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Biodiesel via in Situ Wet Microalgae Biotransformation: Zwitter-Type Ionic Liquid Supported Extraction and Transesterification

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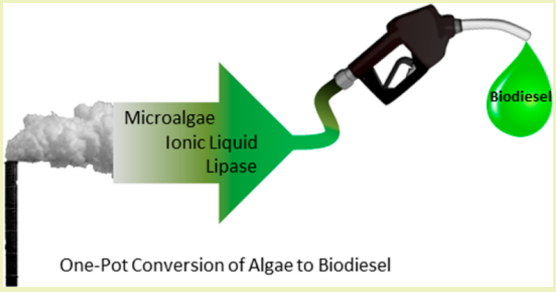
Supporting Information

ABSTRACT: The production of biodiesel derived from microalgae is among the most forthcoming technologies that provide an ecologic alternative to fossil fuels. Herein, a method was developed that enables the direct extraction and conversion of algal oil to biodiesel without prior isolation. The reaction occurs in aqueous media catalyzed by immobilized *Candida antarctica* lipase B (Novozyme 435). Zwitter-type ionic liquids were used as cocatalyst to improve the selectivity and reactivity of the enzyme. In a model reaction with sunflower oil, 64% biodiesel was obtained. Applying this method to a slurry of whole-cell *Chlorella zofingiensis* in water resulted in 74.8% of lipid extraction, with 27.7% biotransformation products and up to 16% biodiesel. Factors that reduced the lipase activity with whole-cell algae were subsequently probed and discussed. This "in situ" method shows an improvement to existing methods, since it integrates the oil extraction and conversion into an one-pot procedure in aqueous conditions. The extraction is nondisruptive, and is a model for a greener algae to biodiesel process.



KEYWORDS: *Zwitter ionic liquid, Immobilized CALB (Novozyme 435), Chlorella zofingiensis, Direct transesterification*

INTRODUCTION

The production of biodiesel as an alternative to fossil fuels has gained increasing interest over the past few decades. Nevertheless, the question of a reasonable feedstock is not yet fully addressed. For example, oils and fats from kitchen wastes are of limited availability as are animal fats from slaughterhouses. Alternatively, the use of vegetable oil could be an available solution due to its higher abundance. However, agro-based sources are controversial; they compete with arable land for food and animal feed production and even encourage deforestation. Moreover, this approach consumes vast amounts of water and fertilizers that will themselves become a considerable cost factor in the near future due to an increasing scarcity. In particular, the availability of phosphate fertilizers will decrease considerably in the foreseeable future.[1]

The cultivation of microalgae proves to be a feasible alternative. It does not compete for arable land and food production. Algae can be facilely cultivated on nonagricultural surfaces, such as roof tops and facades.[2] Due to this flexibility, the cultivation can be moved to sites with increased CO_2 emission. In comparison to oil crops, such as palm kernels or canola, microalgae show a higher turnover, yielding more oil per hectare and year.[3,4] Appropriate cycling techniques make the cultivation of microalgae independent of external nutriments and fertilizer addition.[5,6]

While the cultivation techniques are constantly improved, the isolation of the produced oil still remains an important challenge. The principal steps in microalgae-biodiesel production are (1) microalgae cultivation, (2) harvest and isolation, (3) cell lysis and oil extraction, and (4) transesterification into biodiesel.[7] All of these steps are usually energy and cost intensive, and are hardly economical. For example, the isolation and harvest of the oil contributes about 20−30% to the total production cost.[8]

