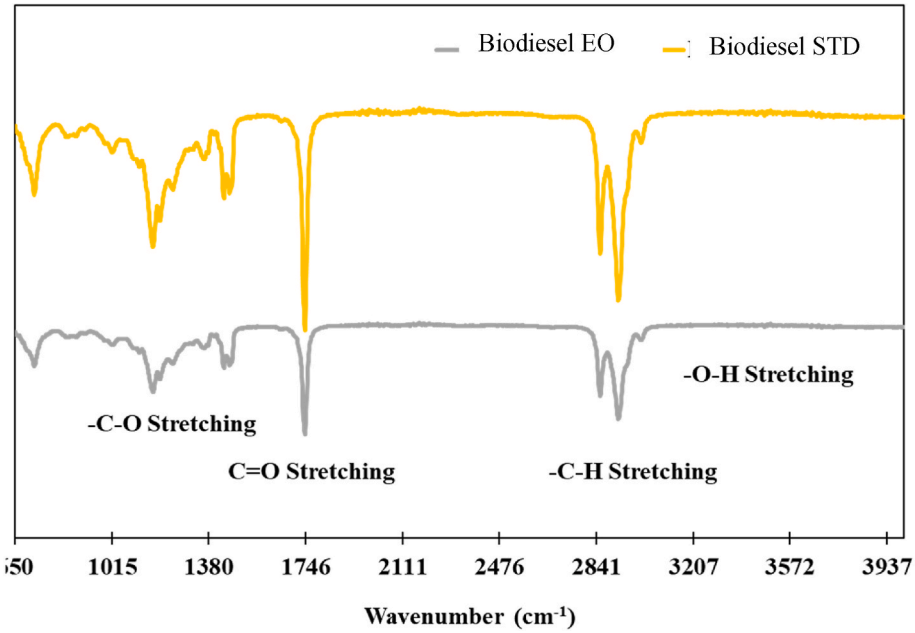

Fig. 3. FTIR spectra of the commercial biodiesel (Standard, B100) and biodiesel from extracted oil from palm oil mill effluent.

Table 2
Fuel properties of biodiesel from extracted oil of palm oil mill effluent in comparison with EN 14214 and ASTM D6751 standards.

Properties	Method	Biodiesel from EO	Biodiesel specification		
			Thai	ASTMD 6751	EN 14214
Methyl ester content (%wt)	EN14103	96.5 ± 0.2	Min 96.5	Min 96.5	Np
Acid value (mg KOH/g oil)	ASTM D664	0.2 ± 0.0	Max 0.5	Max 0.5	Max 0.5
Water content (mg/kg)	EN ISO12937	<10[a]	Max 500	Np	Max 500
Saponification (mg KOH/g)	AOAC920.160	183 ± 4.2	Np	Np	Np
Density at 15 °C (kg/m^3)	EN14111	857 ± 3.8	860–900	860–900	870–900
Viscosity at 40 °C (cSt)	ASTM D445	4.1 + 0.2	3.5-5.0	1.9-6.0	3.5-5.0
Flash point (°C)	ASTM D93	160 ± 2.1	Max 120	Min 120	Min 160

[a] <10 mg/kg indicates that the measured value was below the detection limit of the instrument (EN ISO 12937). Min: Minimum; Max: Maximum; Np: No Report.

