# Enhanced production of microalgal biomass and lipid as an environmentally friendly biodiesel feedstock through actinomycete co-culture in biogas digestate effluent

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## HIGHLIGHTS

- Actinomycete co-culture successfully enhanced both microalgal biomass and lipid.
- *Piscicocus intestinalis* WA3 produced high algal growth-promoting agents.
- Microbial lipids are composed of C16-C20 fatty acids with good fuel properties.
- Biogas effluent can be used as cost-effective media for actinomycete co-culture.

## GRAPHICAL ABSTRACT
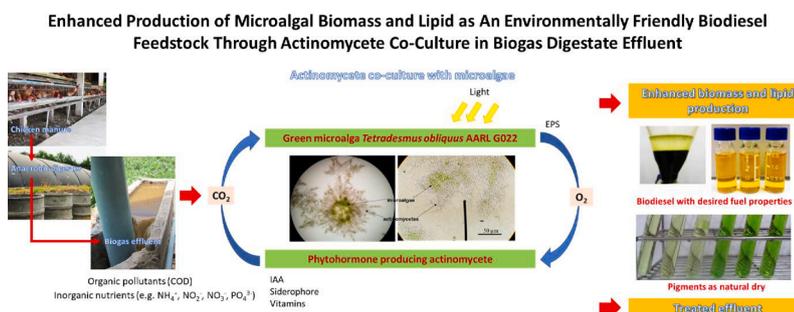


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## ARTICLE INFO

## ABSTRACT

In this study, an innovative approach to enhance the production of microalgal biomass and lipid as a promising sustainable feedstock for biodiesel was proposed using an actinomycetes co-culture with microalgae in the biogas digestate effluent (BDE) that can be employed as an environmentally friendly and cost-effective strategy. Among tested actinomycete isolates, *Piscicocus intestinalis* WA3 produced indole-3-acetic acid and siderophores as algal growth promoting agents and showed effective lipid accumulation with satisfying fatty acids composition. During co-cultivation of *P. intestinalis* WA3 with microalga *Tetradesmus obliquus* AARL G022 in the BDE, biomass production, chlorophyll *a* content, and lipid productivity were significantly increased by 1.30 folds, 1.39 folds, and 1.55 folds, respectively, compared to microalgae monoculture. The accumulated lipids contained long-chain fatty acids with better fuel properties that could potentially be used as biodiesel feedstock. The overall results evidenced that actinomycete co-culture would contribute greatly to the cost-effective production of environmental-friendly microbial-based biofuel.

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## 1. Introduction

Over the past few decades, microbial lipids generated by oleaginous microorganisms, including bacteria, yeasts, molds, and algae, are receiving more attention than conventional fuels like petroleum, coal and natural gas, because it has the potential to be an efficient, environment-friendly, renewable, and sustainable replacement to conventional fuels (Salama et al., 2017). Contrarily, the extensive usage of conventional fuels has caused environmental issues in particular greenhouse gas emissions (Yen et al., 2015). Among all oleaginous microorganisms, microalgae are the most sustainable and eco-friendly source for biodiesel feedstock because they can effectively utilize light and carbon dioxide to generate lipid-rich biomass that could be potentially transformed into biodiesel with satisfactory fuel properties (Jusoh et al., 2015). Advantageously, they are easy to mass-cultivate for scaling-up of microalgae cultivation allow us to extract rich lipid. Microalgae lipids have a comparable fatty acid composition or much higher than plant oil (Jusoh et al., 2015). However, the industrialized biodiesel production from microalgae lipid is still far from successful because of costly large-scale microalgae cultivation. Many researchers have therefore worked hard to reduce the costs involved in the production of microalgal biodiesel by developing various strategies and methods, such as selection of high-lipid producing microalgae strain, microalgae cultivation using low-cost medium, and enhancement of microalgae growth coupling with lipid accumulation by chemicals and/ or environmental factors (Salama et al., 2017). Through these methods, it has partially successful in improving the microalgal growth, lipid production, and fatty acid profiles. Despite these challenges, an alternate approach needs to be pursued for obtaining a higher production rate of both microalgal biomass and lipid that will contribute to sustainable commercial development of biodiesel-based biofuels.

Recently, researchers have used phytohormones to stimulate the microalgal cells for enhancing both biomass and lipid (Singh et al., 2020; Sivaramakrishnan and Incharoensakdi, 2020). Phytohormones (plant hormones) are natural or synthetic chemical messengers that have profound impact on growth and development of plant (Jusoh et al., 2015; Salama et al., 2017). The main phytohormonal categories include auxins, cytokinines, abscisic acid and gibberellins, and among these, auxins, especially indole acetic acid (IAA) is vital for microalgal growth and metabolism (Salama et al., 2017; Singh et al., 2020; Sivaramakrishnan and Incharoensakdi, 2020). For example, IAA act as a microalgal growth promoting agent when microalga *Chlamydomonas reinhardtii* was cultured in the nutrient medium containing IAA by increasing the growth up to 54–69% (Park et al., 2013). The IAA supplementation had been employed to stimulate biomass and lipid accumulation in microalga *Acutodesmus obliquus* (Jusoh et al., 2015) and *Desmodesmus* sp. (Singh et al., 2020). Similar changes on chlorophyll contents in microalga *Chlorella pyrenoidosa* were observed with IAA supplementation (Dao et al., 2018; Rajapitamahuni et al., 2019). However, although numerous studies have indicated that IAA promotes the microalgae growth and production of some valuable compounds, the addition of IAA may increase the overall production cost.

Symbiotic relationships via co-culture have been developed to promote both microalgal biomass and lipid production (Kumsiri et al., 2018). Previous studies have found that co-cultivation of microalgae with IAA-producing bacteria could save the cost of IAA supplementation in the culture medium (Dao et al., 2018; Kumsiri et al., 2018; Rajapitamahuni et al., 2019; Lakshmikandan et al., 2021). During co-cultivation, the microalgae can produce oxygen via photosynthesis while actinomycete produced carbon dioxide to the microalgae for biomass and lipid production (Yen et al., 2015). This discovery is intriguing, as an increment in both biomass and lipid productivity can have a favorable impact on the overall biodiesel yields positively. Recently, Lakshmikandan et al. (2021) also suggested that co-cultivation of bacteria and microalgae can save the microalgal harvesting cost, indicating the cost-effectiveness of the co-culture system. Moreover, few

studies on the feasibility of utilizing industrial wastewater for co-cultivation of microalgae (such as *Chlorella vulgaris*, *Tetradesmus obliquus*, and *Scenedesmus* sp.) and IAA-producing bacteria (such as *Nocardia* spp., *Pseudomonas* spp., *Bacillus* spp., *Acinetobacter* spp., and *Stenotrophomonas* spp.) have been documented (Dao et al., 2018; Kumsiri et al., 2018; Xu et al., 2020). In the literature, actinomycetes are among phytohormones-producing bacteria that have been well-studied on IAA production (Lasudee et al., 2018). The use of actinomycetes could not only produce phytohormone but also generate siderophores and solubilize phosphate (Lasudee et al., 2018; Myo et al., 2019) that might enhance the nutrient availability in the industrial wastewater, especially dissolved organic matter, and then resulting in a higher yield of microalgal biomass and lipid (Krug et al., 2020). Biogas effluent from anaerobic digestion with huge amount of essential organic and inorganic substances could be one of the low-cost media for co-cultivation of microalgae and actinomycetes. Duangjan et al. (2016) and Kumsiri et al. (2018) suggested that the biogas effluent must be diluted into an appropriate dilution to avoid substrate inhibition and to enhance light penetration for maximizing microalgal biomass and lipid yield. This indicated that the technique for the co-culture of actinomycetes and microalgae was not only successful in the production of both microbial biomass and lipid and pollution control/treatment but was also costly reduced.

