ORIGINAL PAPER

The Potential of Single-Cell Oils Derived from Marine Fungus (*Aspergillus pseudofelis* MMERU 25) as Alternative Feedstock Sources for Biodiesel Production

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Received: 20 October 2023/ Revised: 13 December 2023/ Accepted: 15 December 2023

Abstract.

The potential of single-cell oils derived from marine fungi can serve as a feedstock for biodiesel production. However, different marine fungal species exhibit distinct capabilities for high lipid production under varying conditions, resulting in variations in biodiesel characteristics. Therefore, the objective of this study is to investigate culture media conditions, the optimal duration for lipid production, and the biodiesel characteristics of *Aspergillus pseudofelis* MMERU 25. Cultivation was conducted using 70% MEB for 20 days and molasses (2.5, 5, 10, and 10.5 ml) mixed with 70% MEB for 15 days. Biodiesel characteristics, including chemical profiles, viscosity, acidity, methyl esters, linolenic acid content, H.H.V., and iodine value were also analyzed. The results indicate that *A. pseudofelis* MMERU 25 achieves the highest lipid production within 10 days in 70% MEB and reaches the maximum lipid yield at a 2.5 ml concentration of molasses in 70% MEB within 15 days. When *A. pseudofelis* MMERU 25 is cultured in 70% MEB for 10 days, the biodiesel quantity reaches 360 ml/kg of dry biomass. The biodiesel characteristics are consistent with previous research and the standards of biodiesel qualities by the DOEB, except for acidity, methyl esters content, and H.H.V. These parameters can be controlled to eliminate residual acids in biodiesel and adjust reaction conditions to enhance its suitability for use as a biodiesel feedstock. Therefore, biodiesel produced from marine fungi has the potential to be an alternative energy source for Thailand in the future.

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Keywords: *Aspergillus pseudofelis*, Biodiesel, Feedstock, Marine fungi, Single-cell oils

1. Introduction

The demand for biodiesel, also known as fatty acid methyl ester (FAME), has experienced a significant increase, which results from petroleum prices and the global adoption of government policies, including Thailand's, that mandate a minimum proportion of biofuel for all petrol and diesel used in transport. Biodiesel, being a renewable fuel, offers compelling advantages compared to conventional diesel fuel. These advantages include enhanced biodegradability, reduced toxicity, and a lower emission profile (Strobel, 2015; Vicente et al., 2004; 2009). Nevertheless, biodiesel production presents certain inherent drawbacks. Traditional feedstocks, including vegetable oils and animal fats, have been linked to an unintended increase in food costs due to the diversion of these valuable resources into fuel production. These limitations have incentive the search for alternative feedstock sources for biodiesel production. Presently, researchers are turning their attention to single-cell oils (SCO) derived from oleaginous microorganisms, such as microalgae can store intracellular lipids in response to stress conditions (Narala et al., 2016). The algal-derived lipid profile is highly variable, consisting of C6-24 with highly unsaturated esters containing up to six double bonds, and depends largely on the cultural conditions and the species of algae. Therefore, the large-scale commercialization of this technology for the production of biofuel-compatible oil remains elusive (Sargeant et al., 2017). Bacteria demonstrate very high growth rates under simple cultivation conditions, but they do not accumulate large quantities of fatty acids

(Meng et al., 2009; Zabeti et al., 2010). Yeast offers several advantages over microalgal feedstocks for lipid production, as they are fully heterotrophic organisms, have shorter generation times, can reach much higher cell densities, are less susceptible to viral infections, and the level of bacterial contamination in the production medium can be minimized by using low pH growth conditions (Kot et al., 2016; Li et al., 2008). Lipid accumulation in oleaginous yeasts is a response to environmental stress, particularly nitrogen limitation, and excess carbon in the growth medium, similar to filamentous fungi (Lamers et al., 2016). These microorganisms offer a promising avenue for sustainable biodiesel production, as they can be cultivated in a controlled environment, independent of climatic dependencies. Fungi, in particular, exhibit significant potential, as they accumulate lipids that closely resemble triglycerides found in vegetable oils (Martins et al., 2019; Singh et al., 2020; Zhang et al., 2014).

Marine fungi are a specific category of fungi capable of surviving in high-salinity environments through the production of compounds like mannitol, arabitol, and lipids, which serve to regulate osmotic balance within and outside their cells. (Wethered et al., 1985). However, to assess the potential of the marine fungus (*A. pseudofelis* MMERU 25) as a viable feedstock for biodiesel production, it is imperative to examine its lipid content in relation to dry biomass weight. When the lipid content exceeds 20% of the dry biomass, the fungus can be classified as an oleaginous fungus (Athenaki et al., 2018). Moreover, it is crucial to investigate the optimal period for maximizing lipid production, as different marine fungi may exhibit distinct peak periods for lipid production. Furthermore, the choice of culture medium plays a pivotal role in establishing the ideal conditions for lipid production in fungi. Typically, nutrient-rich media with a substantial glucose content are preferred, as marine fungi can efficiently utilize them for both biomass and lipid production. Examples of such media include malt extract broth and agricultural byproducts like molasses (Kumar et al., 2020; Mhlongo et al., 2021). Therefore, this research is in accordance with bioeconomy development policies and presents a viable option for future research and development in this field.

