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Biodiesel from green alga *Scenedesmus acuminatus*

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Abstract: Renewable fuels for alternative energy sources have been paid a great attention in recent years. Biodiesel has been gaining worldwide popularity as an alternative energy source. The production of biofuels from microalgae, especially biodiesel, has gained huge popularity in the recent years, and it is assumed that, due to its eco-friendly and renewable nature, it can replace the need of fossil fuels. *Scenedesmus* genus was discussed by phycologists as promising microalgae for biofuel production based on its biomass and fatty acid productivity. In the present study, *S. acuminatus* was cultivated in piggery wastewater effluent to couple waste treatment with biodiesel production. The batch feeding operation by replacing 10% of algae culture with piggery wastewater effluent every day could provide a stable net biomass productivity of 3.24 g L^{-1} day^{-1}. The effect of acid hydrolysis of lipids from *S. acuminatus* on FAME (fatty acid methyl esters) production was investigated. Direct transesterification (a one-stage process) of the as harvested *S. acuminatus* biomass resulted in a higher bio-diesel yield content than that in a two-stage process. This study results revealed that it is feasible to produce biodiesel from wet microalgae biomass directly without the steps of drying and lipid extraction.

Keywords: Biodiesel, Fresh Water, *Scenedesmus acuminatus*, Piggery Wastewater Effluent

1. Introduction

Biofuel is a renewable energy, which may be instead of the fossil fuel resources in the future with decreasing of the fossil fuel on a daily basis. Biodiesel is a renewable fuel alternative for diesel engines [1]. It can be produced in any climate. Biodiesel is biodegradable, nontoxic and a low emission profiles, environmentally friendly biofuel, also contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than conventional diesel fuel [2–4]. Due to these merits, biodiesel fuel has received considerable awareness in recent years. Biodiesel is a mix of monoalkyl esters of long-chain fatty acids, obtained by chemical reaction (transesterification), coming from renewable feedstock such as vegetable oil or animal fats, and alcohol with a catalyst [5]. It is called the biodiesel fuel, which consists of the simple alkyl esters of fatty acids, is presently making the transition from a research topic and demonstration fuel to a marketed commodity. Various biomasses can be used to produce biodiesel. Traditional feedstock of biodiesel contains plant oils and animal fats [6,7]. Nevertheless, such raw materials may compete with food supply, increase the utilization of limited farmland, and require long time to harvest which is hard to satisfy the large and long-term global energy demand; the most commonly used are rapeseed, canola, corn, soybean, oil palm, coconut and soybean, but also other crops such as mustard, hemp and waste vegetable oil animal fats [8]. Even algae are promising source of biodiesel in nature, primarily to highlight the order-of-magnitude differences present in the oil yields from algae when compared with other oilseeds. For example, the Tallow tree could yield significantly higher quantities of oil than current crops, and microalgae offer the potential for triglyceride production rates some 200 times higher than terrestrial biomass [9]. Hence, microalgae are considered a promising alternative and a renewable feedstock source for biofuels.

In addition, microalgae are believed to be excellent candidates for fuel production because of their high photosynthetic efficiency, high growth rate, and high area-specific yield. Moreover, microalgae can be cultivated in saline/brackish water and on non-arable land; therefore, this precludes competition for the conventional crop land [10]. Consequently, microalgae have received more attention in the recent decades.

Biodiesel, which is produced from biomass by transesterification of triacylglycerols, is one of the most

prominent renewable energy sources [5]. Microalgae are emerging as one of the most promising resources of biodiesel, with a projected yield of 58,700 to 136,900 liter ha^{-1} $year^{-1}$ [9]. For microalgae cultivation, the huge consumption of water resources and inorganic nutrients is costly. Addition of organic carbon, though found highly stimulatory for microalgal growth, increases the feedstock cost. Thus, an economically acceptable and environmentally sustainable carbon source for alga-based biodiesel is currently needed. The use of micro-algae for biodiesel in itself is particularly attractive because it is up to 30 times [10], more efficient in producing oil for biodiesel compared to conventional methods, and micro-algae can be cultured in poor quality salty water or nutrient loaded water. Micro-algae are much more efficient converters of solar energy than any known terrestrial plant, because they grow in suspension where they have unlimited access to water and more efficient access to CO_2 and dissolved nutrients [11–14].