However, few reports are available on this topic. The co-culture of microalgae and phytohormone-producing actinomycete in biogas effluent was not well-investigated. Thus, this research aimed to select and identify actinomycetes having ability to produce microalgal growth promoting agents and their capacity for growth in biogas effluent as well as lipid accumulation. The growth-promoting effect of actinomycete on microalga *T. obliquus* AARL G022 growth in the biogas digestate effluent (BDE) was studied, compared to microalgae or actinomycete monoculture. Furthermore, the fatty acid composition of the obtained microbial lipid and fuel qualities of the generated biodiesel were also estimated. This study will also provide supporting findings in databases regarding the cost-effective and environmentally friendly production of microorganism-based biofuel.

## 2. Materials and methods

### 2.1. Microorganisms, culture conditions and media

Actinomycetes were isolated from biogas effluent of chicken manure biogas plant at Huai Nam Rin farm, Lamphun province, Thailand using the dilution plate technique. Plates were incubated at 30 °C for 4–8 weeks and examined periodically and presumptive actinomycete colonies were isolated. Pure culture of actinomycetes were transferred to ISP2 agar slants and then incubated at 30 °C before use. Green microalga *Tetradesmus obliquus* AARL G022 obtained from Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. Algal culture was inoculated in Jaworki's medium (JM) and incubated at 25 °C under continuous light feeding of 10.8 $\mu$mol/m$^2$/s for 5–7 days before use.

### 2.2. Screening and identification of high phytohormones-producing actinomycetes

Actinomycete isolates were firstly screened for their growth on ISP2 agar under incubation temperature at 30 °C for 7 days. Morphological characteristics of isolates, including color of mycelium, colony morphology, and morphology under microscopic, were assessed. Then, the ability to grow in 25% BDE agar containing 25% biogas digestate effluent medium (pH = 7.1 $\pm$ 0.0, nitrate-nitrogen = 26.0 $\pm$ 7.5 mg/L, soluble phosphate-phosphorus = 34.8 $\pm$ 8.5 mg/L, ammonium-nitrogen = 90.9 $\pm$ 13.6 mg/L, and chemical oxygen demand = 203.0 $\pm$ 12.3 mg/ L) and 1.5% agar were evaluated. The growth was recorded after 7 days of cultivation at 30 °C using three level scores: well-growth (++),

moderate-growth (+), and no growth (−).

The phytohormones and lipid-producing ability of isolates was determined by a step-wise screening approach. Firstly, isolates having ability to grow in 25% BDE agar were cultivated in ISP2 medium containing 0.2% L-tryptophan at 30 °C with shaking speed of 125 rpm. After 7 days of cultivation, culture was centrifuged at 11,000 rpm for 15 min. The supernatant (1 mL) was mixed with 2 mL of Salkowski reagent and incubated at room temperatures for 20 min. The mixture was then subjected to spectrophotometer for measuring the optical density ($OD_{530}$). The indole-3-acetic acid (IAA) production of isolates was calculated using the standard curve of pure IAA (Kumsiri et al., 2018; Lasudee et al., 2018). Then, isolates having IAA production ability (>10 ug/mL) were tested for their siderophores production according to the method of Lasudee et al. (2018). Briefly, isolates were cultured in chrome azurol S (CAS) agar under incubation temperature at 30 °C for 7 days. The orange halo zone diameter surrounding the colony was measured. The phosphate solubilization potential of isolates having>10 μg IAA/mL were also evaluated using the method of Lasudee et al. (2018). Isolates were cultivated on PVK agar containing with 0.5% tricalcium phosphate under incubation temperature at 30 °C for 7 days. The capability to solubilize phosphates was demonstrated by clear areas around the colony.

Selected isolates having high phytohormones were molecularly identified using 16S rRNA gene analysis. The genomic DNA of selected isolates was extracted using the FavoPrepTM Tissue Genomic DNA Extraction Mini Sample Kit (Taiwan) and was then amplified by PCR using two universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), according to the PCR protocol described by Lasudee et al. (2018). The nucleotide sequences of the obtained 16S rDNA were subjected to BLAST analysis with the EzBiocloud database and submitted to GenBank.

### 2.3. Co-cultivation of actinomycetes and microalgae

There are two experiments for evaluating co-cultivation of actinomycetes and microalgae, consisting of the following:

- Experiment I: co-culture in JM medium. This experiment was performed by co-cultivating microalga *T. obliquus* AARL G022 and selected actinomycete isolates at microalgae to bacteria ratio of 3:1 on 250 mL JM medium under continuous light feeding of 10.8 μmol/m$^2$/s, shaking speed at 125 rpm, and cultivation temperature at 25 °C for 10 days. Mono-cultivation of microalgae was also conducted as a control experiment. Co-culture that has the highest growth performance was selected for the next experiment.
- Experiment II: co-culture in 25% BDE medium. Selected co-culture was conducted in 25% BDE medium (as the suitable concentration of BDE for cultivating microalgae *T. obliquus* AARL G022 reported by Duangjan et al. (2016)) with and without 0.2% L-tryptophan supplementation for 12 days. The culture conditions were the same as Experiment I. Mono-cultivation of microalgae and/or selected actinomycete isolate was also performed as a control experiment.

### 2.4. Analytical methods

Total biomass was calculated in term of dried biomass (g/L). Culture broth (10 mL) was filtered using 4.7 cm Whatman GF/C glass fiber filters and washed with distilled water twice. Filtrated biomass was then dried at 60 °C until constant weight. Total biomass was determined gravimetrically. Actinomycete's growth was measured by dilution plate counting on ISP2 agar and colony-forming units were provided as the number of cells (CFU) after 24 h of incubation at 30 °C. Microalgal growth was monitored by determining chlorophyll *a* content. The chlorophyll *a* of dried microalgal biomass was extracted using 90% MeOH and then boiled at 70 °C for 20 min. Centrifugation was done at 6000 rpm for 10 min to obtain the supernatant. The supernatant was

measured the optical density (OD) at 630 nm ($A_{630}$), 645 nm ($A_{645}$), 665 nm ($A_{665}$) and 750 nm ($A_{750}$) with a spectrophotometer (Kumsiri et al., 2018). The following equations were used to compute the chlorophyll *a* (mg/L):

$$\text{Chlorophyll } a \, (\text{mg/L}) = (11.6A - 1.31B - 0.14C) \times \left[ (V/V_f) \times (1/L) \right] \tag{1}$$

where A is $A_{665} - A_{750}$, B is $A_{645} - A_{750}$, C is $A_{630} - A_{750}$, V is the total volume of extract (mL), $V_f$ is the volume of sample (mL), and L is the light path length of width of cuvette (cm).