2. Materials and Methods

2.1 Culture media

Five different culture media were used for this research incliding, (1) malt extract agar with 70% seawater (70% MEA) containing: malt extract powder 20 g, peptone 1g, glucose 20 g, agar 15 g, seawater 700 ml, and distilled water 300 ml (Add streptomycin sulfate at a concentration of 10 mg/L into media after autoclaving and cooling to a temperature between 45-50°C before pouring it into a plastic petri dish), (2) Czapek-dox agar with 70% seawater (70% CZA) containing: Czapek-dox agar 49.9 g, seawater 700 ml, and distilled water 300 ml, (3) malt extract broth with 70% seawater (70% MEB) containing: malt extract powder 20 g, peptone 1 g, glucose 20 g, seawater 700 ml, and distilled water 300 ml, and (4) malt extract agar (MEA) containing: malt extract powder 20 g, peptone 1 g, glucose 20 g, agar 15 g, and distilled water 1,000 ml (5) malt extract agar with 70% seawater mixed molasses (70% MEAM) containing: malt extract powder 20 g, peptone 1 g, glucose 20 g, agar 15 g, seawater 700 ml, distilled water 300 ml, and different volume of molasses (2.5, 5, 10, and 15 ml) (Buaruang et al., 2015; Reis et al., 2019; 2020).

2.2 Sample collection and isolation of marine fungus

A marine sponge (*Cliona albimarginata*) was randomly sample collected by SCUBA diving on a coral reef in Koh Aeo, Andaman Sea, Phuket Province, Thailand, in July 2020. A sample photograph was taken with a waterproof digital camera, and a label with a sample number and

location was placed on waterproof paper. The labeled sample was then placed in a polyethylene bag containing seawater. Finally, the sample was placed in an icebox for one night before isolation and was immediately transported to the laboratory. The sponge was washed with a 0.06% sodium hypochlorite solution for 1 minute, followed by rinsing with sterilized seawater three times. The sponge was dried on sterile filter paper, cut into small pieces (5 x 5 mm^2), and placed on a 70% MEA. It was then incubated at 28°C for 7 days. The hyphal tips emerging from the sponge pieces were individually transferred onto MEA slants and maintained as pure cultures at the Marine Microbe Environment Research Unit (MMERU) of the Division of Environmental Science, Faculty of Science of Ramkhamhaeng University, Bangkok, Thailand (Buaruang et al., 2015; Cardona et al., 2021).

2.2 Morphological studies

The fungus was cultivated on 70% CZA and identified based on morphological characteristics such as colony growth rate, growth pattern, and texture. Colony colors were recorded according to the mycological color chart (Rayner, 1970). Microscopic characteristics were examined under stereo and compound microscopes. Ascospore ornamentation studies were conducted using scanning electron microscopy (SEM), JEOL JSM 6400 (Manoch et al., 2009; Buaruang et al., 2015; Cardona et al., 2021).

2.3 DNA extraction and sequencing analysis

A. pseudofelis MMERU 25 was also confirmed by the analysis sequence of the internal transcribed spacer (ITS) gene. Briefly, 2–15 mg of mycelia were ground in liquid nitrogen. DNA was extracted using the DNeasy™ Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The entire nuclear ITS regions were amplified with the primers ITS1F-5′ (Gardes and Bruns, 1993) and ITS4-3′ (White et al., 1990). PCR reactions were conducted on a thermal cycler, and the amplification process consisted of initial denaturation at 95 °C for 5 min, 34 cycles at 95 °C for 1 min (denaturation), at 55 °C for 1 min (annealing), and at 72 °C for 1.5 min (extension), followed by a final extension at 72 °C for 10 min. PCR products were cleaned using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), then examined by agarose gel electrophoresis (1% agarose with 1× TBE buffer) and visualized under UV light after staining with ethidium bromide. DNA sequencing analyses were carried out by Macrogen Inc., Seoul, South Korea (Cardona et al., 2021).

2.5 The suitability of different culture media and durations for the production of biomass and lipids in A. pseudofelis MMERU 25

2.5.1 The biomass production of A. pseudofelis MMERU 25 in 70% MEB

A. pseudofelis MMERU 25 was incubated in 70% MEB in a total of 60 flasks without shaking. Subsequently, the cultures were harvested by filtration using Whatman No.1 filter paper each day, with three flasks collected daily until reaching 20 days. The dried biomass was obtained by placing it in a hot air oven at 50°C for 24 hours, followed by weighing the biomass of *A. pseudofelis* MMERU 25 (Carvalho et al., 2015; Reis et al., 2019).

2.5.2 The lipid production of A. pseudofelis MMERU 25 in 70% MEB

A. pseudofelis MMERU 25 was incubated in 200 ml of 70% MEB contained in 500 ml Erlenmeyer flasks for 10 and 15 days. The cultures were harvested by filtration using Whatman No.1 filter paper and rinsed thoroughly with distilled water to remove the media from the cells. The dried biomass was obtained by placing it in a hot air oven at 50°C for 24 hours, followed by crushing and filtering through a 0.045 mm sieve, resulting in a dried biomass weight of 500 mg. This dried biomass was used for lipid extraction, which was performed using a mixture of 2:1:0.8 (v/v) chloroform, methanol, and water with a total volume of

18 ml (Vicente et al., 2009; Zhang et al., 2014). After the extraction was carried out by an ultrasonicator for 20 minutes with three replications, the percentage of lipid content per dried biomass weight was calculated using Eq. 1, along with the standard deviation (SD) (Longmatcha et al., 2022; Reis et al., 2019).

$$\text{Lipid content (\%)} = \frac{\text{Total extracted lipids (mg/200mL)}}{\text{Dry biomass weight (mg/200mL)}} \times 100\% \quad \text{Eq.1}$$

2.5.3 The biomass production of A. pseudofelis MMERU 25 in 70% MEBM

A. pseudofelis MMERU 25 was incubated in 70% MEB mixed with molasses at concentrations of 2.5, 5, 10, and 15 ml in 500 ml Erlenmeyer flasks containing 200 ml of 70% MEBM for 15 days. Subsequently, the cultures were harvested by filtration using Whatman No.1 filter paper. The dried biomass was obtained by placing it in a hot air oven at 50°C for 24 hours, followed by weighing the biomass of *A. pseudofelis* MMERU 25 (Carvalho et al., 2015; Reis et al., 2019; 2020).