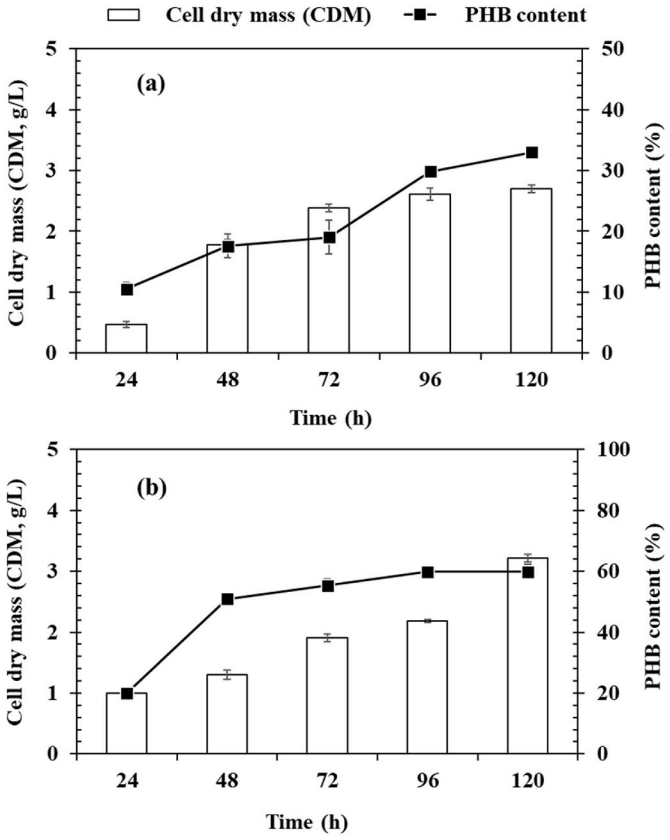

Fig. 4. The production of polyhydroxybutyrate by *Caldibacillus thermoamylovorans* PHA005 was studied under incubation at 45 °C, pH 7.0, and agitation at 150 rpm: (a) shows results using wastewater remaining after oil extraction without the addition of extra carbon, while (b) presents results with 20 g/L molasses as a supplementary carbon source.

molasses under controlled conditions [41]. These findings collectively underline the role of molasses in enhancing PHA or PHB biosynthesis by improving carbon availability. The results of this study further highlight the potential of *C. thermoamylovorans* PHA005 as a viable candidate for sustainable PHB production, particularly when supplemented with low-cost carbon sources, such as molasses. Nevertheless, the ability of this strain to synthesize PHB without additional carbon supplementation also emphasizes its promise for cost-effective biopolymer production from waste-derived substrates. Overall, WR-POME represents a renewable and economical feedstock that supports microbial growth and PHB synthesis even without added carbon, reinforcing its value for bioplastic production.

GC analysis revealed that the monomer composition of PHA varied depending on the carbon source (Table 3). When WR-POME was used as the sole carbon source, the resulting PHA consisted of both 3-hydroxybutyrate (3HB, 78 mol%) and 3-hydroxyvalerate (3HV, 22 mol%), indicating the formation of a copolymer (hydroxybutyrate-co-hydroxyvalerate, PHBV). The detection of 3HV suggests that volatile fatty acids such as propionic and valeric acids in the POME were incorporated into the polymer chain. The results are consistent with previous studies. Setiadi et al. [42] reported that *Ralstonia eutropha* could synthesize PHA containing 3HB and 3HV monomers when cultivated with volatile fatty acids derived from POME. Meanwhile, Junpadit et al. [43] demonstrated the production of a terpolymer PHA using *Rummeliibacillus pycnus* strain TS8 with POME as a carbon source, highlighting the influence of complex waste substrates on monomer composition. Conversely, when WR-POME was supplemented with molasses, the PHA consisted almost exclusively of 3HB, confirming the formation of PHB homopolymer [39]. These findings indicate that the carbon source composition strongly influences PHA monomer structure, with complex substrates, such as WR-POME, promoting copolymer formation, while sugar-rich supplementation enhances PHB purity.

Table 3
The polyhydroxyalkanoate monomer composition and retention time from wastewater remaining after oil extraction with and without molasses supplementation.

Monomer (mol %)	Carbon number	Retention time (min)	WR-POME	
			Without molasse	With molasse
3-hydroxybutyrate (3-HB)	C_4	5.6	78	100
3-hydroxyvalerate (3-HV)	C_5	7.5	22	0

FTIR characterized the polymeric functional groups in PHB obtained from WR-POME by PHA005 using molasses as a supplementary carbon source. The principal infrared absorption bands (peaks) were observed in the 4000–650 cm^{-1} (Fig. 5).