Lipids of dried biomass were extracted with a mixture of dichloromethane/methanol (2:1, v/v) and sonicated for 15 min. The suspension was centrifugated to achieve a clear supernatant at 6000 rpm for 10 min. The solvent layer was collected and evaporated to dry at 30 °C and the extracted lipid was determined gravimetrically (Kumsiri et al., 2018). The following equations were used to compute the lipid content (%) and lipid production (mg/L):

$$\text{Lipid content} \, (\%) \ = \ \left[ \text{dried lipid (g)/dried biomass (g)} \right] \times 100 \tag{2}$$

$$\text{Lipid production} \, (\text{mg/L}) = \left[ \text{lipid content} \, (\%) \times \text{biomass production (g/L)} \right. \\ \left. \times 1000 \right]/100 \tag{3}$$

Microbial lipids were transformed into fatty acid methyl esters (FAME) by transesterification reaction (Kumsiri et al., 2018). Briefly, the extracted lipid was mixed with 1 mL of 15 g NaOH in 100 mL 50% MeOH, boiled for 30 min, mixed with 2 mL of 1:1.18 v/v MeOH: 6 N HCl, and boiled again at 80 °C for 20 min. Cooled samples are subjected to 1 mL of 1:1 v/v hexane: diethyl ether and the sample was mixed thoroughly. The remaining upper layer (FAME) was harvested and washed with 3 mL of 1.2% w/v NaOH. FAME was subjected to GC–MS (6890A GC System with a 5975C inert XL EI/CI MSD with a Triple-Axis Detector operated at 70 eV; Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5-ms capillary column (30 mm × 0.25 mm GC 6890, Agilent Technology, USA). The fatty acid composition was used to estimate the fuel qualities such as saponification value (SV; mg KOH), cetane number (CN), cold filter plugging point (CFPP; °C), iodine value (IV; g I$_2$/100 g), degree of unsaturation (DU; %), pour point (PP; °C) and Cloud point (CP; °C) using BiodieselAnalyzer Version 2.2 (Talebi et al., 2014).

### 2.5. Statistical analysis

Triplicate analyzes were performed and the results were analyzed by means of descriptive statistical analysis of the data using one-way ANOVA (analysis of variance) and Duncan's multiple range tests (p < 0.05) using SPSS statistics 17.0.

### 3. Results and discussion

#### 3.1. Screening and identification of high phytohormones-producing actinomycetes

Actinomycetes can be considered as a promising source of bacteria capable to effectively produce phytohormones, especially indole-3-acetic acid (IAA) which positively promote the microalgae growth and biochemicals production (Kumsiri et al., 2018). The efficacy to produce siderophores and solubilize phosphate enhanced the potential use of actinomycetes for solubilizing insoluble nutrients/pollutants in industrial wastewater (Myo et al., 2019) such as biogas digestate effluent (BDE). These demonstrated that actinomycetes can directly or indirectly increase the availability of nutrients that could be afterward used for phytoremediation of wastewater. In this study, we focused on the screening and identification of potent phytohormone-producing actinomycetes growing in BDE. Isolates having great potentiality of IAA and

siderophores production and the phosphate solubilization ability were chosen for their microalgal growth promotion. Twelve actinomycetes isolated from biogas effluent of chicken manure biogas plant were morphologically characterized in the ISP2 agar. All isolates showed well-developed substrate mycelium, branching filaments, and most aerial mycelia appeared floccose, granular, or powdery. The presence of soluble pigments affects the different colors of aerial mycelium.

Sequential screening of high phytohormone-producing actinomycetes was performed to evaluate the growth performance on the 25% BDE, the capacity for the production of microalgal growth promoting agents, and the ability to solubilize phosphate. The results found that isolates WA7 and WA10 did not grow in the 25%BDE, and only isolate WA2 showed moderate growth. Importantly, nine isolates namely, WA1, WA3, WA4, WA5, WA6, WA8, WA9, WA11, and WA12, grew well and be selected to test their ability to produce IAA. At this stage, all isolates were effective for IAA production in the range of 0.4–63.6 μg/mL. The highest IAA production was by isolate WA3 with IAA leveled at 63.6 μg/mL followed by isolate WA12 (15.9 μg/mL). Fu et al. (2015) and Kumsiri et al. (2018) reported that IAA is a key phytohormone that governs actinomycetes' physiologic reaction and gene expression. These IAA levels depend on the different pathways (indole-3-acetamide pathway, indole-3-pyruvate pathway, tryptamine pathway, tryptophan side-chain oxidase pathway, indole-3-acetonitrile pathway) to synthesize IAA using tryptophan as a key precursor in actinomycetes (Spaepen et al., 2007). Thus, isolates WA3 and WA12 having higher IAA production of > 10 μg/mL were chosen for tertiary screening step to ensure their capacity to produce siderophores.

Table 1 shows that both isolates WA3 and WA12 exhibited siderophores production as seen from the orange zone on CAS agar around their colonies with the diameter of 19.7 and 7.0 mm, respectively. Moreover, only isolate WA12 solubilized tricalcium phosphate on Pikovskaya medium agar with the clear zone dimeter of 11.7 mm (Table 1), indicating that the potential use for enhanced nutrient availability (Kumsiri et al., 2018). Although isolate WA3 did not show the ability to solubilize phosphate, this strain is capable of producing IAA and siderophores. Hence, both isolates were then subjected to evaluating their lipid accumulation.

According to Table 1, the lipid content of both isolates was comparable by up to 6.5% (for isolate WA3) and 5.6% (for isolate WA12). Table 1 also reported the proportion of fatty acid compositions in the isolates WA3 and WA12 lipid. Oleic acid (C18:1n9c, 32.0%), palmitic acid (C16:0, 25.9%), heptadecanoic acid (C17:0, 21.8%), and stearic acid methyl ester (C18:0, 15.8%) were four major fatty acids discovered in the isolate WA3 lipids, whereas palmitic acid (C16:0, 48.6%) and pentadecanoic acid (C15:0, 38.4%) were the major fatty acids detected in isolate WA12 lipids. Similarly, Zhuang et al. (2020) also found that C15-C18 fatty acids are naturally found in actinomycete lipids. It is noteworthy that the greater amount of saturated fatty acids (SFAs) in isolates WA3 and WA12 lipids suggested their prospective usage as biodiesel feedstocks since the oxidative stability and cetane numbers might be increased along with reduced $NO_X$ emissions and minimized time-out for ignition (Srinuanpan et al., 2018)

Actinomycete isolates WA3 and WA12 were then identified as *Piscicoccus intestinalis* WA3 (base pair length 1357; maximum identity 100%; similarity to *Piscicoccus intestinalis* NBRC104926) and *Streptomyces leeuwenhoekii* WA12 (base pair length 1344; maximum identity 99%; similarity to *Streptomyces leeuwenhoekii* C34), respectively. The 16S rRNA sequencing genes was submitted to the GenBank sequence database under the accession numbers MW888512 for *P. intestinalis* WA3 and MW888513 for *S. leeuwenhoekii* WA12. The identity of the species was further validated by phylogenetic analysis of 16S rRNA sequence by comparing it with data from the EzBiocloud database (Fig. 1). As demonstrated by a phylogenetic tree, isolates WA3 and WA12 were clearly grouped with *P. intestinalis* and *S. leeuwenhoekii*, respectively. *P. intestinalis* and *S. leeuwenhoekii* were previously reported for phytohormone production (Razmilic et al., 2018; Shan et al., 2018). However, no attempt for *P. intestinalis* co-culture with microalgae is recorded but only one research reported by Lakshmikandan et al. (2021) have demonstrated *Streptomyces* co-culture with green microalga *Chlorella vulgaris*.