The controversy of high ecological relevance of algae based oil with the low economic efficiency of the production inspired us to focus on in situ extraction and transformation of oil from the whole-cell microalgae. Current methods mainly focus either on the in situ lysis of the cells with subsequent transesterification,[9,10] or enhanced extraction techniques of prior concentrated algal slurry. Such a process was discussed, inter alia, using dry and milled microalgae in a suspension in MeOH with KOH as a catalyst. Microwave irradiation assisted in the liberation of the oil into the reaction mixture, due to an increased diffusion through the cell wall and cell disruption.[11] In general, the outer cell wall of microalgae is relatively robust, and a total mechanical rupture is not easily achieved.[12] An alternative to disruptive methods is the lipid extraction of whole cells.[13,14] Respectively, the most promising procedures up to date rely on extraction with super critical CO_2,[15,16] switchable solvents,[17,18] and ionic liquids (IL).[19] All methods are efficient, and the extracted oil can be easily separated from the extracting fluid. Nevertheless, they rely on a precedent isolation/concentration of the biomass. Conversely, an integration of in

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situ extraction and transesterification into a one-pot procedure would considerably reduce the overall cost and environmental impact.[20,21] The combination of both steps is difficult to achieve. For example, water was found to be problematic especially in the chemical transesterification, where it undergoes side reactions or acts as a retardant or inhibitor to the catalyst. In this respect, ionic liquids could combine both processes. ILs enhance the extraction of algal oil through the outer cell membrane without disruption.[19] The cell membrane is used as a filter membrane which subsequently reduces the amount of cell debris in the extracted lipids. Additionally, ionic liquids were shown to be beneficial to the lipase activity,[22,23] and were thought to be key elements for transesterification in aqueous media.

Herein we describe for the first time a method that combines both steps: extraction of oil from a whole-cell microalgae mixture of *C. zofingiensis* with the subsequent enzyme catalyzed biotransformation to biodiesel in aqueous medium with zwitter-type ionic liquids as cocatalysts. The generality of the method will first be examined with sunflower oil in an aqueous suspension using zwitterionic liquids with surfactant properties as additives. These conditions are then transferred to a slurry of *C. zofingiensis* cells in water. The final objective was a process development that enables the possibility to directly transform algal oil in the bioreactor to increase the overall efficiency of biodiesel production from microalgae.

EXPERIMENTAL SECTION

Microalgae Cultivation Preparation. *Chlorella zofingiensis* (SAG 211-14 *Chromochloris zofingiensis*) were obtained from the Culture Collection of Algae (SAG) at the Georg-August-Universität Göttingen (Göttingen, Germany). The cultivations were performed in CZ-M1 medium.[24] Before inoculation, a 100 mL preculture was prepared in a baffled Erlenmeyer flask and incubated for 4 days in an LED shaker (Lab-Therm LT-XC, Kuhner; 5% p_{CO2} at 26 °C with 311 μmol m^{-2} s^{-1} continuous lightning at 115 rpm).

Autotrophic Cultivated Algae. *Method A (LED Shaker: Lab-Therm LT-XC, Kuhner).* All glassware and media were sterilized prior to use. The growth medium was transferred to a 2 L baffled Erlenmeyer flask and inoculated with 1% (v/v) of preculture. The flask was closed with a cellulose stopper and placed in an LED shaker under the aforementioned conditions for either 14 or 20 days (the culture medium was not replenished during this period) with 5% p_{CO2} at 26 °C with 311 μmol m^{-2} s^{-1} continuous light at 115 rpm.

Method B (Flat Panel Photobioreactor: Labfors 5 Lux, Infors). In deviation from method A, the CZ-M1 medium was enriched with Chloramphenicol (34 μg/mL) prior to the inoculation in the flatbed reactor. The reactor was filled with 1.8 L of medium and inoculated with 1% (v/v) preculture. The LED light source was then set to approximately 300 μmol m^{-2} s^{-1}, and the air flow was set to 1 mL min^{-1} with 1% p_{CO2}. Throughout the cultivation, the temperature was kept constant at 25 °C, and the pH was held at 7.0. The cultivation was stopped after 10 days.

Heterotrophic Cultivated Algae. Sterile heterotrophic cultivations were processed under corresponding conditions in a standard incubator (SI6R-2 Shel Lab). A 20 g L^{-1} solution of glucose was added to the medium for heterotrophic cultivation. The flasks were wrapped with aluminum foil to protect the cultivations from light. The algae were cultivated at 26 °C and agitated with 115 rpm.