Continued only increasing use of petroleum will intensify local air pollution and magnify the global warming problems caused by CO_2 [14]. One of the most serious environmental problems today is that of global warming, caused primarily by the heavy use of fossil fuels. Photosynthetic microalgae are potential candidates for utilizing excessive amounts of CO_2 [13], since when cultivated these organisms are capable of fixing CO_2 to produce energy and chemical compounds upon exposure to sunlight [12]. The derivation of energy from algal biomass is an attractive concept in that unlike fossil fuels, algal biomass is rather uniformly distributed over much of the earth's surface [5]. In a particular case, such as the emission of pollutants in the closed environments of underground mines, biodiesel fuel has the potential to reduce the level of pollutants and the level of potential or probable carcinogens [4].

Biodiesel is a renewable fuel that is produced by chemically reacting algal oil with an alcohol such as methanol. The reaction requires a catalyst, usually a strong base, such as sodium or potassium hydroxide, and produces new chemical compounds called methyl esters. It is these esters that have come to be known as biodiesel. There are four primary ways to make biodiesel, direct use and blending, microemulsions, thermal cracking (pyrolysis) and transesterification. The most common way is transesterification as the biodiesel from transesterification can be used directly or as blends with diesel fuel in diesel engine [8–10].

One promising approach is to couple biodiesel production with wastewater treatment, as algae can be successfully cultivated in wastewaters. The algal cultivation process was focused on in this study for reducing the cost. Microalgae can be cultivated in wastewaters because they can utilize the nutrients contained in most wastewaters. By using wastewater as a nutrient source, the cost of nutrients and water in biodiesel production can be reduced, and also the quality of wastewater discarded after treatment can be improved simultaneously. Cultivation of microalgae in swine wastes, dairy manure, and other animal residues has been reported by several literatures [7,15,16]; by using *Scenedesmus* Sp., which is part of our ecosystem and is very accessible, a more environmental friendly and renewal fuel can be produced. In terms of oil production, of the published algal species, members of the *Scenedesmus* genus have been identified as potential oil-producing species, with both rapid growth, as well as relatively high lipid content [17, 18].

The present article reports simultaneous biodiesel production and waste recycling with the green microalga *S. acuminatus* isolated from the fresh water fish pond. During the study the alga was subjected and compared with different methods of transesterification for maximum FAME yield.

2. Materials and Methods

2.1. Microalgal Isolation, Purification and Identification

Algae samples were collected by plankton net (20-µm pore size) from freshwater fish pond (18° 55′4.2″N; 99° 0′41.1″E) at a location near Maejo University, Sansai, Thailand. The collected samples were samples of about 5 ml were inoculated into 5-ml autoclaved Bold Basal Medium (BBM) [19] in 20-ml test tubes and cultured at room temperature (25±1°C) under 67.50 ± 2 $\mu mol^{-1}m^2$ sec^{-1} intensity with 16:8 h photoperiod for 10 days. After incubation, individual colonies were picked and transferred to the same media for purification in 250 mL conical flask. The culture broth was shaken manually for five to six times a day. The pre-cultured samples were streaked on BBM medium-enriched agar plates and cultured for another 10 days with cool white fluorescent light using the same light intensity.

The single colonies on agar were picked up and cultured in liquid BBM medium, and the streaking and inoculation procedure was repeated until pure cultures were obtained. The purity of the culture was monitored by regular observation under microscope. The isolated microalgae were identified microscopically using light microscope with standard manual for algae [20, 21]. Green microalga *S. acuminatus* structure shown in figure 1.