Following several screening stages employed in this study, these indicated that actinomycetes could not only effectively produce microalgal promoting agents but may also promote the overall lipid yield. Thus, it can be considered that both isolates WA3 and WA12 might be useful in co-culture with microalgae to enhance microalgal biomass coupling with lipid production.

### 3.2. Performance of co-culturing actinomycetes with microalgae

To increase the performance of selected actinomycetes isolate WA3 and WA12, co-culture with microalga *Tetradesmus obliquus* AARL G022 were evaluated in JM medium for 10 days. During actinomycetes cocultivation, the growth of microalgae increased rapidly and reached the maximum growth within 8 days of cultivation with providing chlorophyll *a* content of 10.20 mg/L (for Co-WA3), 9.67 mg/L (Co-WA12), and 7.24 mg/L (for G022) (Fig. 2a) while the actinomycete growth of Co-WA3 ($9.87 \times 10^5$ CFU/mL) was higher that of Co-WA12 ($3.02 \times 10^5$ CFU/mL) (Fig. 3b). These observations indicate the obvious productiveness of the two actinomycetes for enhancing the growth of microalga *T. obliquus* AARL G022. The possible reason would be because the phytohormone produced by actinomycetes might be directly utilized by the microalgae and lead to an increase in chlorophyll *a* content.

As established by literature, the presence of IAA and siderophores in the culture medium might potentially cause a rise in the amount of microalgae chlorophyll *a* (Trinh et al., 2017; Rajapitamahuni et al., 2019). Han et al. (2018) and Sivaramakrishnan and Incharoensakdi (2020) reported that IAA has a significant function in the development and metabolization of microalgae and even small levels of IAA stimulated the production of microalgae and increased biomolecular biosynthesis. Siderophores containing N molecule in the structure can be a potential supply of nitrogen for microalgal growth and can also firmly attach to iron to boost its solubility. It is hypothesized that IAA and siderophores may stimulate the activity of photosynthesis by boosting the pigments synthesis pathway (Dao et al., 2018; Rajapitamahuni et al., 2019) and by activating cellular redox systems (Piotrowska-Niczyporuk and Bajguz, 2014). Moreover, Gangwar et al. (2014) suggested that actinomycetes did not produce only phytohormones but also secrete other compounds such as enzymes, vitamins and other nutrients that may improve the microalgal biomass production and biochemical accumulation in their cells such as pigments like chlorophylls and carotenoids.

**Table 1**
Indole-3-acetic acid (IAA) and siderophores production, phosphate solubilization ability, lipid production and fatty acid composition of actinomycete isolates WA3 and WA12 cultured in 25% biogas effluent.