2.5.4 The lipid production of A. pseudofelis MMERU 25 in 70% MEBM

A. pseudofelis MMERU 25 was incubated in 70% MEB mixed with molasses at concentrations of 2.5, 5, 10, and 15 ml in 500 ml Erlenmeyer flasks containing 200 ml of 70% MEBM (5 flasks) for 15 days. The cultures were harvested by filtration using Whatman No.1 filter paper and rinsed thoroughly with distilled water to remove the media from the cells. The dried biomass was obtained by placing it in a hot air oven at 50°C for 24 hours, followed by crushing and filtering through a 0.045 mm sieve, resulting in a dried biomass weight of 500 mg. This dry weight was used for lipid extraction, which was performed using a mixture of 2:1:0.8 (v/v) chloroform, methanol, and water with a total volume of 18 ml. After the extraction was carried out by an ultrasonicator for 20 minutes with three replications, the percentage of lipid content per dried biomass weight was calculated using Eq. 1, along with the SD (Reis et al., 2019; 2020; Vicente et al., 2009; Zhang et al., 2014).

2.6 Transesterification Reaction

A. pseudofelis MMERU 25 was incubated in 70% MEB for 10 days. The cultures were harvested by filtration using Whatman No.1 filter paper and rinsed thoroughly with distilled water to remove the media from the cells. The dried biomass was obtained by placing it in a hot air oven at 50°C for 24 hours, followed by crushing and filtering through a 0.045 mm sieve. A transesterification reaction was performed using a mixture of methanol, sulfuric acid, and chloroform in a volume ratio of 10:1:1, which was added to 200 mg of the dried biomass. The reaction was conducted at a temperature of 65°C for 48 hours. Fatty acid methyl ester (FAME) or biodiesel was separated using hexane in a separation funnel (with FAME and hexane in the upper layer and glycerol in the lower layer), and hexane was removed using a rotary vacuum evaporator (Freedman et al., 1986; Talebian-Kiakalaieh et al., 2013).

2.7 Biodiesel Analysis

2.7.1 Chemical profile

Chemical profile analysis of a 20 mg sample of FAME, dissolved in deuterated chloroform ($CDCl_3$), was performed using the Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR), AVANCE NEO 500 MHz from Bruker, Germany.

2.7.2 Viscosity

Viscosity measurement of FAME at 40°C was carried out using a 100-sized viscometer. The measurements were

performed in triplicate, using a modified method derived from the ASTM D445 standard (In-house method based on ASTM D445).

2.7.3 Acidity

Measurement of the acidity of the biodiesel sample was conducted using a Potentiometric Autotitrator, 794 Titrino from Metrohm, Switzerland. The measurements were performed three times using a modified method based on the ASTM D664 standard (In-house method based on ASTM D664) with KOH as the titrant.

2.7.4 Chemical composition, quantity of methyl ester and linolenic acid from FAME

Analysis of the composition and quantities of FAME and the quantity of linolenic acid was performed using a Gas Chromatography-Flame Ionization Detector (GC-FID), Agilent (GC7820A) from the USA. The measurements were performed three times with methyl heptadecanoate as an internal standard, using a modified method based on the EN14103 standard (In-house method based on EN14103).

2.7.5 Higher Heating value (H.H.V)

The H.H.V of the biodiesel was measured using a Bomb Calorimeter, model C5000 from IKA, Germany. The measurement was performed three times using a modified method based on the ASTM D240 standard (In-house method based on ASTM D240).

2.7.6 Iodine value

The iodine value of the biodiesel was measured by a modified method based on the EN 14111 standard (In-house method based on EN 14111).

2.8 Statistical analysis

The experiments were conducted in triplicate, and the findings are reported as the mean value ± the standard deviation (SD). Statistical analysis was performed using Microsoft Excel 2019 software.

3. Results

3.1 Identification of A. pseudofelis MMERU 25

The morphology of *A. pseudofelis* MMERU 25 is as follows: In Figure 1A, the colony of *A. pseudofelis* MMERU 25 on 70% CZA after 7 days appears white, and it abundantly produces superficial cleistothecia, which are white to pale cream and globose to subglobose in shape. Figure 1B shows conidiophores that are typically long and columnar, with a colorless (hyaline) and smooth appearance. These conidiophores give rise to sub-spherical vesicles that are biseriate. Figure 1C, the asci are globose to subglobose in shape. Figures 1D-1E depict ascospores that are hyaline to pale yellow. These ascospores have two widely separated, flexuous equatorial crests, and their convex surfaces are irregularly roughened by fine spines and appear corrugated (Eamvijarn et al., 2013; Horie et al., 2003). Based on DNA analysis, a BLAST search of ITS gene sequences, and phylogenetic analysis, *Aspergillus* sp. is closely related to *A. pseudofelis* with a 99% similarity. In summary, *Aspergillus* sp. MMERU 25 identified as *A. pseudofelis*, exhibits specific morphological characteristics and has been genetically confirmed as a member of the genus *Aspergillus* within the family Aspergillaceae in the kingdom Eukaryota.