Mixotrophic Cultivated Algae. The mixotrophic cultures were cultivated following method A, and in addition, 20 g L^{-1} of glucose was added to the medium.

Microalgae Harvesting. The growth medium was transferred to centrifugation vessels and centrifuged at 6000 *g* for 30 min (MF 20-R centrifuge, Awel). The sedimented algae biomass was then transferred to falcon tubes and lyophilized (Alpha 2-4 LD freeze-dryer, Christ) until the weight remained constant. The dry algae mass was ground in a mortar and stored in the dark and dry place at ambient temperature. Optical microscopy showed that the obtained powders consisted of whole cells with different sizes depending on cultivation conditions.

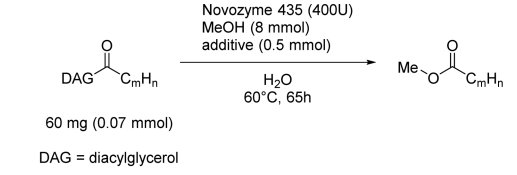
Oil Content Microalgae. The method was adapted to Lamers et al.[25] for the determination of the total lipid content of microalgae. A 200 mg portion of algal dry mass was suspended in 4 mL of 50 mM Tris (pH 7.5) containing 1 M NaCl, and glass beads were added the cells, and then lysed (Precellys24, Bertin Technologies; 3 cycles at 30 s, with 30 s pause). The lysed suspension was then extracted one time with 4 mL of chloroform/methanol (2:2.5) solution, and then twice with 4 mL of neat chloroform. The obtained crude product was resuspended in hexane and filtered over Celite. (There is evidence that the extraction of whole-cell microalgae in acetone is equally effective. However, the reaction conditions have not been optimized to confirm the correctness of the method.)

Average Molecular Weight of the Oil. *NMR Method.* Oil was weighed into an NMR tube, and a known amount of dimethylsulfone (MSM) was added. The ^{1}H NMR spectrum for the analysis was recorded using a Bruker Avance 400 MHz NMR spectrometer. These experiments were performed in triplicate.

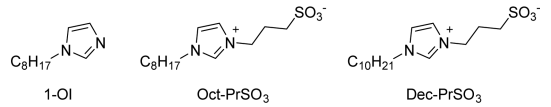
GC Method. Algal oil was transesterified with sodium methoxide ($NaOCH_3$) to the corresponding fatty acid methyl esters. The esters were then isolated and analyzed by GC (Agilent 6850; column, Optimawax 0.25 μm). The average mass was determined by correlating the detected fatty acids weight with its content in the oil.

Lipase Mediated Biodiesel Synthesis. *General Procedure.* Lyophilized whole-cell microalgae (300 mg) or sunflower oil (60 mg) was added to a 10 mL screw cap glass vial containing 4 mL of deionized water, 256 mg (8 mmol) of MeOH, enzyme, and additive (1-octylimidazole, Oct-PrSO$_3$, Dec-PrSO$_3$; Table 1). The mixtures were vortexed and placed in triplicate in a rotary hybridization oven preheated to 60 °C and processed for 65 h/8 rpm.

Table 1. Model Reaction Development of the Enzymatic Transesterification with Sunflower Oil in Aqueous Mixture



Additive:



1-OI Oct-PrSO$_3$ Dec-PrSO$_3$

entry	additive	biodiesel [%][a]	rest oil [%][a]	free FA [%][a]
1[b]	none	24.8 ± 3.4%	61.1 ± 2.0%	14.2 ± 0.5%
2[b]	1-OI	17.6 ± 4.0%	82.5 ± 3.5%	0%
3[b]	Dec-PrSO$_3$	8.2 ± 1.5%	91.8 ± 1.5%	0%
4[b]	Oct-PrSO$_3$	61.9 ± 0.9%	38.1 ± 0.8%	0%

[a]Yields were normalized to 100% (raw data are found in the Supporting Information). [b]Duplicates.