| Isolate | Growth in 25% biogas effluent | Total indole (μg/mL) | Siderophore (mm) | Phosphate soluble (mm) | Lipid production (%) | Relative content of total fatty acid (%) | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | | C14:0 | C15:0 | C16:0 | C16:1 | C17:0 | C18:0 | C18:1n9c | C20:0 |
| WA3 | ++ | 63.6 ± 1.0 | 19.7 ± 0.6 | – | 6.5 ± 0.4 | – | 3.3 | 25.9 | 1.0 | 21.8 | 15.4 | 32.0 | 0.6 |
| WA12 | ++ | 15.9 ± 0.9 | 7.0 ± 1.0 | 11.7 ± 0.6 | 5.6 ± 1.4 | 7.8 | 38.4 | 48.6 | – | 5.2 | – | – | – |
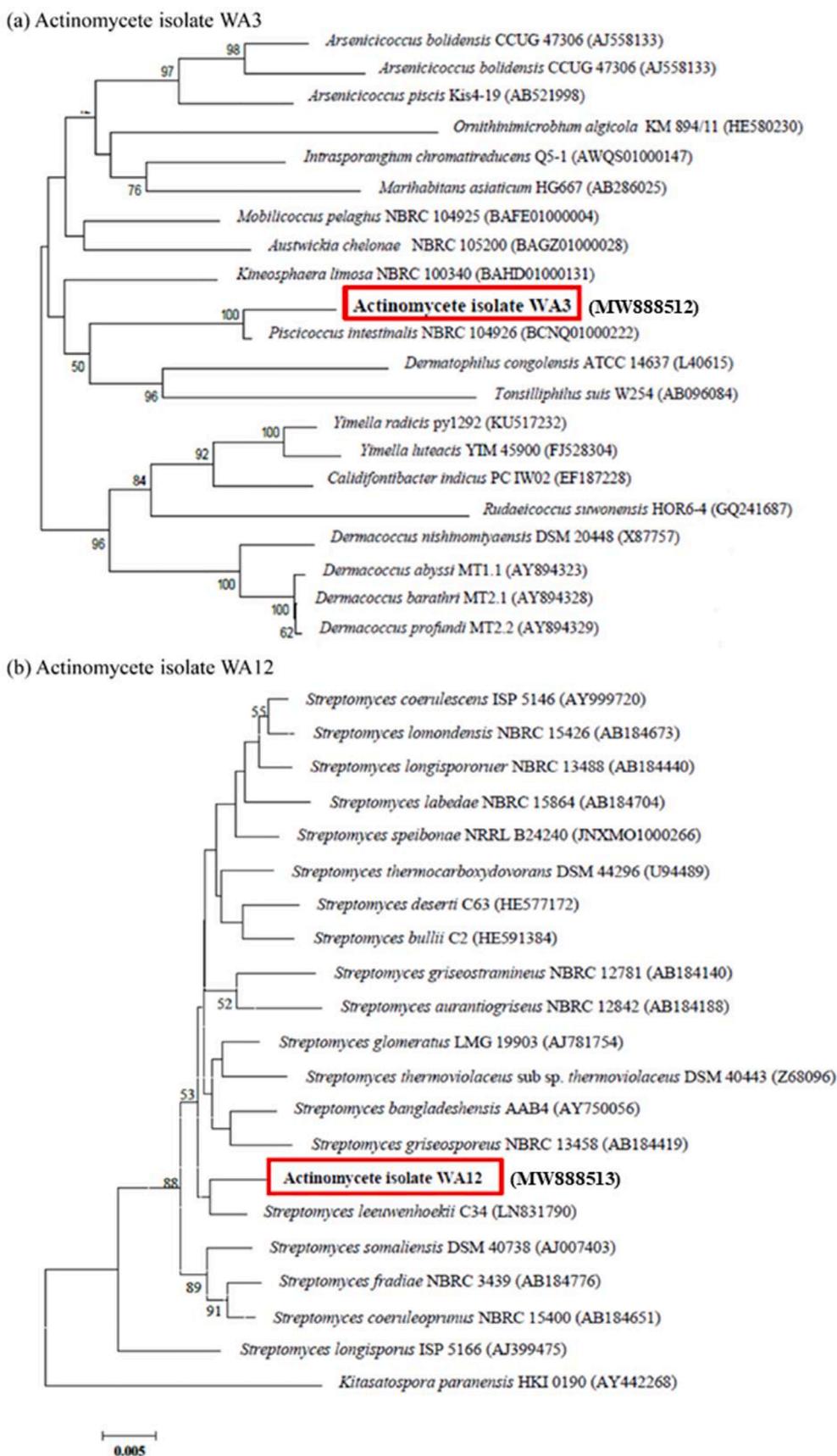
**Fig. 1.** Neighbor-joining phylogenetic trees of actinomycete isolates WA3 and WA12 with their closely related strains using MEGA6.1 software after 1000 rounds of bootstrap resampling related information.
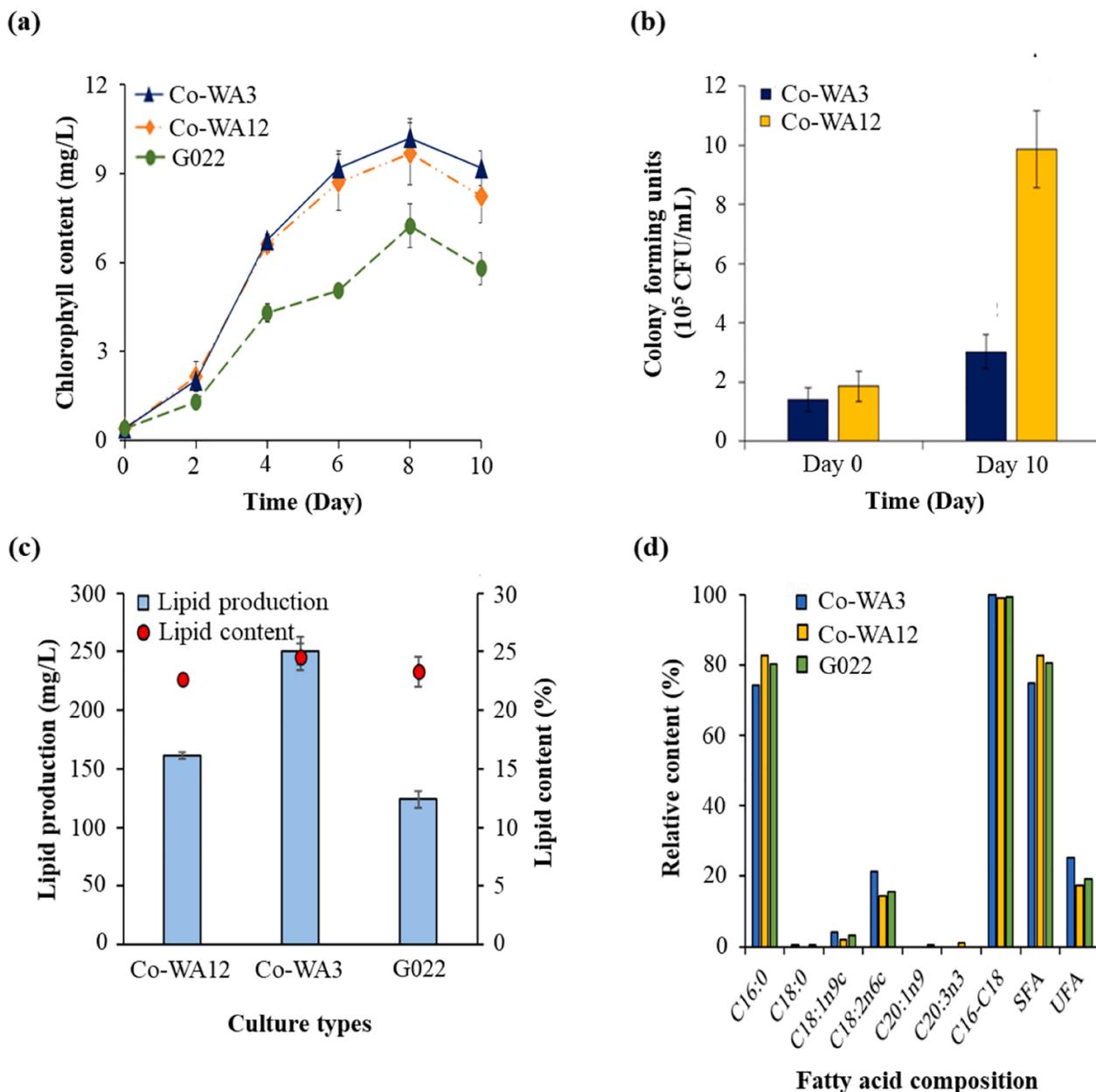
**Fig. 2.** Growth, lipid production, and fatty acid composition of *T. obliquus* AARL G022 (G022) monoculture, co-culture with actinomycete isolate WA12 (Co-WA12), and co-culture with actinomycete isolate WA3 (Co-WA3) in JM medium.

The significant enhancement of both actinomycete and microalgae growth during Co-WA3 cultivation was also feasible because of the effective gas exchange between $O_2$ produced from microalgae and $CO_2$ generated from actinomycetes that could easily be utilized by both microorganisms (Sforza et al., 2018). Unfortunately, the actinomycete isolate WA12 did not grow well during Co-WA12 cultivation, although more microalgal growth may produce more $O_2$ (Fig. 2b). It could be possible that the imbalance growth of the two microorganisms led to the lower growth of actinomycete isolate WA12 in the Co-WA12 cultivation. Culture conditions may be benefit to the growth of microalgae, it might have an advantage in the co-culture system and have detrimental effect on the actinomycete isolate WA12 growth. Similar observations were documented by Yen et al. (2015), Dao et al. (2018), and Berthold et al. (2019) who found that the microorganisms' equitable growth would maximize co-cultural advantages.

The lowered growth in actinomycete isolate WA12 and the fact of synergistic microalgal-bacterial contacts can be explained by inhibitory chemicals produced from the microalgae (Berthold et al., 2019). A negative interaction between microalgae and bacteria has been observed by Kouzuma and Watanabe (2015) who found that algae can control bacterial colonization via the release of toxic metabolites such as volatile halogenated compounds and fatty acids. In addition, chemical signals produced by the microalgae might be employed for cell–cell signaling, are not nutrients, they may suppress gene expression and/or physiological activities (Dao et al., 2018), resulting in the lower growth of actinomycete. Conclusively, actinomycete co-culture with microalgae might exhibit both positive and negative interactions.