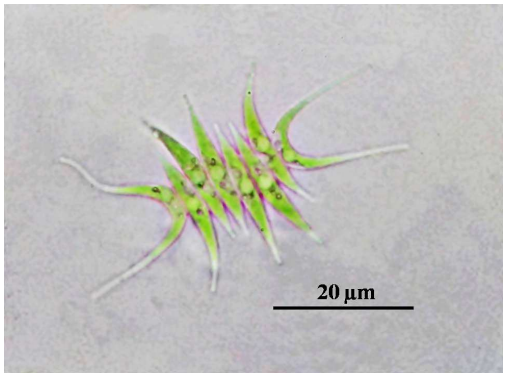

Figure 1. *Scenedesmus acuminatus.*

2.2. Inoculums Preparation

Isolated and purified microalgae were inoculated in 250-ml Erlenmeyer flasks containing 125 ml culture medium (BBM).

Flasks were placed on a reciprocating shaker at 120 rpm for 7 d at room temperature of 25±1 °C. Light was provided by cool white fluorescent lamps at an intensity of 37.5 $\mu mol^{-1}m^2$ sec^{-1}. The inoculums were then transferred to 1000-ml Erlenmeyer flasks (photo-bioreactor). All experiments were carried out in triplicate.

2.3. Medium Preparation

The raw piggery wastewater effluent was collected from the Faculty of Animal Science and Technology (18°54'55.36"N; 99° 1'6.61"E), Maejo university near the laboratory was used as a substrate to cultivate *S. acuminatus*. Pre-treatment was carried out by sedimentation and filtration with a filter cloth to remove large, non-soluble particulate solids. After filtration the substrate was autoclaved for 20 min at 121°C, after which the liquid was stored at 4 °C for 2 days for settling any visible particulate solids and the supernatant was used for microalgae growth studies. The characteristics and features of the autoclaved wastewater are summarized in Table 1.

Table 1. *Characteristics of autoclaved piggery wastewater effluent (means ± SD).*

Parameter	Autoclaved
pH	7 ± 0.0
COD (mg L^{-1})	3200± 63
TN (mg L^{-1})	748.0 ± 3.0
TP (mg L^{-1})	128 ± 43
Suspended solid (mg L^{-1})	288 ± 43

2.4. Photo-Bioreactor Set Up

The standard reactor of continuous stirred tank reactor (CSTR) was used. The batch-fed algal cultures were grown in photo-bioreactor (CSTR), cultured at room temperature (25±1°C) under 67.50 ± 2 $\mu mol^{-1}m^2$ sec^{-1} intensity with 16:8 h photoperiod. The triplicate reactors were operated at 10 days detention time and other operational factors were list in Table 2 and methods were presented in Table 3.

Table 2. *Operational parameters*

Operational parameter	Photo-bioreactor
Scale	Lab
Detention time	10 days
Reactor design	1L up-flow flask
Water volume	1L
Feeding	Batch feed daily
Filter size	0.45 µm
Mixing speed	Magnetic mixer
Light intensity	67.05 ± 2 µmol s^{-1} m^{-2}
Operation period	30 days

2.5. Analytical Methods

All the indices including pH, chemical oxygen demand (COD), total nitrogen (TN), total phosphorous (TP) and algal biomass of total suspended solids were continuously monitored throughout the study, following the standard protocols of APHA [20].