After the biotransformation, the reaction mixtures were diluted with acetone and the solids filtered off and washed with acetone. The organics were removed with a rotary evaporator at 60 °C. The remaining aqueous phase was extracted 3 times with 4 mL of ethyl acetate. The combined organic phases were dried over Na_2SO_4. The obtained crude product was analyzed and quantified by ^{1}H NMR (Bruker UltraShield 400).

Method A. This method used Novozyme, 435:240 mg (400 U).

Method B. This method used Coated Enzyme,[22] 384 mg (400 U).

Method C. This method used Novozyme, 435:240 mg (400 U), with 0.5 mmol of additive.

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Algae−Oil Conversion and Biodiesel Yield Determination by ^{1}H NMR. A known quantity of dimethylsulfone (MSM) was added to a weighted crude oil extract and dissolved in $CDCl_3$. The proton spectrum was measured using a Bruker Avance 400 MHz NMR spectrometer. For quantification, the following peaks were taken into consideration: 3.65 ppm (s, 3-H), methyl group of the ester moiety of the biodiesel molecule; 2.99 ppm (s, 6-H), methyl group of MSM; 2.2−2.4 ppm (m, 2-H), methylene group of the C_2 carbon of the fatty acid (FA) moiety. There is overlap of the signals for free fatty acid (fFA), oil (TAG), and biodiesel. The electron density of the protons of the methylene group increased in the order free fatty acid < TAG < biodiesel, which can be seen with a slight upfield shift in the NMR spectrum. Therefore, the amount of the fFA can be estimated by integration of single peak (at 2.35 ppm) corresponding to the left-most peak of the fFA triplet signal. The full area of the signal was then estimated by extrapolation to a triplet signal. The amount of TAG was estimated by subtracting the amount of biodiesel and the amount of fFA from the full area

Synthesis of 1-octyl-3(propyl-3-sulfonyl)imidazolium. The synthesis was realized in two steps and in as green a manner as possible.

1-Octyl-1H-imidazole. The synthesis was based on a literature procedure.[26]

Imidazole [10.21 g (150 mmol, 1.04 equiv)] was dissolved in 160 mL of 30% NaOH in water, and THF (green sourcing possible)[27] (200 mL) was added. A 27.77 g (144 mmol, 1.00 equiv) portion of 1-bromooctane was added, and the mixture was heated to reflux for 72 h.

The formed organic phase was separated from the aqueous phase, which was re-extracted twice with 50 mL of ethyl acetate. The combined organic phase was extracted with HCl (10% in water) and dried over Na_2SO_4. The solvent was evaporated, and the crude product was distilled under reduced pressure (10 mbar, 145−150 °C). Yield: 22.0 g (85%), yellowish oil. The results are according to the literature.[28]

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.48 (s, 1H), 7.04 (s, 1H), 6.89 (s, 1H), 3.92 (t, 2H, J = 6.89 Hz), 1.80−1.71 (m, 2H), 1.32−1.20 (m, 10H), 0.86 (t, 3H, J = 7.25 Hz).

Oct-PrSO$_3$. A 3.03 g (16.8 mmol, 1.02 equiv) portion of 1-octyl-1*H*-imidazole was weighed into a Schlenk flask. The flask was degassed and set under N_2 atmosphere. A 2.01 g (16.5 mmol, 1.00 equiv) portion of propane-1,3-sulfone was added, and the reaction mixture was heated to 80 °C for 16 h. Afterward, the mixture was heated to 100 °C and evacuated (2 Torr) for 8 h. Yield (isolated): 4.47 g (90%), yellowish resin.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.65 (t, 1H, J = 1.63 Hz), 7.66 (t, 1H, J = 1.77 Hz), 7.25 (t, 1H, J = 1.79 Hz), 4.55 (t, 2H, J = 6.99 Hz), 4.23 (t, 2H, J = 7.44 Hz), 2.83 (t, 2H, J = 7.02 Hz), 2.38 (quint, 2H, J = 7.03 Hz), 1.85 (quint, 2H, J = 7.32 Hz), 1.34−1.17 (m, 14H), 0.85 (t, 3H, J = 6.91 Hz).

