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In Situ Enzymatic Conversion of *Nannochloropsis oceanica* IMET1 Biomass into Fatty Acid Methyl Esters

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Abstract Conventionally, production of methyl ester fuels from microalgae occurs through an energy-intensive two-step chemical extraction and transesterification process. To improve the energy efficiency, we performed in situ enzymatic conversion of whole algae biomass from an oleaginous heterokont microalga *Nannochloropsis oceanica* IMET1 with the immobilized lipase from *Candida antarctica*. The fatty acid methyl ester yield reached 107.7% for dry *Nannochloropsis* biomass at biomass to *t*-butanol to methanol weight ratio of 1:2:0.5 and a reaction time of 12 h at 25 °C, representing the first report of efficient whole algae biomass conversion into fatty acid methyl esters at room temperature. Different forms of algal biomass including wet *Nannochloropsis* biomass were tested. The maximum yield of wet biomass was 81.5%. Enzyme activity remained higher than 95% after 55 days of treatment (equal to 110 cycles of reaction) under the conditions optimized for dry algae biomass conversion. The low reaction temperature, high enzyme stability, and high yield from this study indicate in situ enzymatic conversion of dry algae biomass may potentially be used as an energy-efficient method for algal methyl ester fuel production while allowing co-product recovery.

Keywords Biofuel · Biomass · In situ enzymatic conversion · Fatty acid methyl ester · Lipase · *Nannochloropsis oceanica*

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Introduction

Biodiesel, composed primarily of fatty acid methyl esters (FAMEs), is widely accepted as an alternative energy source to replace fossil fuels. Oils from food sources have been transesterified to produce FAMEs, which are not sustainable or economically feasible [1]. In recent years, microalgal biomass has emerged as a promising feedstock for FAME production owing to their fast growth rate, high lipid content, and ability to grow with non-arable land and non-potable water [2, 3]. Generally, algae biodiesel is produced through a two-step process: solvent-based chemical extraction of lipids from algal biomass followed by transesterification of extracted lipids into FAMEs (see Fig. 1), catalyzed by an alkali or an acid at 60–80 °C in the presence of methanol [4, 5].

High energy input and low biomass productivity are two primary bottlenecks for algae biodiesel production [1, 6]. The energy requirement for drying, extraction, and conversion processes accounts for 10.7, 14.5, and 14.8%, respectively, of the total energy consumption for algae biodiesel production [7]. In the two-step oil extraction and conversion process, there are some inevitable

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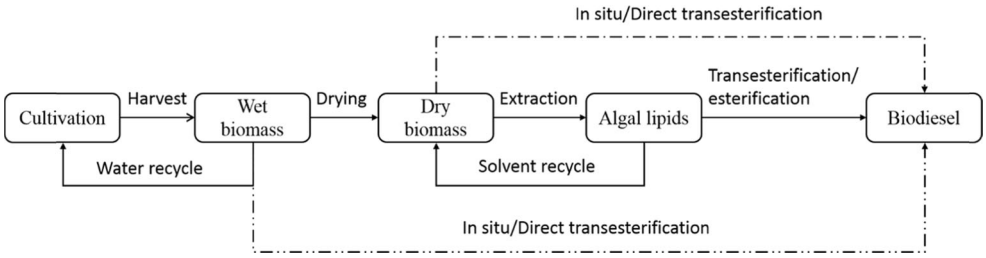

Fig. 1 Diagram of FAME production routes from algal biomass. Possible routes to eliminate drying and/or oil extraction steps by in situ wet and dry algae biomass conversion were depicted by *dashed lines*

losses of oils during lipid extraction, separation, and purification processes, reducing the overall yield [4, 5]. In order to reduce the energy input associated with the lipid extraction process and minimize lipid loss, in situ transesterification of algal biomass was proposed to streamline the production process (See Fig. 1). Several previous studies have attempted to transesterify dry algal biomass in situ [8–11]. However, pretreatment methods such as sonication are required and thus the conversion processes is still energy intensive [8, 9, 12, 13]. To convert wet algal biomass into biocrude, supercritical and thermochemical liquefaction techniques were used [10, 11, 14]. Despite their advantages, supercritical and thermochemical liquefaction techniques operate under high temperature and high pressure, which were considered cost-prohibitive to scale-up [15].

Enzymes are alternatives to chemical catalysts for lipid transesterification reaction. The enzymatic conversion process is less energy intensive and more environmentally benign compared with chemical conversion since it usually requires less heating and generates less wastewater [4, 16–19]. However, previous reports of enzymatic conversion of algal feedstock still required moderate heating at 35–45 °C [18, 19]. Moreover, in situ conversion experiments on algal feedstocks often involved physical means of cell disruption like sonication or microwave [8, 9]. Such processes are energy intensive and the high reaction temperature may lead to oxygenation or degradation of high-value products that may otherwise be separated or produced after the conversion process [4].

Previously, an enzymatic platform was established to convert lipids from *Nannochloropsis oceanica* IMET1, a widely employed strain for FAME and eicosapentaenoic acid production [4, 20, 21]. In this study, we achieved in situ enzymatic conversion of a dry or wet form of whole *Nannochloropsis* biomass without pretreatment. The biomass to co-solvent ratio, methanol ratio, temperature, and reaction time were optimized for FAME production, and the enzyme stability under optimized reaction conditions was also investigated. This work represents a novel way to enzymatic convert whole algal biomass at room temperature, thus eliminating any oxygenation or degradation problems associated with heating while minimizing energy input.