Table 3. *Environmental and algal biomass measurements*

Parameter	Equipment or method
Light intensity	LI-COR light meter (LI-250)
Water temperature	Thermometer
Settleable solid	Imhoff cone
Species	Microscope
Operation period	30 days
pH	Method 423 (Standard Methods)
COD	Method 508B (Standard Methods)
NH_4^+-N	Method 417D (Standard Methods)
TKN	Method 420A (Standard Methods)
NO_2^--N	Method 419 (Standard Methods)
NO_3^--N	Method 418A (Standard Methods)
TN	= Total of N species
TP	Method 424D (Standard Methods)
TSS	Method 209 (Standard Methods)

2.6. Nutrient Removal

Every day 50 mL microalgae culture was decanted from the reactors then by centrifugation at 4000 rpm, 10°C for 10 minutes and filtration by 0.45 µm membranes. After these preprocessing, the supernatant was used to monitor the concentration of NO_3-N, NH_4-N, TN and TP following the standard testing methods [20]. The removal efficiency can be calculated following formula:

$$\text{Removal efficiency} = (C_i - C_0)/C_0 * 100\%$$

where C_o and C_i are defined as the mean values of nutrient concentration at initial time t_0 and time t_i, respectively..

2.6. Lipid Extraction

Total lipids from 100 mg microalgae were extracted using 2mL chloroform/methanol (v/v: 2/1) [21], ultrasonic treatment for 10 min and centrifugation at 4000 rpm for 5 min. The supernatants were then collected into pre-weighted centrifuge tubes. This process was repeated three times. The collected supernatants were dried under nitrogen flow and then at 60°C until the weight of samples remained constant.

2.7. Fatty acid Methyl Ester (FAME) Content Analysis and Transesterification

Biodiesel samples were analyzed quantitatively and qualitatively to determine the biodiesel yield and FAME composition. The samples were weighed and moved to 10-mL flasks. Then, 5 mL H_2SO_4–methanol (v/v H_2SO_4/methanol) was added, and the flask was stirred at a specific temperature for a specific amount of time with refluxing. After the specific time period, the flask was cooled to room temperature. Next, 2 mL of hexane and 0.75 mL of distilled water were added to the flask and mixed for 30 s on a vortex mixer. The mixture formed two phases, and the upper hexane layer contained the fatty acid methyl esters (FAMEs). The hexane layer was

transferred to a new vial and mixed with the internal standard C17-ME for analysis by gas chromatography (GC). The fatty acid methyl esters (FAME) were then extracted with hexane and analyzed by GC-MS as described by Thomæus et al. [22]. FAME analysis was performed using GC-MS (Agilent 6890 -HP5973 model, Australia). All the measurements of the values used in the tables and figures represent the average ± SD of four individual replicates during the whole experiment.

3. Results and Discussion

3.1. Microalgal Growth in Piggery Wastewater Effluent Medium

Algal growth is directly affected by the availability of nutrients and light, the pH stability, temperature, and the initial inoculum density [11,13]. Microalgae can grow an abundantly under suitable conditions and with sufficient nutrients. They often double their biomass within 3.5 h or, at the longest, 24 h during their exponential growth phase. The green microalga, *S. acuminatus* was chosen for the study because, it was found to be dominant among other algal species in their natural environment. Instead of huge differences in the climatic conditions of the places where collection has been done and the place where all the experimental work was carried out, the microalga showed luxurious growth, which reveals its flexible nature to adapt the wide range of the environmental condition. There were several indexes for algal biomass measurement and roughly we could classify into two groups, (1) direct index such as dry weight and (2) indirect index such as chlorophyll, so-called proxy index [11–14]. According to Ramaraj et al. [12], TSS was applied to be an index of algal biomass measured in this study. The average biomass was 3.24 and ranged 3.07–3.42 g/L (Table 4).

Culturing of microalgae in wastewater also substantially reduces the need of chemical fertilizers and their related burden on life cycle. Through the utilization of wastewater, the zero-waste concept is further implemented, and thus stimulates a more sustainable practice for the microalgae biofuel industry. It has even been proposed that integrated phyco-remediation and biofuel technology appears to be the only source of sustainable production of biofuels [15, 16, 23].