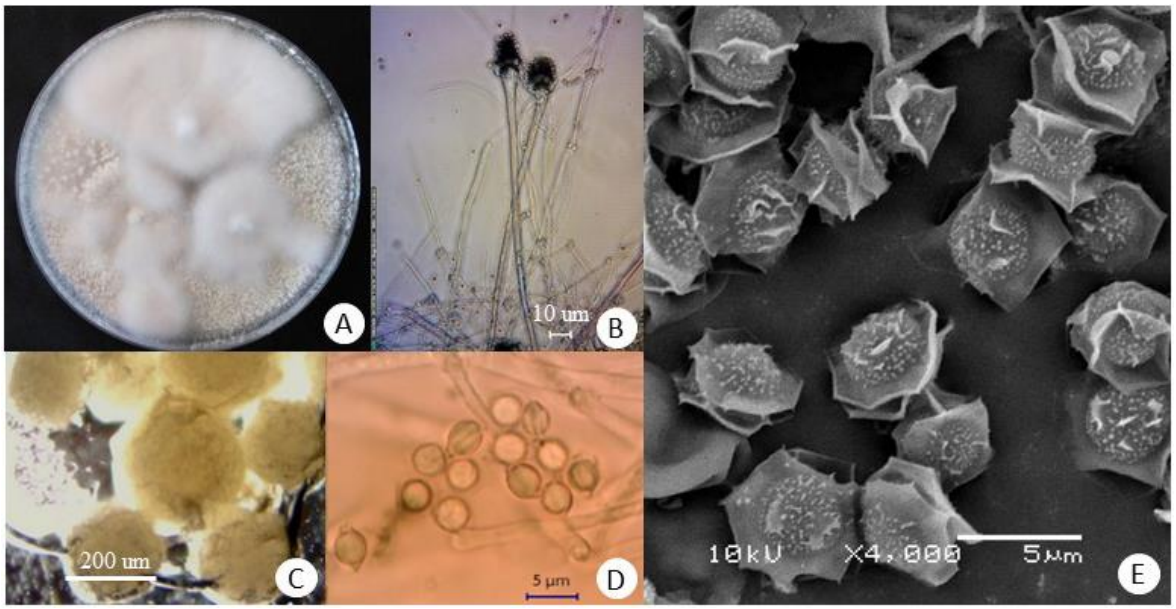

Figure 1. (A) *Aspergillus pseudofelis* MMERU 25 on 70% CZA for 7 days, (B) conidiophores and conidia, (C) cleistothecia, (D) ascospores under the light microscope, and (E) ascospores under SEM

3.2 The suitability of different culture media and durations for the production of biomass and lipids in A. pseudofelis MMERU 25

3.2.1 The biomass production of A. pseudofelis MMERU 25 in 70% MEB

The results of the biomass production of *A. pseudofelis* MMERU 25, cultivated in 70% MEB, were obtained using a total of 60 flasks. The cultivation was carried out at room temperature without shaking. Subsequently, fungal mycelium was harvested daily, with three flasks per day, for a total of 20 days. The results revealed that *A. pseudofelis* MMERU 25 exhibited a consistent growth rate, with biomass reaching 3.14 g per dried weight on day 10 and an average biomass of 3.11 g per dry weight from day 10 to day 20 (Figure 2).

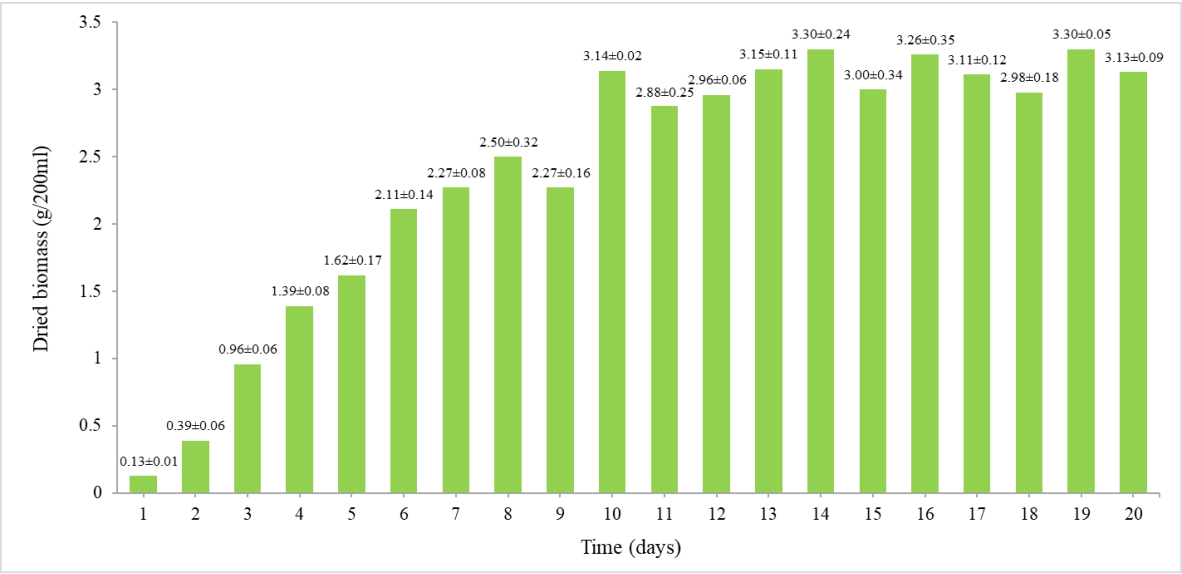

Figure 2. The growth rate of *A. pseudofelis* MMERU 25 cultivated in 70% MEB for 20 days (dried weight ± SD)

3.2.2 The lipid production of A. pseudofelis MMERU 25 in 70% MEB

Lipid extraction from 500 mg of dried *A. pseudofelis* MMERU 25 using a solvent system of methanol, chloroform, and water

(2:1:0.8). The results showed that the total lipid yields for 10 days and 15 days were 37.68% and 30.94%, respectively, relative to the dry weight of the mycelium, as presented in Table 1.