Materials and Methods

Strains and Culture Conditions

N. oceanica IMET1 was maintained in the Aquaculture Research Center of the Institute of Marine and Environmental Technology, University of Maryland, USA. Biomass of *N. oceanica* IMET1 was harvested by centrifugation (5000*g*, 10 min) after 6 days of cultivation in nitrogen-depleted f/2 medium as previously described [4]. *Scenedesmus* sp. HTB1 was isolated from the Chesapeake Bay by Dr. Feng Chen at the University of Maryland, which has been used as a robust strain for carbon sequestration and biofuel production. These strains were grown in BG-11 culture medium under continuous light illumination of 45 μE m^{-2} s^{-1} at 26 °C, with aeration by compressed air mixed with 1.5% CO_2.

Lipid Content of the Algal Biomass

Wet algal biomass was freeze-dried and the total lipids (crude algal oils) were extracted from dry algal biomass as previously described [4]. Briefly, algal biomass was extracted by methanol/dimethyl sulfoxide (DMSO) (9:1 volume to volume ratio) for 30 min, followed by extraction with hexane/ether (1:1 volume ratio) for 30 min. Supernatants from the two extraction steps were collected and combined, and then an equal amount of water was added into the extracts (water/methanol/hexane/ether, 1:1:1:1 volume ratio). The lipid-containing organic layer was collected and the solvents in the extracts were evaporated under a stream of nitrogen. The solvent-extractable material was weighed to determine the lipid content of the algal biomass.

In Situ Enzymatic Conversions of Algal Biomass into FAMEs

Enzymatic conversion was performed in glass vials with polytetrafluoroethylene caps on an incubator shaker (Innova 4080, New Brunswick Scientific, Enfield, CT) at 200*g*. Novozyme 435 (N435, the immobilized lipase from *Candida antarctica*, >5000 U g^{-1} Sigma-Aldrich Co., St. Louis, MO) was used for lipid conversion as previously

described [4]. Wet biomass or dry biomass was first added into a reaction vial, followed by *t*-butanol and methanol. N435 (about 2 mg or 10–20% weight of total lipids, which was the minimum amount that could be accurately weighed under our experimental conditions) was then added into the mixture. We used relatively large percentages of N435 because of the scale (mg scale) of our experiment, which could be reduced drastically in scale-up experiments. When the reaction was completed, chloroform was added to stop the reaction. Then, methanol and 0.75% KCl (chloroform/methanol/0.75% KCl, 8:4:3 volume ratio) were used to stratify the mixture into three layers. Chloroform layer containing FAMEs was collected and the solvent was evaporated in a nitrogen gas stream. For further analysis, FAMEs were dissolved again in chloroform and separated by thin-layer chromatography (TLC). The molar ratio of triacylglycerol (TAG) to methanol was calculated using a separate experiment, in which an equal amount of algae biomass was solvent-extracted and TLC-quantified.

Chemical Conversions of Crude Algal Oil into FAMEs

To compare our in situ conversion method with the traditional two-step chemical extraction and conversion, 15 mg algal oils (adjusted according to the lipid content of different algae samples) were converted chemically into FAMEs and used as the control assuming 100% efficiency. Crude algal oils were converted with 1 mL methanol and 1% H_2SO_4 at 85 °C for 2.5 h (methanol to oil molar ratio was about 500:1 to guarantee a complete reaction). The vial was shaken every 30 min. Then, the mixture was extracted with chloroform and 0.75% KCl (chloroform/methanol/0.75% KCl, volumetric ratio of 8:4:3). The chloroform layer containing FAMEs was separated and the solvent was then evaporated in nitrogen gas stream.

Determination of the FAME Yield

FAMEs were separated by TLC as previously described [22, 23]. Equal amounts of samples and controls were loaded onto the silica gel 60 F_{254} plates (EMD Millipore, Billerica, MA) and developed in a hexane/t-butyl methyl ether/acetic acid (80:20:2, volume ratio) solvent system. Besides, 50 μg of FAME standard (Sigma) was loaded on each TLC plate as an internal control. For lipid visualization, the developed TLC plates were sprayed uniformly with 8% H_3PO_4 containing 10% copper (II) sulfate pentahydrate, air dried, and then baked at 180 °C for 3 min. The yield was calculated using densitometry and an image analysis software (ImageJ2x, National Institutes of Health, USA). Similar values were obtained by gas chromatography–mass spectrometry quantification, indicating the reliability of this method. The product yield is calculated as: Yield $= \frac{As}{Ac}\frac{Oc}{Os} \times 100\%$, As = densitometry area value of the sample, Ac = densitometry area value of the internal standard, Oc = the amount (weight) of the internal standard loaded, and Os = the amount of the sample loaded.

Microwave Irradiation to HTB1 Cells

A Frigidaire 900 W microwave was utilized for the microwave irradiation. It was equipped with a slide holder (Lyse-it™ LLC, MD) and a standard Lyse-it™ slide prepared with an adhesive isolator (Grace BioLabs). One milliliter cell suspension in distilled water containing 10^9 HTB1 cells was added to a standard Lyse-it™ slide where the microwave was applied to. Two microwave powers (30% power or 40% power) with a constant 60-s irradiation time were tested. In a 60-s irradiation, 30% power (270 W) and 40% power (360 W) provide 16.2 and 21.6 kJ, respectively. After irradiation, the sample (lysate) was removed from the isolator and collected in a 50-mL conical tube. An additional 1 mL distilled water was added to the isolator slide to collect any remaining lysate. A total of 10 trials were performed in the same manner in order to collect sufficient lysate. The lysate was centrifuged at 14,000*g* for 10 min to separate the pellet (algal biomass) and the supernatant. The supernatant was discarded, and the pellet was freeze-dried before being used for in situ enzymatic conversion.