Table 4. *Algal biomass and lipid measurements*

Parameter	Average	Minimum	Maximum
Total biomass (g/L)	3.24	3.07	3.42
Lipid production (mg/L)	710.11	543.5	844
Productivity of lipid (mg/L/d)	71.01	54.35	84.4

3.2. Characterization of Piggery Wastewater Effluent and Nutrient Removal Efficiency of S. scuminatus

Growing algae depends on the availability of principal nutrients like nitrogen, phosphorus, carbon, sulphur and micronutrients including silica, calcium, magnesium, potassium, iron, manganese, sulphur, zinc, copper, and cobalt. Algal cells have the capability to uptake nitrogen and phosphorus from water. Nitrogen and phosphorus are the two important nutrient compounds to analyze a water source for potential algae growth [23, 24]. Many treatments of piggery wastewater by microalgae have been investigated, as a means of providing environmental protection from and recovery of nutrients. Neglecting the cost, processing of piggery wastewater by microalgae with the simultaneous production of oil would seem to be a good choice. The removal of nitrogen, phosphorus, calcium and inorganic carbon from piggery wastewater by microalgae cultivation as a function of incubation time has been studied [23, 24].

The algal species such as *C. mexicana*, *M. reisseri*, *C. vulgaris*, *N. pusilla*, *S. Obliquus*, and *O. multisporus* shown the maximum nitrogen, phosphorus and inorganic removal (62%, 28%, and 29%) were obtained with C. mexicana, respectively, while the maximum calcium removal (71%) was obtained with *C. vulgaris*. The lowest nitrogen, phosphorus and inorganic carbon removal (8%, 3%, and 1.3%) were obtained with *M. reisseri* after 20 days of cultivation [23].

Table 5. *Nutrient removal Piggery wastewater and effluent*

Microalgal species	Wastewater type	Nitrogen	Phosphorus	Reference
C. Mexicana	Piggery wastewater	62%	28%	23
S. obliquus	Piggery effluent	41%	59%	24
S. acuminatus	Piggery effluent	75%	88%	This study

The nutrient removal efficiency of *S. acuminatus* was analyzed in this study and results shown in Table 5. Microalgae can be efficiently used to remove significant amount of nutrients because they need high amounts of nitrogen and phosphorus for protein (45–60% of microalgae dry weight), nucleic acid, and phospholipid synthesis [16]. The nitrogen in sewage effluent arises primarily from metabolic interconversions of extra derived compounds, whereas 50% or more of phosphorus arises from synthetic detergents [23]. Consequently, this study results demonstrated that *S. acuminatus* was highly utilized the macronutrients from the piggery in wastewater effluent medium.

3.3. Lipid Yield and Fatty acid Methyl Ester of S. Acuminatus

Algae grown on wastewater media are a potential source of low-cost lipids for production of liquid biofuels. This study investigated lipid productivity and nutrient removal by green algae grown during treatment of wastewaters effluent without supplemented CO_2. The lipid productivity and lipid content were presented in Table 4. The major fatty acid composition of

each isolate was determined using GC analysis. Table 6 shows the fatty acids (FA) profile of *S. abundans* grown under large scale cultivation using indigenously made photobioreactor. The FA profile of alga was determined by the quantification of FAME content which reveals the abundance of FA with carbon chain length of C16 and C18. Oleate (C18:1), palmitate (C16:0), linolenate (C18:3), linoleate (C18:2), palmitoleate (C16:1) and stearate (C18:0) were contributing over 90% of the total FAME content. The properties of biodiesel are highly influenced by the FA profile of the algae.