Table 1. The dried biomass weight and the percentage of the lipid content of *A. pseudofelis* MMERU 25 cultivated in 70% MEB for 10 and 15 days

No.	10 days		15 days	
	Dried biomass weight (g)	**Lipid (g)**	**Dried biomass weight (g)**	**Lipid (g)**
1.	0.5000	0.1896	0.5000	0.1583
2.	0.5000	0.1740	0.5000	0.1303
3.	0.5000	0.2017	0.5000	0.1756
Average		0.1884		0.1547
SD		0.01		0.02
Lipid yields (%)		37.68		30.94

3.2.3 The biomass and lipid production of A. pseudofelis MMERU 25 in 70% MEBM

The biomass production results of *A. pseudofelis* MMERU 25 in 70% MEBM were divided into four sets, each with five repetitions. Each experimental set involved added molasses of different concentrations, ranging from 2.5, 5, 10, and 15 ml, respectively. The cultures were incubated for 15 days at room temperature. The first experimental set (2.5 ml) exhibited an average dried biomass weight of 2.65 g and an accumulated lipid content of 45.35%. In the second experimental set (5 ml), the average dried biomass weight was 2.51 g with a lipid content of 48.44%. The third experimental set (10 ml) showed an average dried biomass weight of 2.36 g and an accumulated lipid content of 42.8%. The fourth experimental set (15 ml) had an average dried biomass weight of 3.33 g and a lipid content of 40.51% (Figure 3 and Table 2).

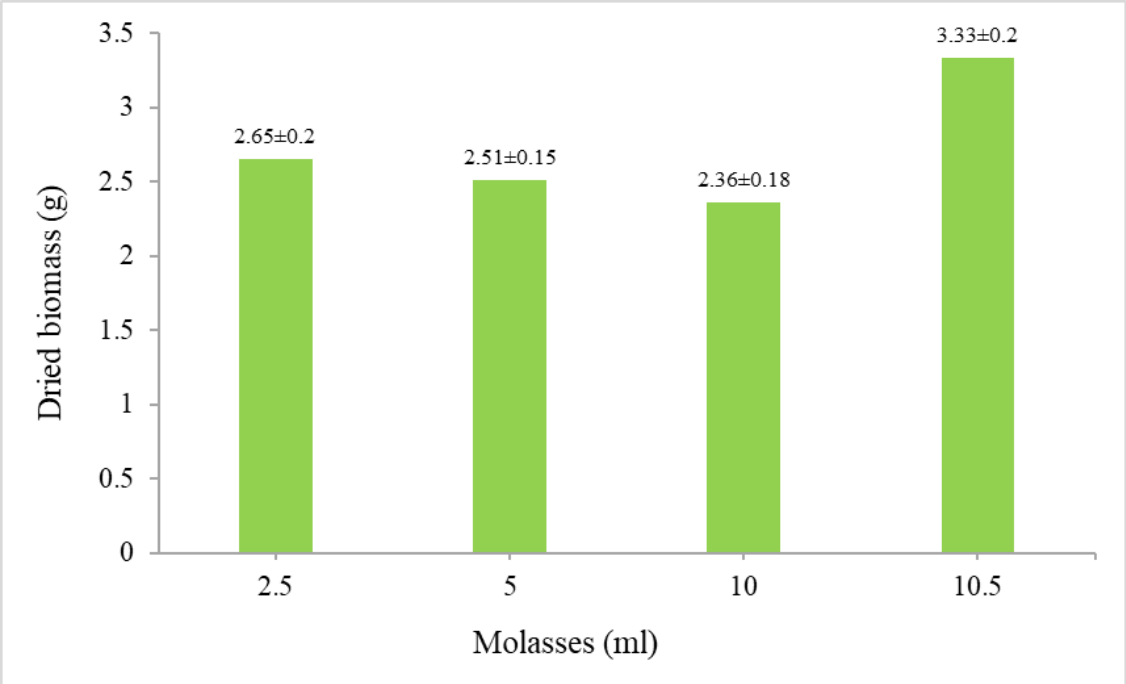

Figure 3. The growth rate of *A. pseudofelis* MMERU 25 cultivated in different concentrations of molasses for 15 days (dried weight ± SD)

Table 2. The percentage of lipid content in *A. pseudofelis* MMERU 25 under various concentrations of molasses for 15 days

Molasses (ml)	Lipid yields (%)	SD
2.5	45.35	2.08
5.0	48.44	2.22
10.0	42.80	0.96
10.5	40.51	0.09

3.3 The potential of biodiesel from A. pseudofelis MMERU 25

3.3.1 The result of biodiesel extraction

The extraction of biodiesel from dried biomass of *A. pseudofelis* MMERU 25, which was cultivated in a 70% MEB for 10 days, was performed using 200 mg of the solvent system of methanol, sulfuric acid, and chloroform (10:1:1) and was conducted at a temperature of 65°C for 48 hours. This procedure resulted in the production of 360 ml of biodiesel per 1 kg of dried biomass (Figure 4).

3.3.2 Chemical profile

This analysis revealed a chemical shift in the ^{1}H NMR spectrum, characterized by singlet (*s*) signals (1 signal), triplet (*t*) signals (2 signals), and multiplet (*m*) signals (5 signals). The FAME spectra corresponding to these findings are presented in Figure 5.