Considering lipid accumulation, the lipid contents after actinomycete co-culture were comparable with level of 22.67–24.60% while the lipid content of the microalgae G022 monoculture was 23.30% (Fig. 2c). The results indicated that IAA and other related phytohormones produced by both actinomycetes are not enough to change carbon metabolism to lipid biosynthesis. Sivaramakrishnan and Incharoensakdi (2020) suggested that the phytohormone level plays an important role to

**(a)**



**(b)**



**(c)**
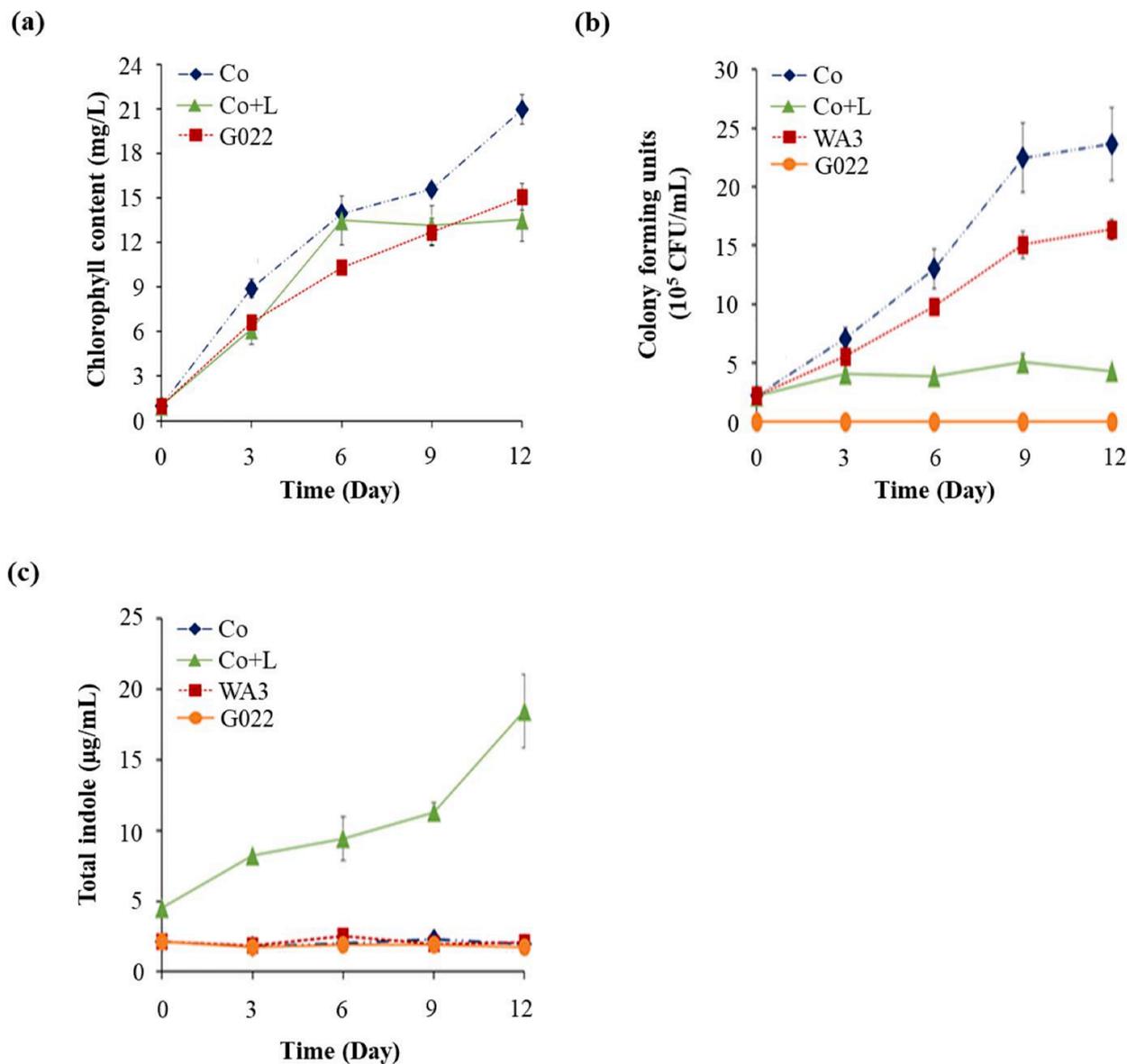


**Fig. 3.** Growth and total indole production of *T. obliquus* AARL G022 (G022) monoculture, actinomycete isolate WA3 (WA3) monoculture, co-culture with actinomycete isolate WA3 (Co-WA3) in 25% biogas effluent, and co-culture with actinomycete isolate WA3 in 25% biogas effluent and 0.2% L-tryptophan (Co-WA3 + L) supplementation.

stimulate the microalgal lipid content and other high-value products. However, the suitable level of phytohormones during actinomycete co-culture with microalgae has not yet been clearly reported. Interestingly, the overall lipid yields were increased up to 250.92 mg/L (for Co-WA3 with lipid productivity of 25.09 mg/L/day) and 161.16 mg/L/day (for Co-WA12 with lipid productivity of 16.12 mg/L/day) while lipid production and lipid productivities of mono-culture microalgae G022 were 124.19 mg/L, and 12.42 mg/L/day, respectively. A substantial increase in lipid yields resulted from increased production of biomass. This suggested that the co-overwhelming culture's growth of both microorganisms would culminate in the highest biomass and total lipid yields. Chu (2017) proposed that the creation of potential biofuel feedstock at cheap prices involves a significant amount of high microalgae biomass and lipid production. The lipid productivity in this study is low compared to the previous report of Wang et al. (2015) who found that lipid productivities of 86.2–87.7 mg/L/day can be obtained during bacteria *Acidovorax facilis* or *Diaphorobacter* sp. co-culture with microalga *Scenedesmus obliquus*. However, this value is still higher than those reported by others using *Nocardia bhagyanarayanae* co-culture with

microalga *T. obliquus* (20 mg/L/day) (Kumsiri et al., 2018), *Streptomyces rosealbus* co-culture with microalga *Chlorella vulgaris* (21 mg/L/day) (Lakshmikandan et al., 2021), and *Pseudomonas composti* co-culture with microalga *Characium* sp. (3.67–8.24 mg/L/day) (Berthold et al., 2019).

The fatty acid compositions of microbial lipids after actinomycete co-culture with microalga *T. obliquus* AARL G022 consisted of C16-C20 fatty acids; among these, C16-C18 (C16:0, C18:0, C18:1, and C18:2) are satisfactory for obtaining great-quality biodiesel (Srinuanpan et al., 2018) and had yielded higher than 99% of the fatty acid (Fig. 2d). C10-C14 fatty acids were not detected in microbial lipids. Similar compositions of fatty acids in microbial lipids have been observed by Salama et al. (2017) and Kumsiri et al. (2018). Compared with microalgae G022 monoculture, the C16:0 fatty acid in Co-WA3 lipid increased by 1.03-fold but decreased in CO-WA12 lipid. Singh et al. (2020) found that phytohormones, especially IAA, effectively enhanced C16:0 fatty acid content in *Desmodesmus* sp. lipid. In contrast, IAA did not promote the C16:0 fatty acid synthesis in lipids of microalga *Scenedesmus obliquus*, *Ourococcus multisporus* and *Chlorella vulgaris* (Salama et al., 2017). In addition, Co-WA12 caused a rise in the levels of C18:1n9c and C18:2n6c

fatty acids in the lipids as well as total unsaturated fatty acids (UFA), while those fatty acids in Co-WA3 lipids were lower compared to microalgae G022 monoculture lipid (Fig. 2d). However, a minor increment in saturated fatty acids (SFA) was observed in Co-WA3 lipids. One possible reason for these contradicting observations is the developmental regulation of fatty acid synthesis. Changes in fatty acid composition might be linked to the expression of four fatty acid biosynthetic genes including β-ketoacyl ACP synthase I (KAS I) gene, stearoyl-ACP desaturase (SAD) gene, omega-6 fatty acid desaturase (ω-6 FAD) gene, and omega-3 fatty acid desaturase (ω-3 FAD) gene (Jusoh et al., 2015).