Data Analysis

All the data represented mean ± standard deviation (SD) of triplicate experiments and significant differences were determined by the *t* test using EXCEL where necessary. A significant difference was defined where *P* value was lower than 0.05.

Results and Discussion

In Situ Enzymatic Conversions of Dry Algal Biomass into FAMEs

Lipid Production in N. oceanica IMET1

To induce TAG production, *N. oceanica* IMET1 was cultivated under N-depleted conditions. As shown in Fig. 2(A), under N-deprivation, the cells grew slower than the control under the N-replete conditions. At day 6, the biomass yield was only half of that under the N-replete conditions. However, under N-deprivation, *Nannochloropsis* TAG content was about four-fold higher than that under N-replete conditions (Fig. 2(B)). TAGs are an ideal feedstock for FAME production [2], and TAG-rich *Nannochloropsis* lipids from N-deprivation conditions were chosen in the following experiments.

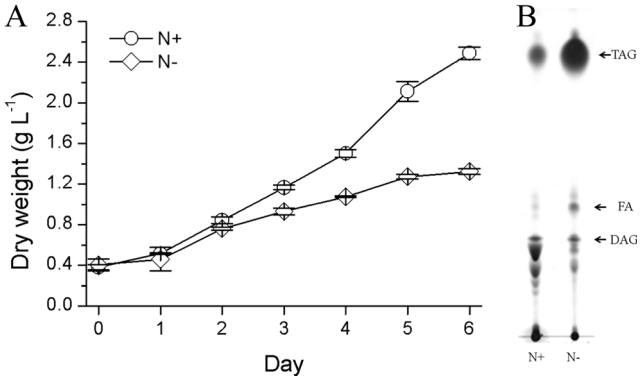

Fig. 2 Growth and lipid production of *N. oceanica* IMET1. *A* cell dry weight, *B* lipid profiles including TAG and fatty acid content as visualized by TLC. N− Oil: lipids from cells grown under N-depleted conditions. *Nannochloropsis* cultures were maintained under continuous light illumination of 105 μE m^{-2} s^{-1} at 26 °C and aerated by air mixed with 1.5% CO_2 (volume ratio), and 45 μg of total lipids were loaded onto a TLC plate. Data represented mean ± SD of triplicate experiments

Impact of Co-solvent and Methanol

Extraction and conversion of lipids need to be conducted in one step for direct conversion of algal biomass. Previous research indicates that *t*-butanol can improve the conversion efficiency of algal lipids into FAMEs, whereas Novozyme 435 (N435), the immobilized lipase from *C. antarctica*, was the best enzyme for conversion of algal oils [4]. In our experiments, no butyl ester was detected, indicating that *t*-butanol did not react with lipids under our experimental conditions, in agreement with previous studies using *t*-butanol in transesterification reactions [24–26]. Therefore, *t*-butanol and N435 were used in our reaction system. We tested different dry *N. oceanica* IMET1 biomass to *t*-butanol weight ratios in order to find the optimal co-solvent ratio. As shown in Fig. 3a, the yield increased significantly as the biomass to *t*-butanol ratio changed from 1:1 to 1:2 (*t* test, $P = 0.001$). Further increase in the *t*-butanol input led to a decrease in the yield, possibly due to a dilution effect as previously reported [4]. We also noticed that the biomass to *t*-butanol ratio of 1:2 was the minimum volume to completely submerse the algae biomass used in our experiment. Thus, this ratio was subsequently chosen in the following experiments. It is noteworthy that *t*-butanol may be recovered from FAMEs by distillation according to their different boiling points. We then tested the ratio of methanol in the reaction. Methanol is a substrate in the transesterification reaction but a high concentration of methanol in the reaction may inactivate enzymes and reduce the FAME yield [27]. As shown in Fig. 3b, the yield increased significantly with the biomass to methanol weight ratio changed from 1:0.05 to 1:0.5 ($P = 0.043$). Changing biomass to methanol ratio further to 1:2 or 1:5 did not significantly impact ($P = 0.21$) the yield. We determined the biomass to methanol ratio of 1:0.5 was optimal for the following experiments.

Effect of Temperature

To determine the optimal reaction temperature for in situ enzymatic conversion of dry *N. oceanica* IMET1 biomass into FAMEs, we carried out the experiment with the biomass to *t*-butanol and methanol ratio of 1:2:0.5 at 25, 35, 45, and 55 °C for 16 h (Fig. 3c). We found the FAME yield increased significantly from 99.3% at 25 °C to 113.2% at 55 °C ($P = 0.0027$), possibly resulting from greater lipid extraction efficiency of algal biomass at a higher temperature. It was unlikely due to a higher yield of algal lipids at higher temperatures, since no significant impact was found on the reaction temperature ranging from 25 to 55 °C during enzymatic conversion of *N. oceanica* IMET1 lipids [4]. To compare the yield of our in situ method with the traditional two-step chemical extraction and conversion process, the latter was included as the control, which was set as 100% yield. There was some inevitable loss of lipids in the control during the lipid extraction, separation, and purification processes. In contrast, in situ conversion of algal biomass avoided any loss of lipids with simultaneous oil extraction and conversion, thus our FAME yield exceeded 100%. In consistence, previous reports [28–30] indicated that FAME yield of in situ conversion was higher than conventional two-step oil extraction and transesterification processes. We achieved a high yield of 99.3% at room temperature (25 °C). Although slightly lower than that at 55 °C, it saved energy for heating as a tradeoff of reaction time and avoided possible oxygenation of high-value natural products under higher temperature [31, 32]. We thus chose room temperature in the following experiments.