Figure 4. The biodiesel from *A. pseudofelis* MMERU 25

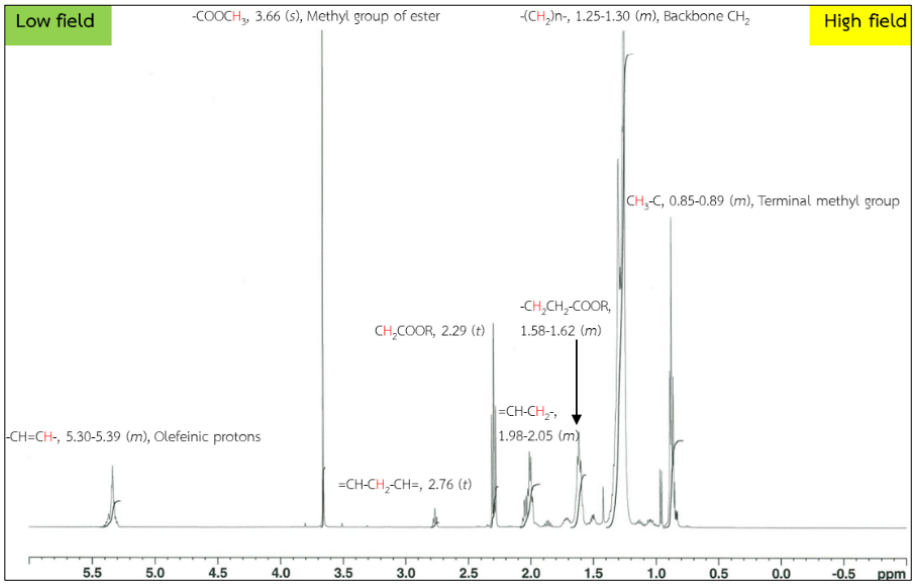

Figure 5. The FAME spectra were analyzed using a ^{1}H NMR

3.3.3 Viscosity

The viscosity of FAME was measured using a modified version of the ASTM D445 standard method. The measurement was conducted three times, and the FAME extracted from *A. pseudofelis* MMERU 25 exhibited a viscosity value of 3.544 cSt (Table 3).

Table 3. The viscosity of FAME extracted from *A. pseudofelis* MMERU 25 at a temperature of 40°C

No.	Flow time (sec)	Kinetic viscosity (cSt) = C*t
1	234.93	3.540
2	235.34	3.547
3	235.2	3.544
Average		3.544
SD		0.003
Relative standard deviation (%)		0.089

3.3.4 Acidity

The analysis of the acidity of the biodiesel sample, using a modified version of the ASTM D664 standard method, revealed that the biodiesel extracted from *A. pseudofelis* MMERU 25 had an acidity value of 1.2910 mg KOH/g of biodiesel (Table 4).

Table 4. Showed the weight of biodiesel (WB), the endpoint (EP), acidity values (AV) and the average acidity values (AAV)

No.	WB (g)	EP (ml)	AV (mg of KOH/g of biodiesel)	AAV (mg of KOH/g of biodiesel)	SD
1.	5.0066	1.266	1.2844		
2.	5.0074	1.269	1.2876	1.2910	0.0088
3.	5.0004	1.279	1.3010		

3.3.5 Chemical composition, quantity of methyl ester and linolenic acid from FAME

The analysis results showed the chemical composition and quantity of methyl ester and linolenic acid methyl ester (C18:3) in the FAME extracted from *A. pseudofelis* MMERU 25 using a modified version of the EN14103 standard method, with methyl heptadecanoate as the internal standard (C17:0, retention time (Rt) = 14.074 minutes). Based on the data from the GC-FID chromatogram (Figure 6 and Table 5), it was observed that the FAME extracted from *A. pseudofelis* MMERU 25 consists of 13 different methyl esters, categorized into seven saturated methyl esters (Methyl myristate, Methyl pentadecanoate, Methyl palmitate, Methyl stearate, Methyl arachidate, Methyl behenate, and Methyl lignocerate) and six unsaturated methyl esters (Methyl palmitoleate, Methyl oleate, Methyl linoleate, Methyl linolenate, Methyl eicosonate, and Methyl erucate). The total quantity of all methyl esters, determined using C17:0 as the internal standard, was found to be 83.202% by weight of the analyzed biodiesel sample. The quantity of linolenic acid methyl ester (C18:3) was calculated to be 0.044% by weight of the analyzed biodiesel samples. Notably, among the methyl esters, four major components were identified: (1) Methyl oleate (C18:1, Rt = 16.026 minutes) had the highest quantity, with an average of 34.66% by weight of the analyzed biodiesel samples. (2) Methyl palmitate (C16:0, Rt = 12.788 minutes) was the second most abundant, with an average quantity of 19.53% by weight of the analyzed biodiesel samples. (3) Methyl stearate (C18:0, Rt = 15.589 minutes) was the third most abundant, with an average quantity of 11.83% by weight of the analyzed biodiesel samples and (4) Methyl linoleate (C18:2, Rt = 16.833 minutes) was the fourth most abundant, with an average quantity of 10.68% by weight of the analyzed biodiesel samples.

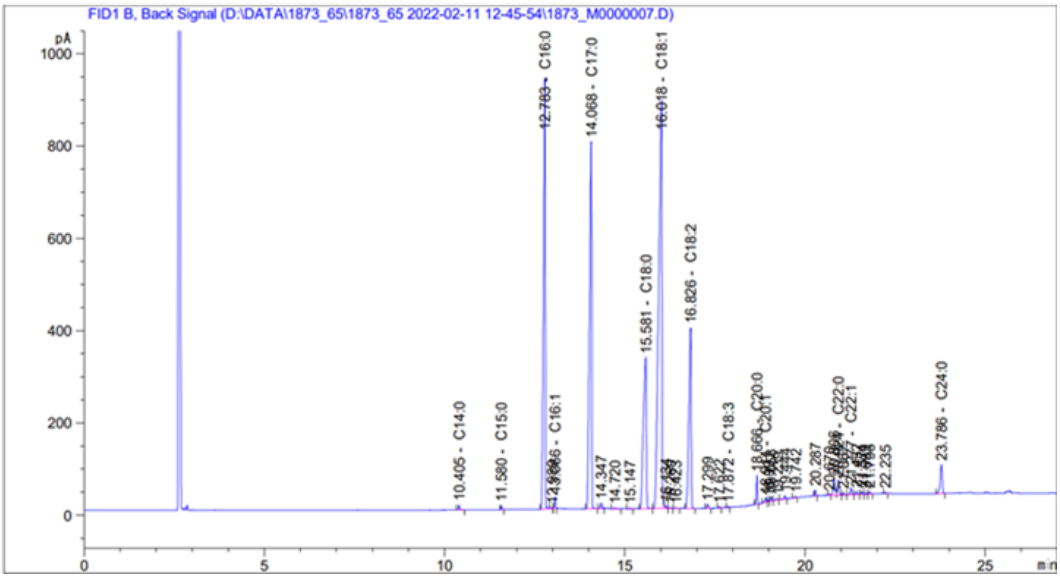

Figure 6. The chromatogram of biodiesel from *A. pseudofelis* MMERU 25 using GC-FID