The extracted polymers from WR-POME showed characteristic peaks at 1730 cm^{-1} and 1273 cm^{-1}, corresponding to specific functional groups of PHB [44]. The absorption band identified the hydroxyl group of the PHB polymer at 2978.19 cm^{-1}. Additionally, peaks corresponding to methyl (CH_3) and methylene (CH_2) groups were detected in the 3000-2600 cm^{-1} region. The asymmetrical methyl (CH_3) group showed an absorption peak at 2932.91 cm^{-1}, while characteristic carbonyl (C=O) stretching was observed at 1723 cm^{-1}, confirming the identity of the extracted polymer as PHB (Table 4). Similar FTIR absorption patterns have been widely reported for PHB in previous studies [38].

3.5. Polyhydroxybutyrate production from palm oil mill effluent in comparison with other studies

Table 5 compares various studies on PHB production using POME and related waste materials as carbon sources, highlighting the use of different microbial strains, which resulted in varying PHA contents and polymer types. For instance, *Pseudomonas putida* Bet001 produced

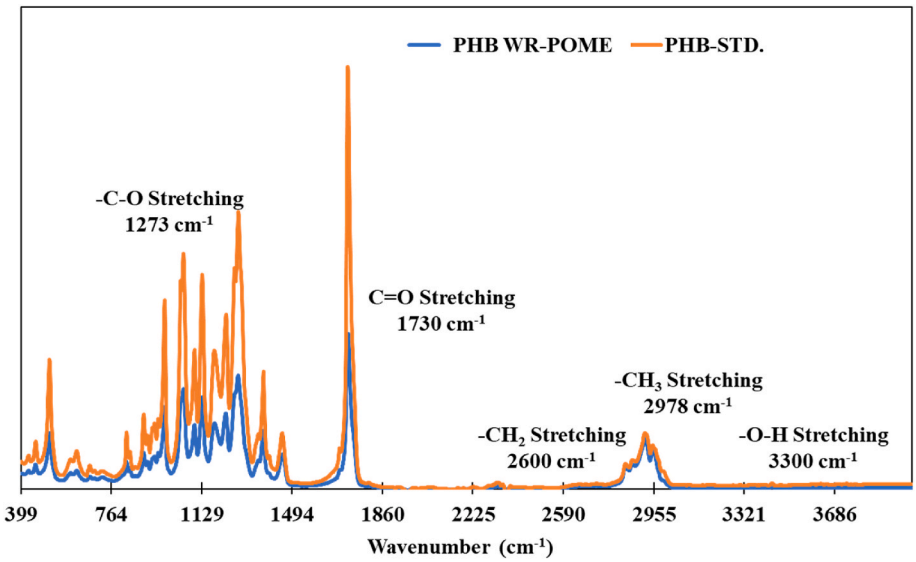

Fig. 5. FTIR spectrum of purified polyhydroxybutyrate (PHB) of wastewater remaining after oil extraction with molasses from *Caldibacillus thermoamylovorans* PHA005 compared to PHB standard (PHB-Std.).

Table 4
Functional group assignments of FTIR peaks from polyhydroxybutyrate extracted from wastewater remaining after oil extraction, in comparison with standard ranges.

Standard range (cm^{-1})	Functional group	Observed peak in PHB (cm^{-1})
1720–1740	C=O stretching (ester carbonyl group)	1730
1227–1279	C-O-C stretching (ester group)	1273
2975–3000	CH_3 stretching (methyl group)	2978
2930–2960	CH_3 asymmetric stretching	2932
2850–2950	CH_2 and CH_3 stretching (methylene and methyl groups)	2600–3000
3200–3550	O-H stretching (hydroxyl group)	3300

Table 5
Comparison of polyhydroxyalkanoate production from palm oil mill effluent and related waste materials.