Reaction Time

We determined the effect of reaction time on conversion efficiencies. At the biomass to methanol weight ratio of 1:0.5 (equal to TAG to methanol molar ratio of 1:30), the yield increased to 99.2% at 8 h and reached a maximum of 107.7% at 12 h; no significant difference was observed between 12 and 24 h ($P = 0.68$) (Fig. 3d). Compared to a chemical process which usually takes 0.5–2 h, the enzymatic reaction time was longer and subsequently raised the concern of increasing CAPEX. However, since enzymatic conversion does not require heating, less energy and cost is thought to be needed as compared with chemical conversion. This remains to be tested in future scale-up trials.

In previous algal oil conversion experiments at high TAG to methanol molar ratios, significant inhibition of enzyme activity was often observed [4]. We did not observe such inhibition at TAG to methanol molar ratio of 1:30 likely because N435 was more stable at room temperature under our experimental conditions; by contrast, previous reports used higher temperature that could lead to enzyme inhibition (see Table 1). Moreover, the methanol used in our in situ conversion reaction

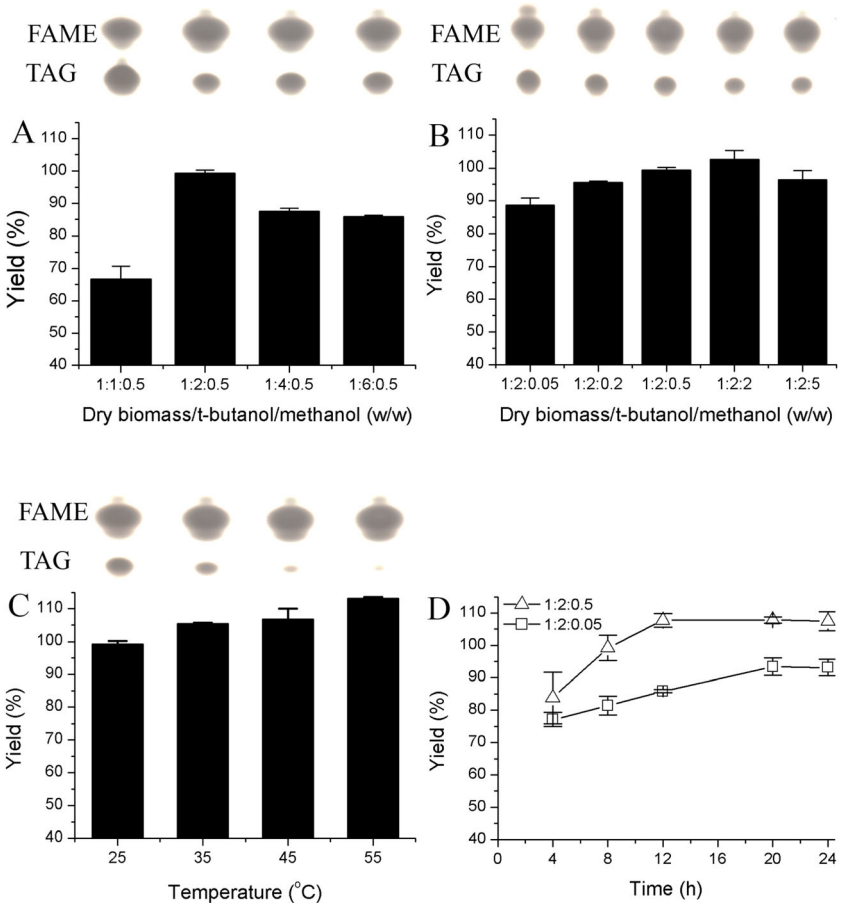

Fig. 3 Optimization of in situ enzymatic conversion conditions for dry *N. oceanica* IMET1 biomass into FAMEs. **a** Effect of *t*-butanol ratio on yield; reaction conditions were biomass to methanol ratio of 1:1 with varying *t*-butanol ratios at 25 °C for 16 h. **b** Effect of methanol ratio on FAME yield; reaction conditions were biomass to *t*-butanol ratio of 1:2 with varying methanol ratios at 25 °C for 16 h. **c** Effect of temperature on FAME yield; reaction conditions were biomass to *t*-butanol to methanol ratio of 1:2:0.5 for 16 h. **d** Time course of dry biomass conversion with two different methanol ratios. Biomass to *t*-butanol to methanol weight ratios were described in (**d**). Lipid content of *N. oceanica* IMET1 biomass was 46.3%. Chemical conversion of extractable lipid from *N. oceanica* IMET1 was included as a control and assumed a yield of 100%. Data represented mean ± SD of triplicate experiments

may largely be absorbed by the algal biomass for lipid extraction and conversion, thus has limited access to N435. We also tested the biomass to methanol weight ratio of 1:0.05 (equal to the TAG to methanol molar ratio of 1:3 and is the theoretic minimum level for complete transesterification of TAG [33]). With the considerably reduced amount of methanol, the maximum conversion still reached 93.4% at 20 h. For future scale-up experiments where overall cost reduction is the priority, we could use these conditions to lower the methanol ratio. In summary, the optimized reaction conditions for the conversion of dry algal biomass were biomass to *t*-butanol to methanol weight ratio of 1:2:0.5, and 12 h reaction at 25 °C.