Thus, it should be noted that our findings reveal actinomycete coculture not only promotes microalgal growth but also enhances the overall lipid yield. Actinomycete isolate WA3 co-culture was chosen for further experiments due to its higher growth performance and lipid productivity with desirable fatty acid composition.

### 3.3. Cost-effective co-cultivation of actinomycetes and microalgae using biogas digestate effluent

Anerobic/biogas digestate effluent represents promising low-cost nutrients for microalgal cultivation, resulting in an efficient wastewater phytoremediation and production of sustainable feedstock for biofuel-based products (Duangjan et al., 2016; Kumsiri et al., 2018). In this study, the actinomycete isolate WA3 co-culture with microalga *Tetradesmus obliquus* AARL G022 was cultured in diluted biogas digestate effluent (BDE) at 25% that is an eco-friendly and cost-efficient process. Dilution of an effluent can improve light penetration (Srinuanpan et al., 2019) and reduce substrate inhibition (Duangjan et al., 2016), resulting in an enhancement of photosynthesis and biomass production. Moreover, the effect of 25%BDE with/without the addition of L-tryptophan was also studied to induce the phytohormone production, especially, indole-3-acetic acid (IAA) during actinomycete co-culture with microalgae. Increment in IAA production via L-tryptophan supplementation has been reported (Khamna et al., 2010; Myo et al., 2019). Hence, in this study, the effect of actinomycete isolate WA3 on the growth of microalga *T. obliquus* AARL G022 with and without L-tryptophan supplementation were evaluated in 25%BDE.

Based on Fig. 3a, the chlorophyll *a* content rapidly increased during co-cultivation in 25%BDE without L-tryptophan supplementation (Co) with the maximum chlorophyll *a* content of 20.96 mg/L at day 12 of cultivation while co-cultivation with L-tryptophan supplementation (Co + L) the chlorophyll *a* was lower at 13.54 mg/L and reached the stationary phase within 6 days of cultivation. The chlorophyll *a* content of microalgal monoculture was also increased up to 15.07 mg/L after 12 days of cultivation. The lower chlorophyll *a* content in Co + L might be due to the negative effect of excessive IAA produced by actinomycete that inhibits the microalgal growth. As tryptophan is a key precursor for the actinomycete IAA biosynthesis pathway, the presence of L-tryptophan in the medium promotes IAA formation (Khamna et al., 2010; Lakshmikandan et al., 2021). It should be noted that increasing the IAA levels > 10 μg/mL at day 9 and 12 of Co + L reduces the chlorophyll *a* content, suppressing the cell growth of microalgae (Fig. 3b). Our findings are in agreement with previous report confirming that a reduced stimulating impact was seen for microalgal growth at concentrations over 10 mg/L IAA as biomass levels were not substantially influenced (Singh et al., 2020). Also, in papers by Sivaramakrishnan and Incharoensakdi (2020), and Lakshmikandan et al. (2021) also reported that tryptophan can negatively affect microalgal growth, thereby decreasing chlorophyll *a* synthesis.

Although L-tryptophan supplementation enhanced the levels of actinomycete IAA, the growth of actinomycete isolate WA3 did not increase during Co + L cultivation, compared to actinomycete monoculture (WA3) (Fig. 3c). This could be because when microalgal growth slows, $O_2$-$CO_2$ exchange between microalgae and actinomycete may also likely to decline. A decrease in the growth of bacteria during co-cultivation with microalgae has been reported previously (Peng et al.,

2021) and is likely a function of cell-to-cell $O_2$-$CO_2$ exchange availability. Interestingly, the growth of actinomycete isolate WA3 was increased during co-cultivation without L-tryptophan supplementation (Co), compared to WA3 (Fig. 3c). This might be due to the symbiotic effects that benefit the growth of both microorganisms mutually. One of the likely explanations was the conveyance of the gaseous in the medium that the oxygen generated by *T. obliquus* AARL G022 could immediately use by an actinomycete isolate WA3, and *T. obliquus* G022 could consume $CO_2$ produced by an actinomycete isolate WA3. In addition to effective gas exchanges, some nutrients produced by both microorganisms may have a synergistic influence on the total co-culture system's biomass improvement (Yen et al., 2015). The significant enhancement in overall biomass and lipid production was recorded at 1.08 g/L and 207.76 mg/L, respectively during co-cultivation without L-tryptophan (Table 3). Conclusively, the co-culture of actinomycetes and microalgae without the addition of L-tryptophan might boost the microalgal biomass, and result in the maximum total lipid production. A similar observation was reported by Wang et al. (2015), Kumsiri et al. (2018), and Peng et al. (2021).

More importantly, the 25% BDE was successful used to replace the commercial JM medium and to promote the co-culture system. The usage of JM medium for cultivation of microalgae is costly due to chemical costs for roughly $45 per m$^3$ culture volume. A cost-effective way to decrease the usage of freshwater/tap waters that should be examined in further studies is to dilute BDE by utilizing recovered secondary effluent. Co-culture of actinomycete and microalgae in industrial effluents is therefore fascinating because it serves two purposes: phytoremediation of effluent and the economical production of huge quantities of microalgal biomass as a viable feedstock for biofuel. The BDE contains various forms of carbon, nitrogen, and phosphorus. During co-culture synergism, both microalgae and actinomycete can consume organic and inorganic carbon contained in the BDE acting as nutrient sources for their growth and resulting in the reduction of COD. Both microorganisms could also utilize $NO_3$-N through denitrification and/or assimilation. The oxygen produced from microalgal photosynthesis was utilized by actinomycete towards the nitrification process while microalgae exploited $NO_3$-N as a nitrogen source for microalgal growth after the $NH_4$-N was depleted (Nguyen et al., 2020). At the same time, the co-culture was able to completely utilize $PO_4$-P which would possibly due to the increased growth influenced by the balance of $O_2$-$CO_2$ exchange (Roberts et al., 2020). Thus, $O_2$-$CO_2$ exchange might have been crucial since both microalgae and actinomycete consumed nutrients in the BDE. Further studies on microalgae-bacteria gas exchange in wastewater should be investigated to expand our understanding on some of the complicated occurrences in co-cultures. Therefore, it should be concluded that actinomycete co-culture with microalgae using the biogas digestate effluent could then be economically attractive for the industrial sector. In addition, without L-tryptophan supplementation could also further save the production cost.