Microorganisms	Substrates	PHA type	PHA content (%)	Ref.
Bacillus sp.	POME	Mcl	55	[45]
Pseudomonas putida S12	Crude Sludge Palm Oil	Mcl	41	[46]
Ralstonia eutropha JMP 134	POME	Scl	51	[42]
Pseudomonas putida Bet001	POME	Mcl	68.9	[47]
Rummeliibacillus pycnus TS8	POME	Scl	59.9	[43]
Mixed microbial culture	POME	Scl	40	[48]
Chroococcus sp.	POME	Scl	17	[49]
C. thermoamylovorans PHA005	POME	Scl	59.9	This study

68.9% medium-chain-length PHA (mcl-PHA), while *R. eutropha* JMP 134 yielded 51% short-chain-length PHA (scl-PHAs). Moreover, although mixed microbial cultures (MMCs) typically yield lower scl-PHA contents (~40%), they represent a cost-effective and scalable strategy for bioplastic production. In contrast, this study demonstrated that *C. thermoamylovorans* PHA005 accumulated 33 to 59.9% scl-PHA from POME under optimized conditions. This result outperforms several previously reported strains and is comparable to *Rummeliibacillus pycnus* TS8, positioning *C. thermoamylovorans* as a promising candidate for biopolymer production from industrial waste.

Despite challenges in using POME for PHA production due to its complex composition, this study addresses these issues by converting POME into biodiesel. This investigation reduces the complexity and removes inhibitory compounds, making the post-biodiesel wastewater a more consistent and accessible carbon source for PHA synthesis. This innovative waste-to-resource strategy not only enhances the reliability of the substrate but also underscores the potential of POME for biopolymer production. In conclusion, this study demonstrates that POME can remain a viable and efficient feedstock for PHA biosynthesis, provided that the right microbial selection and process optimization are employed. This approach aligns with the circular economy model, contributing to waste valorization and reducing environmental impact.

4. Conclusion

This study demonstrates the potential for converting POME into biodiesel and bioplastics, offering a sustainable waste management solution and reducing the cost of bioproduct. The highest biodiesel (97.7 ± 0.6%) with concomitant PHB production (59.9 ± 0.45% CDM) was achieved. The bioconversion of POME into biodiesel and bioplastics holds significant potential for the future, particularly as environmental concerns intensify and the demand for sustainable, bio-based products increases. Utilizing waste materials such as EO and wastewater from the palm oil industry presents an attractive solution for waste management and resource recovery. The enzymatic transesterification process for biodiesel production shows promise for optimization, which could lead to higher yields and more efficient production methods. Furthermore, producing bioplastics from POME wastewater, particularly through microorganisms such as *C. thermoamylovorans* PHA005, presents a valuable opportunity to meet the growing demand for biodegradable plastic alternatives. Using low-cost carbon sources could further enhance the economic viability of bioplastic production. The use of immobilized lipases, which demonstrated the ability to be reused up to three times in this study, significantly reduces costs and enhances sustainability. This approach reduces waste and supports the circular economy by transforming byproducts into valuable, environmentally friendly products. The reuse of enzymes will further improve the economic feasibility of these processes, fostering broader adoption in industrial settings and contributing to a more sustainable future.

CRediT authorship contribution statement

Narisa Binhayeeding: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Aophat Choonut:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Atipan Saimmai:** Methodology, Investigation, Formal analysis. **Kanokphorn Sangkharak:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Financial Support

The authors would like to thank Thailand Science Research and Innovation for Research and Researcher for Industries Grant (RRi) (project number PHD 61I0033) for financial support.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kanokphorn Sangkharak reports financial support was provided by Thailand Science Research and Innovation (TSRI) for Research and Researcher for Industries Grant (RRi) (project number PHD 61I0033). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to thank Thailand Science Research and Innovation for Research and the Researcher for Industries Grant (RRi) (project number PHD 61I0033) for financial support, and the Faculty of Science and Digital Innovation (Thaksin University), the Faculty of Science (Princess of Naradhiwas University), and the Faculty of Agricultural Technology (Phuket Rajabhat University) for laboratory equipment. We also express our appreciate to Univanich Palm Oil Public Company Limited (Krabi, Thailand) for providing us with palm oil mill effluent.

Data availability

Data will be made available on request.

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