Table 5. Show the retention time, name, and chemical formula of the biodiesel analyzed using the GC-FID technique

Peak	Retention time (min)	Name	Chemical formula
1.	10.411	Methyl myristate, C14:0	$CH_3(CH_2)_{12}COOCH_3$
2.	11.585	Methyl pentadecanoate, C15:0	$CH_3(CH_2)_{13}COOCH_3$
3.	12.788	Methyl palmitate, C16:0	$CH_3(CH_2)_{14}COOCH_3$
4.	13.071	Methyl palmitoleate, C16:1	$CH_3(CH_2)_5CH=CH(CH_2)_7COOCH_3$
5.	14.074	*Methyl heptadecanoate, C17:0	$CH_3(CH_2)_{15}COOCH_3$
6.	15.589	Methyl stearate, C18:0	$CH_3(CH_2)_{16}COOCH_3$
7.	16.026	Methyl oleate, C18:1	$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$
8.	16.833	Methyl linoleate, C18:2	$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOCH_3$
9.	17.878	Methyl linolenate, C18:3	$CH_3(CH_2CH=CH)_3(CH_2)_7COOCH_3$
10.	18.671	Methyl arachidate, C20:0	$CH_3(CH_2)_{18}COOCH_3$
11.	18.916	Methyl eicosonate, C20:1	$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOCH_3$
12.	20.928	Methyl behenate, C22:0	$CH_3(CH_2)_{20}COOCH_3$
13.	21.283	Methyl erucate, C22:1	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOCH_3$
14.	23.793	Methyl lignocerate, C24:0	$CH_3(CH_2)_{22}COOCH_3$

* Internal standard

3.3.6 The H.H.V

The H.H.V measurement results showed that the biodiesel extracted from the marine fungus *A. pseudofelis* MMERU 25 has an H.H.V of 7933 Kcal/Kg of biodiesel, which is equivalent to 33.1903 MJ/Kg of biodiesel (Table 6).

3.3.7 Iodine value

The result of the iodine value for the biodiesel extracted from the marine fungus *A.*

Table 6. The H.H.V of the biodiesel extracted from *A. pseudofelis* MMERU 25

No.	Sample weight (g)	H.H.V (MJ/kg)
1	0.0495	34.4594
2	0.0446	32.2628
3	0.0466	32.8486
Average	0.0469	33.1903
SD	0.0025	1.14

pseudofelis MMERU 25 is 54.100 g Iodine/100g of biodiesel (Table 7). In summary, the analysis results for the viscosity at 40°C, acidity, iodine value, H.H.V, and methyl ester and linolenic acid content of the biodiesel extracted from *A. pseudofelis* MMERU 25 are presented in Table 7.

Table 7. Summarize the analysis results for viscosity at 40°C, acidity, iodine value, H.H.V, and methyl ester and linolenic acid content

No	Parameter	Unit	Method (In-house method)	Result ± SD	Standard (DOEB)
1.	Viscosity at 40°C	cSt	ASTM D 445	3.544 ± 0.003	> 3.5
2.	Acidity	mg KOH/g	ASTM D 664	1.291 ± 0.0088	< 0.5
3.	Methyl ester content	% wt	EN 14103	83.202 ± 0.018	> 96
4.	Linolenic acid content (C18:3)	% wt	EN 14103	0.044 ± 0.003	< 12
5.	H.H.V	MJ/kg	ASTM D 240	33.1903 ± 1.14	41.230 ± 0.0200 (palm)
6.	Iodine value	g Iodine/100g	EN 14111	54.100 ± 1.5	< 120

*Department of Energy Business (DOEB)

3.4 A Comparative analysis of biomass and lipid accumulation in A. pseudofelis MMERU 25, A. tubingensis TSIP9, and Mucor circinelloides

Furthermore, a comparison of biomass and lipid accumulation percentages was conducted among *A. pseudofelis* MMERU 25, *A. tubingensis* TSIP9, and *M. circinelloides*. The results revealed that *A. pseudofelis* MMERU 25 exhibited the highest biomass at 3.14 g/200mL or 15.70 g/L, surpassing *A. tubingensis* TSIP9 with a biomass of 0.79 g/L and *M. circinelloides* with a biomass of 3.73 g/L. Moreover, *A. pseudofelis* MMERU 25 demonstrated the highest lipid accumulation, reaching 37.69% of the dry biomass weight, while *A. tubingensis* TSIP9 and *M. circinelloides* accumulated 20.50% and 19.90% of the dry biomass weight, respectively. The analysis of the main chemical components for biodiesel production from all three fungi types revealed consistent compounds, namely methyl palmitate (C16:0), methyl stearate (C18:0), methyl oleate (C18:1), and methyl linoleate (C18:2), as presented in Table 8.