In Situ Enzymatic Conversions of Wet Algal Biomass into FAMEs

Effects of Co-solvent and Methanol

It was estimated that for algal FAME production, about 11% of energy may be used for the drying process [7]. To test if the biomass drying process can be eliminated, wet *N. oceanica* IMET1 biomass obtained after centrifugation (with a solid content of 39.4%) was used directly as the substrate for enzymatic conversion. FAME yield increased significantly by adding *t*-butanol as a co-solvent and reached a maximum level of 73% at a wet biomass to *t*-butanol weight ratio of 1:1 at 12 h. As for methanol, the yield increased by changing the wet biomass to methanol ratio of 1:0.25 to 1:2. Further increase in methanol ratio to 1:4 ($P = 0.51$) did not result in further improvement in the yield (Fig. 4b). Thus, biomass to methanol ratio of 1:2 was chosen for the following experiments.

Effects of Temperature and Reaction Time

The effect of temperature on enzymatic conversion of wet algae biomass into FAMEs was investigated. A maximum yield of 65.2% was obtained when the reaction temperature was at 45 °C (Fig. 4c). However, when temperature increased further to 55 °C, the yield dropped significantly, possibly due to heat inactivation of the enzyme. Therefore, 45 °C was chosen in the following experiments. After optimization of the methanol to *t*-butanol ratios, a maximum yield of 81.5% was attained at 16 h with the biomass to *t*-butanol to methanol weight ratio of 1:1:2 at 45 °C (Fig. 4d, no difference between 16 and 20 h, $P = 0.49$). The yield of wet *Nannochloropsis* biomass was lower than that of the dry biomass, though the methanol input and reaction temperature were higher. This is in line with previous reports showing the presence of water in

Table 1 Comparison of in situ conversion of dry biomass into FAME

Feedstock	Lipid[a]	Yield[b]	Methanol[c]	Catalyst	Temp. (°C)	Time (h)	Co-solvent[d]	References
Chaetoceros gracilis	44	82	15.8	1.8 v% H_2SO_4	80	0.33[e]	–	[8]
Chlorella pyrenoidosa	21	~40	0.78	20 wt% H_2SO_4	100	4	–	[45]
Chlorella pyrenoidosa	56.3	95	3.16	0.5 M H_2SO_4	90	2	3.95 C_6H_{14}	[46]
Chlorella sorokiniana	23.5	77	15.8	1.8 v% H_2SO_4	80	0.33[e]	–	[8]
Chlorella sp.	27.6	~90	3.16	27.6 wt% H_2SO_4	60	4	–	[47]
Commercial algal biomass	20.9	98	4.43	1.68 M H_2SO_4	65	2	–	[48]
Lipomyces starkeyi (yeast)	50.2	96.8	15.8	0.2 M H_2SO_4	70	20	–	[49]
Mixed culture from wastewater lagoon	14.4	74	15.8	1.8 v% H_2SO_4	80	0.33[e]	–	[8]
Mortierella isabellina (fungi)	53.2	91	15.8	0.2 M H_2SO_4	70	20	–	[49]
Mucor circinelloides (fungi)	22.9	~82.5	N/A[f]	HCl or H_2SO_4	65	8	0.1 $CHCl_3$[g]	[50]
Nannochloropsis oceanica IMET1	46.3	107.7	0.5	N435	25	12	2 *t*-butanol	Current work
Nannochloropsis salina	50–55	~80	11.9	3 wt% KOH	N/A[e]	0.17[e]	–	[9]
Rhodosporidium toruloides (yeast)	58	98.1	15.8	0.2 M H_2SO_4	70	20	–	[49]
Schizochytrium limacinum	51	72.8	2.69	7.5 wt% H_2SO_4	90	0.67	5.92 $CHCl_3$	[51]
Synechococcus elongatus	17.7	40	15.8	1.8 v% H_2SO_4	80	0.33[e]	–	[8]
Synechocystis sp. PCC 6803	18.4	39	15.8	1.8 v% H_2SO_4	80	0.33[e]	–	[8]
Tetraselmis suecica	23	78	15.8	1.8 v% H_2SO_4	80	0.33[e]	–	[8]

[a] Lipid content per cell dry weight (%)

[b] Conversion yield based on extractable lipid (%)

[c] Methanol/biomass weight ratio

[d] Co-solvent/biomass weight ratio

[e] Reactions conducted by microwave

[f] Not mentioned

[g] $CHCl_3$ to methanol volume ratio was 0.1

biomass feedstock inhibits transesterification reaction [8, 34]. Taken together, it is likely that the lower lipid extraction efficiency and more severe enzyme inhibition under the wet biomass conversion conditions result in lower yield compared with those of the dry biomass conversion.