^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ (ppm) 136.93, 123.19, 121.84, 49.94, 48.42, 47.59, 31.66, 30.19, 29.00, 28.95, 26.66, 26.26, 22.54, 14.03.

HRMS (ESI) m/z [M + H]$^+$ calcd for [$C_{14}H_{26}N_2O_3S$ + H]$^+$: 303.1742. Found: 303.1740

Dec-PrSO$_3$. Dec-PrSO$_3$ was synthesized following the literature procedure[22] and recrystallized from ethanol and acetone prior to use giving a white powder.

The results are in accordance with the literature.[29]

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.58 (s, 1H), 7.72 (t, 1H, J = 1.68 Hz), 7.25 (t, 1H, J = 1.75 Hz), 4.49 (t, 2H, J = 6.89 Hz), 4.17 (t, 2H, J = 7.39 Hz), 2.76 (t, 2H, J = 7.11 Hz), 2.32 (quint, 2H, J = 7.06 Hz), 1.79 (quint, 2H, J = 6.70 Hz), 1.27−1.10 (m, 14H), 0.80 (t, 3H, J = 6.89 Hz).

RESULTS AND DISCUSSION

Direct Transesterification of Sunflower and Isolated Microalgae Oil in Aqueous Solutions. The reactivity of immobilized *Candida antarctica* lipase B (Novozyme 435) was probed in a suspension of sunflower oil in distilled water and an excess of methanol (relative to the oil). The influence of various additives was probed at 60 °C for 65 h (Table 1). The immobilized enzyme was filtered off after the reaction, and the crude products were isolated and analyzed. Two isolation methods were tested, lyophilization and extraction with ethyl acetate. Both were equally effective (Table 1).

First, the reactivity of Novozyme 435 was probed in the absence of additives. Concomitant to 61.1 ± 2.0% unconverted sunflower oil and 24.8 ± 3.4% biodiesel, a third product could be observed (Table 1, entry 1). The comparison of the crude ^{1}H NMR with a reference spectrum of lauric acid (Figure 1C)