Table 8. Comparative analysis of biomass and lipid accumulation in *A. pseudofelis MMERU 25*, *A. tubingensis* TSIP9, and *M. circinelloides*

No.	Parameters	Fungal		
		***A. pseudofelis* MMERU 25 (This thesis)**	***A. tubingensis* TSIP9 (Cheirsilp & Kitcha 2015)**	***M. circinelloides* (Vicente et al., 2009)**
1	Culture media	70% MEA	Carboxymethyl cellulose broth	Solid minimal medium
2	Incubation time	10 days	5 days	3 days
3	Temparatue	Room temperature	Room temperature	26°C
4	Biomass	15.70 g/L	0.79 g/L	3.73 g/L
5	Lipid accumulation (% of dry biomass weight)	37.69	20.50	19.90
6	Main chemical components (% of biodiesel weight)			
	C16:0	19.53	21.50	20.00
	C18:0	11.83	9.60	2.00
	C18:1	34.66	35.80	37.00
	C18:2	10.68	18.80	14.30

4. Discussion

Based on the results of the study on the cultivation of the marine fungus *A. pseudofelis* MMERU 25 in 70% MEB for 20 days, it was observed that the growth rate or biomass remained relatively constant from day 10, with a value of 3.14 g. The average biomass from day 10 to day 20 was found to be 3.11 g. Additionally, it was found that *A. pseudofelis* MMERU 25 cultured for 10 days accumulated a lipid content of 37.68% per dried biomass weight, while the accumulation reduced to 30.68% when cultured for 15 days. Therefore, it can be concluded that the optimal cultivation period for *A. pseudofelis* MMERU 25 in 70% MEB is 10 days.

The results of the study on the growth of *A. pseudofelis* MMERU 25 in 70% MEBM, were conducted in a total of four experimental sets and cultured for 15 days. The second set exhibited the highest lipid accumulation, while the fourth set had the highest biomass weight. However, the results of the first set displayed biomass and lipid accumulation values that closely resembled those of the second experimental set, despite the addition of only 2.5 ml of molasses per liter of 70% MEB. Therefore, the addition of 2.5 ml of molasses per liter of 70% MEB appears to be the most suitable approach for fungal cultivation. In summary, the results of the study indicate that *A. pseudofelis* MMERU 25 is capable of producing lipids in a 70% MEBM feed, making it suitable for further commercial development and research.

The potential of biodiesel from *A. pseudofelis* MMERU 25 in 70% MEB for 10 days, as demonstrated in the aforementioned study, yields 360 ml of biodiesel per 1 kg of dry biomass. Moreover, the chemical profile of FAME obtained from *A. pseudofelis* MMERU 25, as determined by the ^{1}H spectrum, aligns with the research conducted by Bardhan et al. (2019). When comparing parameters such as viscosity, acid value, methyl ester content, and linolenic acid content to the biodiesel standards by the Department of Energy Business (DOEB) in 2019-2020, it was observed that the viscosity at 40°C measured at 3.544 cSt, the linolenic acid content was 0.04% by weight, and the iodine value was 54.100 g Iodine/100g of biodiesel. These values are in accordance with the DOEB established criteria, which specify a minimum viscosity of 3.5 cSt, a maximum linolenic acid content of 12% by weight, and an iodine value not exceeding 120 g Iodine/100g of biodiesel. However, the acid value, methyl ester content, and H.H.V. were determined to be 1.291 mg KOH/g of biodiesel, 83.202% w/w, and 33.19 MJ/kg, which does not comply with the DOEB biodiesel standards. The DOEB determined an acid value not exceeding 0.5 mg KOH/g of biodiesel, a minimum methyl ester content of 96% w/w, and H.H.V. of palm of 41.230 MJ/kg. The H.H.V of the biodiesel is measured at 33.19 MJ/kg of biodiesel, which is lower than the H.H.V of biodiesel produced from palm, which is 41.23 MJ/kg of biodiesel. This is because the purity of the biodiesel extracted from *A. pseudofelis* MMERU 25, as found in this study, is 83.202% w/w. If there are future improvements in the purity of biodiesel from *A. pseudofelis* MMERU 25, achieving a purity greater than 96% w/w, as followed by the DOEB, will help increase the H.H.V. Regarding the acidity value of the biodiesel extracted from *A. pseudofelis* MMERU 25, it is measured at 1.291 mg KOH/g of biodiesel, which is higher than the biodiesel standard. This may be attributed to the presence of free fatty acids and sulfuric acid, which are catalyst residues that remain in the biodiesel. In the future, removing these impurities will reduce the acidity value.

Moreover, a comparison of biomass and lipid accumulation percentages was conducted among *A. pseudofelis* MMERU 25, *A. tubingensis* TSIP9, and *M. circinelloides*. The results revealed that *A. pseudofelis* MMERU 25 exhibited the highest biomass at 3.14 g/200mL or 15.70 g/L, surpassing *A. tubingensis* TSIP9 with a

biomass of 0.79 g/L and *M. circinelloides* with a biomass of 3.73 g/L. Moreover, *A. pseudofelis* MMERU 25 demonstrated the highest lipid accumulation, reaching 37.69% of the dry biomass weight, while *A. tubingensis* TSIP9 and *M. circinelloides* accumulated 20.50% and 19.90% of the dry biomass weight, respectively. The analysis of the main chemical components for biodiesel production from all three fungi types revealed consistent compounds, namely C16:0, C18:0, C18:1, and C18:2. Based on this information, it is evident that *A. pseudofelis* MMERU 25 holds promise for further exploration and development as a biodiesel production source in the future. Subsequent research could focus on optimizing growth conditions for the fungus to enhance lipid production and explore the utilization of agricultural waste, such as palm fruit bunches, molasses, etc., aiming for greater efficiency and cost reduction in the culture media of the fungus.

Acknowledgments

This project is funded by the National Research Council of Thailand (NRCT) and the Program Management Unit for Human Resources and Institutional Development, Research and Innovation (PMU-B). We also extend our thanks to the Plant Genetic Conservation Project under the Royal initiative of HRH Princess Maha Chakri Sirindhorn, as well as the Naval Special Warfare Command, the Royal Thai Fleet, and the Royal Thai Navy for their assistance in collecting marine organism samples. We are most grateful to the staff of the Marine Microbe Environment Research Unit (MMERU), Division of Environmental Science, Faculty of Science, Ramkhamhaeng University, for their fieldwork assistance

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