Enzyme Stability

Cost of the enzyme is a primary expense in an enzymatic conversion process. In enzymatic production of bioethanol, the cost of enzyme represented 18.5–22.7% of the total cost [35], and even at the optimal level, enzyme still cost up to 10% of the total expense [36]. For enzymatic FAME production, enzyme made up 22% of raw material costs [37]. Therefore, recycling of enzyme represents a meaningful approach to reduce the production cost of an enzymatic conversion process. An economic analysis showed that cost savings through enzyme recycling may be as great as 50% if the enzyme recover efficiency remained to be 90% in a biomass-to-ethanol process [36]. At an industrial scale (g or kg of enzyme used in a reaction), immobilized enzymes could be separated from reaction mixture through phase separation or centrifugation for enzyme recycling. Considering the scale of reaction in this study (about 2 mg immobilized N435 was used, which was not practical for direct enzyme recycling), the enzyme stability was investigated with the dry and wet biomass of *N. oceanica* IMET1 as substrates (Fig. 5). N435 was pretreated under the optimized conditions (biomass/t-butanol/methanol ratio of 1:2:0.5 at 25 °C for dry biomass or biomass/*t*-butanol/methanol ratio of 1:1:2 at 45 °C for wet biomass, respectively) for a certain amount of time before algal biomass was added for enzymatic conversion. As shown in Fig. 5, after 55 days of the treatment (equal to 110 cycles) under the conditions optimized for dry algae biomass, enzyme activity remained higher than 95%. These data support that N435 is stable under our reaction conditions and may be recycled for conversion of dry biomass. Previous report suggests that the enzymatic production of FAME will be competitive if the enzyme price reduces to \$44 Kg^{-1} [38]. In this study, we showed high enzyme stability after a 55-day treatment (equal to 110 cycles) for dry algae biomass conversion, which could in theory reduce the enzyme cost from the current level of ca. \$1000 Kg^{-1} [38] to \$9.1 Kg^{-1}. It is worth noting that despite the inference on how the enzyme could be reused through the enzyme stability test, it is not the same as enzyme recycling [4]. In the future, the enzymatic reaction

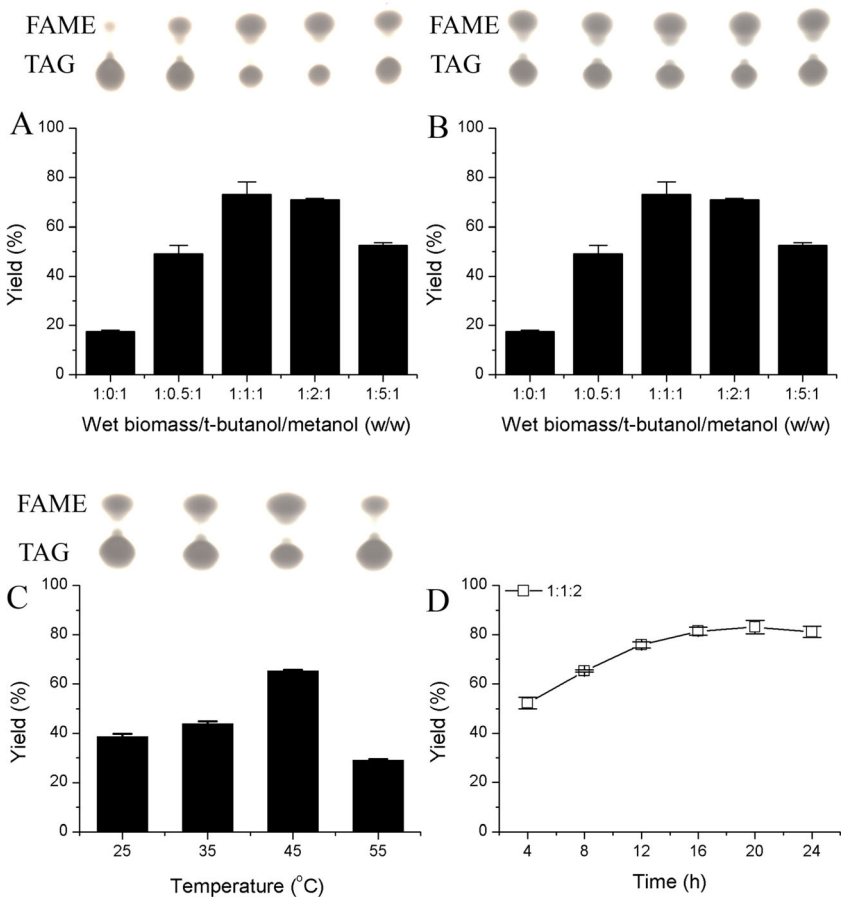

Fig. 4 Optimization of in situ enzymatic conversion conditions for wet *N. oceanica* IMET1 biomass into FAMEs. **a** Effect of *t*-butanol ratio on FAME yield; reaction conditions were biomass to methanol ratio of 1:1 with varying *t*-butanol ratios at 45 °C for 8 h. **b** Effect of methanol ratio on yield; reaction conditions were biomass to *t*-butanol ratio of 1:1 with varying methanol ratios at 45 °C for 8 h. **c** Effect of temperature on FAME yield; reaction conditions were biomass to *t*-butanol to methanol ratio of 1:1:2 for 8 h. **d** Time course of wet biomass conversion. Reaction conditions were biomass to *t*-butanol to methanol weight ratio of 1:1:2 at 45 °C. Chemical conversion of extractable lipid from *N. oceanica* IMET1 was included as the control. Water content and lipid content of wet algal biomass is 60.6 and 56.9%, respectively. Data represented mean ± SD of triplicate experiments

needs to be scaled up for actual enzyme recycling experiment through phase separation and centrifugation, and the enzyme lost during the recycling process needs to be considered.