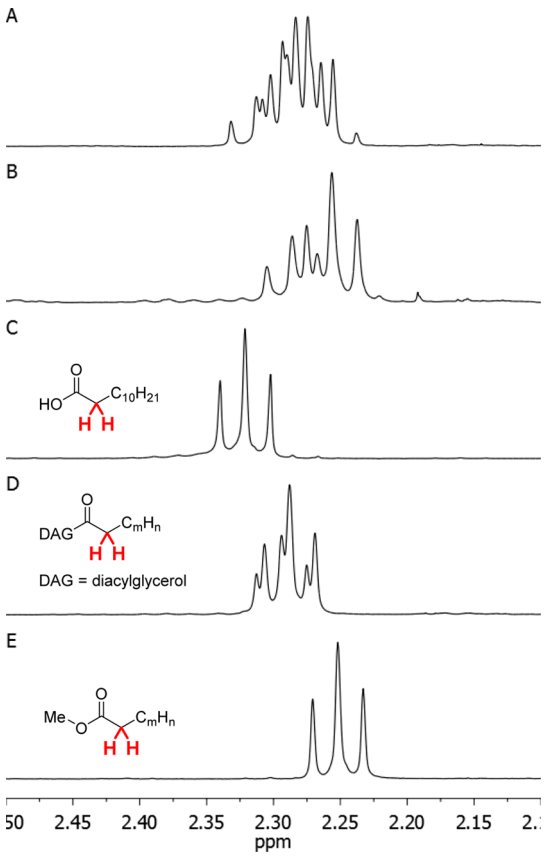

Figure 1. ^{1}H NMR ($CDCl_3$, 400 MHz) spectra to compare α-methylene signals of the fatty acid moiety: (A) transesterification reaction without Oct-PrSO$_3$ additive (mix of free fatty acid, unconverted oil, and FAME), (B) transesterification reaction (mix of unconverted oil and FAME), (C) lauric acid, (D) sunflower oil, and (E) fatty acid methyl ester. General reaction conditions: 60 mg sunflower oil, 400U Novozyme 435, 8 mmol MeOH, 4 mL H_2O (4.0 mL), 60 °C for 65 h.

revealed the formation of 14.2 ± 0.5% of free fatty acid. The proton signals of the α-methylene group of the fatty acid moiety were taken to distinguish both esters, triacyl glycerol and fatty acid methyl ester, from the free fatty acid. While the signals for the oil and the biodiesel form an overlapping signal between 2.28 and 2.33 ppm, the signal for the free fatty acids was sufficiently shifted downfield (2.30−2.34 ppm) to be able to estimate their content. To further enhance the conversion rate, different ionic liquids were probed for their effect on the lipase activity. ILs were already reported to improve enzyme stability, reactivity, and selectivity.[30−32] In addition to the ILs, the nonionic surfactant 1-octylimidazole (1-OI) was used. 1-OI should increase the availability of the suspended oil in solution,

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Table 2. Effect of the Oct-$PrSO_3$ Concentration on the Conversion of Sunflower Oil into Biodiesel[a]

entry	Oct-$PrSO_3$ [mg]	ratio (w/w) Oct-$PrSO_3$/H_2O	molar ratio oil/Oct-$PrSO_3$	biodiesel[b] [%]	rest oil (TAG)[b] [%]	free FA[b] [%]
1[c]	0	0:1	1:0	24.8 ± 4.9%	61.1 ± 4.2%	14.1 ± 0.7%
2[d]	coated enzyme	n/a[e]	n/a[e]	20.0%	80.0%	0%
3[c]	50	1:80	1:1.8	61.5 ± 4.8%	38.5 ± 4.8%	0%
4[c]	100	1:40	1:4.0	63.8 ± 8.2%	36.2 ± 8.2%	0%
5[c]	150	1:27	1:5.5	61.9 ± 0.9%	38.1 ± 0.9%	0%
6[c]	200	1:20	1:7.8	55.4 ± 3.4%	44.6 ± 3.4%	0%

[a]Sunflower oil (~60 mg), Oct-$PrSO_3$ (var), Novozyme 435 (400 U), MeOH (8.0 mmol), and H_2O (4.0 mL) at 60 °C for 65 h. [b]Yields were normalized to 100% (raw data are found in the Supporting Information). [c]Duplicates. [d]Single experiment. [e]Not accessible.

due to its surfactant properties. Furthermore, it was thought to trap the free fatty acids which were formed by the lipase to give an in situ formed IL.

The use of 1-OI showed an improvement of the selectivity toward biodiesel formation (the formation of free fatty acid was not observed), but it showed no increase in overall reactivity. Only 17.6 ± 4.0% biodiesel was obtained (Table 1, entry 2). In contrast, the addition of Dec-$PrSO_3$ resulted in a lower oil conversion with 8.2 ± 1.5% biodiesel (Table 1, entry 3). During the reaction it was observed that the Dec-$PrSO_3$ was not entirely miscible with the oil−water suspension. The reduced miscibility of long chain zwitter-type IL was described before and found here as the limiting factor for its applicability.[29,33] In a further trial, the shorter chain IL Oct-$PrSO_3$ was employed to avoid the solubility problem. Indeed, the biodiesel yield increased to 61.9 ± 0.9% (Table 1, entry 4). Blank reactions in the absence of Novozyme 435 with both ILs, Oct-$PrSO_3$ and Dec-$PrSO_3$, showed no conversion of the sunflower oil.