### 3.4. FAME characterization and its fuel properties

Since greater biomass and lipid yield were obtained in the actinomycete co-culture with microalgae in 25%BDE without L-tryptophan supplementation (Co), it is suggested that co-culture might play a significant influence in the industrialization of the production of biofuel based on microorganisms. High lipid yield is a possible alternative source for cost-effectiveness of the production of biodiesel with satisfied fuel properties (Srinuanpan et al., 2018). According to Table 2, the fatty acids of microbial lipids extracted from co-cultivation without L-tryptophan supplementation (Co) are C16-C18 including palmitic acid (C16:0, 29.61%), hexadecadienoic acid (C16:2, 2.24%), hexadecatrienoic acid (C16:3, 15.49%), stearic acid (C18:0, 2.03%), oleic acid (18:1n9, 23.02%), linoleic acid (C18:2n6c, 23.43%), linolenic acid (C18:3n3, 2.22%), and γ-linolenic acid (C18:3n6, 1.96%), implying that microbial lipids offer potential as a feedstock of biodiesel. Talebi et al.

**Table 2**

Biomass, lipid production and fatty acid composition of *T. obliquus* AARL G022 (G022) monoculture, actinomycete isolate WA3 (WA3) monoculture, co-culture with actinomycete isolate WA3 (Co-WA3) in 25% biogas effluent, and co-culture with actinomycete isolate WA3 in 25% biogas effluent and 0.2% L-tryptophan (Co-WA3 + L) supplementation.

| Parameters | Treatments | | | |
|---|---|---|---|---|
| | WA3 | Co-WA3 | Co-WA3 + L | G022 |
| Biomass (g·L⁻¹) | 0.11 ± 0.02 | 1.08 ± 0.09 | 0.64 ± 0.07 | 0.83 ± 0.04 |
| Lipid content (%) | 4.40 ± 1.70 | 19.60 ± 1.14 | 18.46 ± 2.56 | 20.03 ± 1.31 |
| Lipid production (mg/L) | 4.84 ± 1.87 | 207.76 ± 12.04 | 118.14 ± 16.02 | 133.98 ± 8.66 |
| Fatty acid | Relative content (%) | | | |
| C16:0 | 57.97 | 29.61 | 27.88 | 80.13 |
| C16:1 | – | – | 1.64 | – |
| C16:2 | – | 2.24 | 4.14 | – |
| C16:3 | – | 15.49 | 9.49 | – |
| C17:0 | 2.75 | – | – | – |
| C17:1 | – | – | – | – |
| C18:0 | 30.87 | 2.03 | 2.31 | 0.53 |
| C18:1n9c | 8.41 | 23.02 | – | 3.14 |
| C18:2n6c | – | 23.43 | 21.93 | 15.66 |
| C18:3n3 | – | 2.22 | 31.35 | 8.93 |
| C18:3n6 | – | 1.96 | 1.26 | – |
| C20:1n9 | – | – | – | 0.54 |
| SFA | 91.59 | 31.64 | 30.19 | 80.66 |
| UFA | 8.41 | 68.36 | 69.81 | 19.34 |
| SFA/UFA | 10.89 | 0.46 | 0.43 | 4.17 |

**Table 3**

Estimated fuel properties based on fatty acid composition derived from *T. obliquus* AARL G022 (G022) monoculture, co-culture with actinomycete isolate WA3 (Co-WA3) in 25% biogas effluent, and co-culture with actinomycete isolate WA3 in 25% biogas effluent and 0.2% L-tryptophan (Co-WA3 + L) supplementation.

| Biodiesel properties | EN 14214 | ASTM D6751-10 | Co-WA3 | Co-WA3 + L | G020 |
|---|---|---|---|---|---|
| Saponification value (SV) (mg KOH) | NA | NA | 142.46 | 133.53 | 172.34 |
| Cetane number (CN) (min) | ≥51 | ≥49 | 70.51 | 65.18 | 57.16 |
| Cold filter plugging Property (CFPP) (°C) | −20 to 5 | NA | −5.97 | −7.20 | 9.53 |
| Iodine value, (IV) (g I₂/100 g) | ≤120 | NA | 62.70 | 97.73 | 56.07 |
| Degree of unsaturation (DU) (%) | NA | NA | 65.77 | 82.85 | 52.86 |
| Pour point (PP) (°C) | NA | −15 to 10 | 1.97 | −0.32 | 33.51 |
| Cloud point (CP) (°C) | NA | −3 to 12 | 8.10 | 5.99 | 37.16 |

NA is not available.

(2014) found that C16-C18 fatty acids has the most beneficial for producing biodiesel. In addition, co-culture could accumulate up to 68.36% of PUFA which was 3.53 times higher than that in the microalga monoculture (G022) (Table 2). Higher levels of unsaturation are widely known to lead to lower melting points that are suitable to enhance the low-temperature performances of biodiesel (Srinuanpan et al., 2017) but a worse oxidative stability would be occurred (Srinuanpan et al., 2018). Changes in the fatty acid composition of co-culture might be caused by the expression of fatty acid biosynthesis–related genes in the microalgal–actinomycete consortium. Jusoh et al. (2015) highlighted that the gene expression, especially KAS I, SAD ω-6 FAD, and ω-3 FAD gene were consistent with production of both SFA and PUFA. Further investigations are necessary to acquire insight into the association between the expression of genes and the microbial lipid fatty acid profiles derived from actinomycete co-cultures with microalgae.

The fatty acid compositions were used to compute the biodiesel fuel characteristics (Table 3). The saponification value (SV), cetane number (CN), cold filter plugging point (CFPP), iodine value (IV), degree of unsaturation (DU), pour point (PP) and cloud point (CP) were found to be 142.47 mg KOH/g oil, 70.51, −5.97 °C, 62.70 g I₂/100 g, 65.77%, 1.97 °C, and 8.10 °C, respectively. These values ensure high stability against oxidation and good ignition quality with shorter time period (Srinuanpan et al., 2018). It is possible to conclude that the fuel qualities of co-culture lipid comply with the international biodiesel standards ASTM D6751 (USA) and EN 14214 (European Organization), suggesting their prospective usage as biodiesel feedstock.

## 4. Conclusion

This study highlighted that an innovative approach using an actinomycete co-culture with microalgae in the biogas digestate effluent (BDE) offers the potential use of microorganisms as a promising environmentally friendly feedstock of biodiesel for the industrialization of microbial-based biofuel that can be employed as a cost-effective strategy. The actinomycete *Piscicoccus intestinalis* WA3 co-culture with green microalga *Tetradesmus obliquus* AARL G022 in the BDE is effective not only in the increment of overall biomass production but also effectively enhances the overall lipid yield. More importantly, the fuel characteristics of the generated microbial biodiesel were in conformity with international requirements.

## CRediT authorship contribution statement

**Bancha Kumsiri:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Project administration, Writing - review & editing. **Jeeraporn Pekkoh:** Investigation, Resources, Writing - review & editing. **Wasu Pathom-aree:** Investigation, Resources, Formal analysis, Writing - review & editing. **Saisamorn Lumyong:** Investigation, Resources, Writing - review & editing. **Kittiya Phinyo:** Investigation, Writing - review & editing. **Chayakorn Pumas:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Supervision, Funding acquisition, Writing - review & editing. **Sirasit Srinuanpan:** Investigation, Formal analysis, Data curation, Visualization, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125446.

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