On the other hand, enzyme activity decreased drastically after recycled with wet biomass (water content is 60.6%) and only 57.8% enzyme activity was attained after 4 days of treatment (equal to 6 cycles), indicating that water and high temperature can largely inhibit enzyme activity. In corroboration, it was reported the enzyme activity dropped to 62.6% after 6 cycles reuse in transesterification of wet algal slurry into FAMEs [13]. This may be explained by the thermal denaturation phenomena [39], which decreases enzyme activity due to the presence of water and higher reaction temperature (45 °C) when using wet algae as the feedstock. To overcome this problem, in the future, molecular sieves may be used in the reaction system to minimize enzyme inhibition in the presence of water. Increase the methanol ratio used in the system may also help [8].

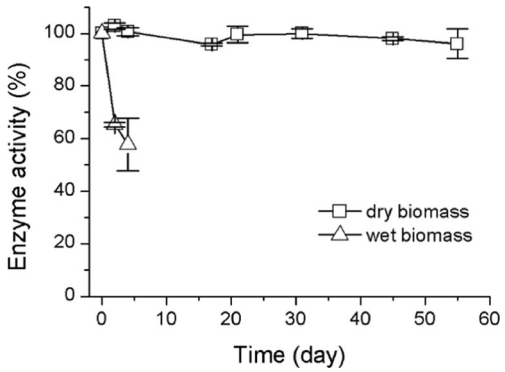

Fig. 5 Enzyme stability under optimized reaction conditions for wet (*open triangle*) or dry algae biomass (*open square*). Reaction conditions were wet biomass to *t*-butanol to methanol weight ratio of 1:1:2 at 45 °C or dry biomass to *t*-butanol to methanol weight ratio of 1:2:0.5 at 25 °C. Data represented mean ± SD of triplicate experiments

Improvement of In Situ Enzymatic Conversion by a Microwave Pretreatment

We also tested the applicability of the in situ enzymatic conversion method with biomass from other algal species. We applied in situ enzymatic conversion using dry *Scenedesmus* sp. HTB1 biomass with the optimal conditions described above, i.e., biomass/*t*-butanol/methanol 1:2:0.5 (*w*/*w*/*w*) at 25 °C for 24 h. In contrast to a complete conversion from dry *Nannochloropsis* biomass by in situ enzymatic conversion (Fig. 3), FAME yield from dry *Scenedesmus* biomass was only 35–45% (Fig. 6a, b) compared with the control (chemical

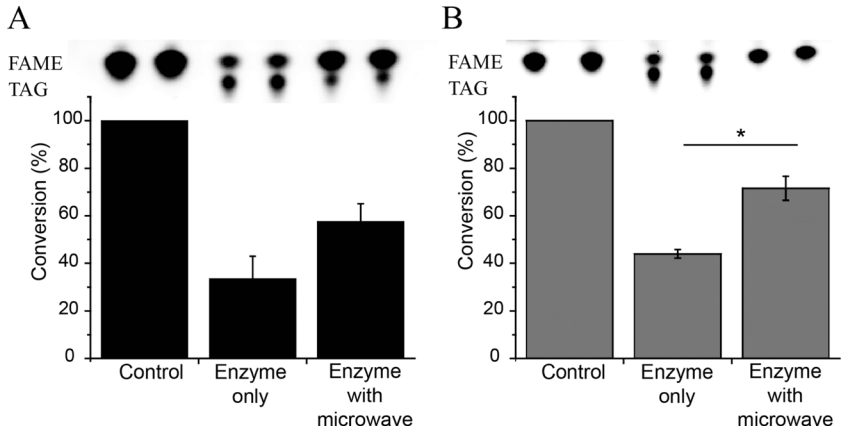

Fig. 6 Effect of microwave pretreatment on in situ enzymatic conversion from *Scenedesmus* sp. HTB1 biomass. **a** Comparison of the FAME yield using HTB1 biomass with and without a 30% power (270 W) microwave pretreatment prior to in situ enzymatic conversion reaction. **b** Comparison of the yield using HTB1 biomass with and without a 40% power (360 W) microwave pretreatment prior to in situ enzymatic conversion reaction. The duration of each microwave treatment was 60 s. Each reaction of in situ enzymatic conversion contained 100 mg dry HTB1 biomass, 5 mg N435, and biomass/*t*-butanol/methanol 1:2:0.5 (*w*/*w*/*w*). The reactions were performed at 25 °C for 24 h. Data represented mean ± SD of at least two independent experiments. The *asterisk* represented *P* value ≤0.05

conversion). We noticed that the color of the *Nannochloropsis* biomass residue after enzymatic conversion became white, indicating a complete extraction and conversion of lipids in the biomass; by contrast, the color of the *Scenedesmus* biomass residue remained green, indicating an incomplete extraction of lipids.