Table 6. *Fatty acid methyl ester profile of S. acuminatus*

Fatty acids	Contents (% of total fatty acids)
Capric acid (C10:0)	0.13
Myristic acid (C14:0)	1.87
Pentadecylic acid (C15:0)	0.53
Palmitic acid (C16:0)	19.34
Palmitoleic acid (C16:1)	11.73
Hexadecadienoic acid (C16:2)	3.81
Strearic acid (C18:0)	19.55
Oleic acid (C18:1)	23.2
Linoleic acid (C18:2)	9.75
Linolenic acid (C18:3)	4.95
Others	5.1
Total saturated fatty acids	34.45
Total unsaturated fatty acids	54.65
Unsaturated/saturated fatty acid ratio	1.55

3.4. Algal Biodiesel Production

Algal biodiesel production consists, primarily of five steps. They are: (a) algae production; (b) algae harvesting; (c) oil extraction; (d) transesterification or chemical treatment; and (e) separation and purification [25]. The oil extraction step includes cell disruption by mechanical, chemical, or biological methods and oil collection by solvent. Major bottlenecks of oil extraction are that the extraction of internal oils is energetically demanding because the cell walls of some species of microalgae are strong and thick and that the oil extraction yield is negatively affected in case of a wet biomass [26]. Extracted microalgal oils are typically converted to biodiesel by transesterification using alcohols and catalysts.

Recently, the combination of oil extraction and biodiesel conversion, called direct (in-situ) transesterification has been studied. Direct transesterification refers to the conversion of algal oils present in biomass to biodiesel. Here, direct transesterification includes both the esterification of free fatty acid and the transesterification of triglyceride from microalgae. This process simplifies the production process and improves the biodiesel yield compared with conventional extraction because of the elimination of an oil extraction step that incurs oil loss. The reactions are simple and comprise the addition of alcohols, catalysts, and biomass and sometimes co-solvents [27]. So far, direct transesterification was carried out with chemical catalysts; lesser reaction time and high yields are the advantages of the chemical method of direct transesterification, though, high energy requirements, difficulties in the recovery of the catalysts and glycerol, and pollution to the environment by these catalysts, are the major disadvantages of this process. Accordingly, the direct transesterification of the oleaginous biomass resulted in a higher biodiesel yield and fatty acid methyl ester (FAME) content than the extraction−transesterification method.

3.5. Analysis of S. acuminatus Biodiesel

Biodiesel consists largely of fatty acid methyl esters, which are produced by the transesterification of biologically-derived lipids [31], and the quality of biodiesel is considerably affected by the composition of the fatty acids in the biodiesel. In Table demonstrated that the palmitic, stearic, oleic, linoleic and linolenic acid were recognized as the most common fatty acids in biodiesel. In addition, the fatty acids profiles of the isolates indicated the presences of lauric (C12:0), myristic (C14:0), palmitic (C16:0), heptadecanoic (C17:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1n9c), α-linolenic (C18:3n3), and γ-linolenic acid (C18:3n6). For the green alga *Scenedesmus*, the fatty acid compositions of 14:0, 16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, α-18-3 have been confirmed under many conditions including photoautotrophic and heterotrophic cultivation, nitrogen starvation, and outdoors in a photobioreactor. Biodiesel fuels enriched in methyl oleate are desirable, relatively small percentages of saturated fatty esters can wreck the cold flow properties of biodiesel. Our finding shows that most of these strains contain 34% of saturated fatty acids (C16 and C18). Among the tested microalgal species, *S. acuminatus* showed the highest oleic acid content, making it the most suitable isolate for the production of good quality biodiesel. Moreover, *S. acuminatus* grown in piggery wastewater effluent showed a rise in palmitic acid content, which is desirable for good-quality biodiesel.

4. Conclusions

Microalgae, *Scenedesmus acuminatus* was feed batch -cultured in a photo-bioreactor to facilitate better culture control and higher biomass productivity. A one-step direct transesterification of microalgal cells was successfully performed which has great significance for fatty acid composition analysis of micro-scale samples in applications such as strain screening. Moreover, this method can be used for direct transesterification of microalgal cells without dehydration beyond centrifugation. Direct transesterification of microalga paste greatly simplifies the process of fatty acid analysis while completely eliminating the drying and oil extraction steps, and thus, the developed method has great potential for a variety of applications. The results of this study indicate that the naturally isolated microalgal strain *S. acuminatus* may be a valuable candidate for biodiesel production.

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