We hypothesized a lysing pretreatment (such as microwave) to the cells may improve the efficiency of in situ enzymatic conversion. With 30% power (270 W) microwave bursting the *Scenedesmus* sp. HTB1 cells for 60 s prior to an enzymatic conversion, the yield increased from 34 to 58% (Fig. 6a). Next, we tested whether an increase of microwave power in the pretreatment can improve the yield in a greater extent. With a 40% power (360 W), 60-s microwave pretreatment, the enzymatic yield increased to 72% (Fig. 6b). The increase of yield with 40% power microwave pretreatment was significant ($P = 0.05$). Moreover, TLC results demonstrated all TAGs in the 40% power microwave-treated biomass were converted into FAMEs (Fig. 6b top panel); by contrast, 30% power microwave did not assist all TAGs being converted to FAMEs, as evidenced by a prominent TAG spot (Fig. 6a top panel). This suggested that 40% power microwave pretreatment worked efficiently on HTB1 biomass and allowed enzyme to convert lipids in the treated biomass. A 72%, instead of 100%, yield resulted from a complete conversion of the pretreated *Scenedesmus* biomass suggested that there might be some lipids leaking from the HTB1 biomass during microwave processing. Therefore, some lipids in the biomass were

Table 2 Comparison of in situ conversion of wet biomass into FAME

Feedstock	Lipid[a]	Yield[b]	Alcohol[c]	Method	Temp. (°C)	Time (h)	Water[d]	References
Chlorella vulgaris	53.3	66	~8[e]	Subcritical water and supercritical ethanol conditions	325	3	~80	[10]
Chlorella vulgaris ESP-31	63.2	92.2[f]	12.35[g]	Sonication + in situ enzymatic transesterification	40–45	48	86–91	[13]
Dunaliella tertiolecta	20.3	30.9	–	Thermochemical liquefaction	340	1	~78.4	[11]
Nannochloropsis oceanica IMET1	56.9	81.5[h]	2	In situ enzymatic transesterification	45	16	60.6	Current work
Nannochloropsis sp. (CCMP1776)	50	~90	7.1	Supercritical methanol conditions	255	0.42	~30	[14]

[a] Lipid content per cell dry weight (%)

[b] Conversion yield based on extractable lipid (%)

[c] Alcohol/wet biomass weight ratio

[d] Water content of wet biomass (%)

[e] Ethanol/wet biomass weight ratio

[f] Hexanes was employed as a co-solvent

[g] Molar ratio of methanol to oil

[h] *t*-Butanol was employed as a co-solvent

lost in the supernatant (too little to be recovered because of our sample size) and not recovered in the subsequent conversion process. Algal cell wall components are diversified, varying in molecular components, intra- and inter-molecular linkages, and overall structure [40]. Our results suggest that for an algae species with a rigid cell wall, such as *Scenedesmus* sp. HTB1, it is necessary to apply a physical or chemical mean of the cell wall disruption to improve in situ enzymatic conversion efficiency. In the future, the amount of algal sample used in the microwave-assisted conversion process needs to be increased to allow recovery of lipids leaked into the supernatant during microwave pretreatment.

Comparison with the State of the Art

Direct transesterification of microalgal biomass could simplify the FAME production process by eliminating a cell disruption and a separate lipid extraction step, thus reducing the energy consumption and cost. Our results were compared with previous reports of in situ conversion of dry biomass (Table 1) and wet biomass (Table 2). Previous reports of in situ chemical conversion of algal biomass used high temperature (60–100 °C) (see Table 1 and [34, 41], whereas enzymatic conversion also required heating at 35–45 °C (see Table 2 and [18, 19]). This work represents the first report of efficient enzymatic conversion of whole algal biomass at room temperature. The maximum yield of 107.7% is the highest among all studies (Table 1). Our room temperature enzymatic conversion method offers the opportunity to recover high-value byproducts from a biofuel production process, such as carotenoids and omega-3 fatty acids [42]. Compared with other in situ conversion methods (Tables 1 and 2), our reaction time is at the medium to high range. In the future, enzyme prospecting work should identify more efficient lipases or better reaction conditions to achieve high yield within a shorter time, ideally within 1 to 2 h.

Water may not only inhibit the transesterification process but also decrease the extraction efficiency from the wet algae biomass. Though conventional methods cannot transesterify wet biomass, some alternative approaches such as supercritical extraction technique, thermochemical liquefaction, and in situ enzymatic conversion do, as shown in Table 2. Hydrothermal liquefaction is a promising technology for microalgae conversion, which processes the whole algae biomass into biocrude without the drying process [43]. Transesterification of wet algae biomass can also be conducted under supercritical conditions [44]. However, these techniques operated under higher temperatures (150–350 °C) and pressure and their cost-effectiveness and energy efficiency remained a matter of debate [44]. Previously, in situ enzymatic conversion of wet *Chlorella* biomass pretreated by sonication has been accomplished [13]. In our study, non-treated wet algal biomass was directly used as a substrate while the yield still reached >80%. However, considering the poor enzyme stability under wet biomass conversion conditions, we concluded the in situ conversion of dry algae biomass is preferable for energy-efficient production of FAMEs.

Conclusions

We conducted in situ enzymatic conversion of microalgal biomass into FAME and achieved a maximum yield of 107.7% with the dry *N. oceanica* IMET1 biomass. The effects of co-solvent, methanol, temperature, and reaction time were investigated and the optimized reaction conditions were biomass to *t*-butanol to methanol weight ratio of 1:2:0.5 and the reaction time of 12 h at 25 °C. The yield reduced to 81.5% when the wet *N. oceanica* biomass was used. The high enzyme stability during in situ enzymatic conversion of dry algal biomass indicates its potential application as a promising method for algae-based FAME production.

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