In order to understand the reaction, the Oct-$PrSO_3$ concentration was varied (Table 2). Precoating[22] of Novozyme 435 with Oct-$PrSO_3$ led to an increased selectivity; biodiesel was observed as the sole product, although the reactivity was lower with 20.0% biodiesel (Table 2, entry 2). An increase of the molar ratio of oil to IL resulted in a change of reactivity (Table 2, entries 3−6). It was observed that the reactivity decreased with higher IL concentrations due to a reduced availability of substrate to enzyme.[34,35] Finally, a maximum conversion around a 1:4 (oil to IL) ratio was the best combination. In general, the IL played two major roles in the reaction: (A) It interacted with Novozyme 435 and therefore increased its selectivity although with lower reactivity (compare Table 2, entries 1 and 2), and (B) it assisted in solubilizing the oil due to its surfactant properties, which increased the substrate availability to the lipase.

In order to screen the developed method for its applicability with microalgae, the protocol was applied to pre-extracted algal oil.[36] In a single experiment, an oil to biodiesel conversion of 39.3% was achieved. The yield was somewhat lower than that with the use of pure sunflower oil but sufficiently close to apply the protocol to algal whole-cell mixtures. Here oil from the microalgae was extracted without disrupting the outer cell membrane and subsequently converted to biodiesel.

Microalgae Cultivation Choice. *Chlorella zofingiensis* was cultivated under four different conditions. The aim was to examine if the cultivation method influences in situ oil extraction and transesterification efficiency. The assumption was that the outer cell surface area of the microalgae depends to a large extent on the cultivation method. However, also, oil content and growth vary with cultivation. The extraction kinetics through the outer cell membrane depends on the available surface (Figure 2). The autotroph cultivation in an

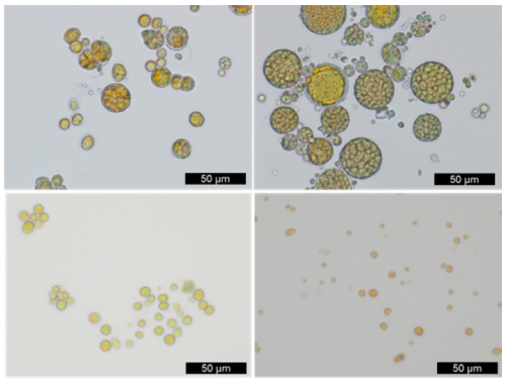

Figure 2. *Chlorella zofingiensis* under different growth conditions. Top left: mixotroph. Top right: heterotroph. Bottom left: autrotroph. Bottom right: air−CO_2 autotroph.

LED shaker using 5% CO_2 resulted in a growth rate of μ = 0.174 and an oil content of 40.9 ± 4.5% after 20 days of cultivation. Heterotrophic cultivation using glucose as carbon and as energy source resulted in an expected higher growth rate of μ = 0.251 and a similar oil content 39.5 ± 1.0%. The mixotrophic cultivation using glucose and LED light was less productive in view of oil content, but also a rather elevated growth rate of μ = 0.246 was observed. Mixotrophic cultivations were less productive with oil content 25.8 ± 1.1%. The molecular weight of the triacyl glycerol (TAG) fraction was determined by chemical transformation into the corresponding fatty acid methyl esters (FAMEs) with subsequent quantification by GC (Agilent 6850). The average molecular weight of the TAG was then determined by relating the molecular weight of the corresponding FAME and their content in the TAG.

In Situ Extraction and Transesterification of Whole-Cell *Chlorella zofingiensis* in Aqueous Medium. The previously developed method was employed to a wet algae slurry.[37] The direct transesterification was thought to replace cell lysis and oil isolation steps as realized in conventional microalgae oil production.[38] The outer cell membrane which served as a filtration membrane was not disrupted (Figure S1, images C and D) during this process as previously observed using ionic liquids.[13] To the best of our knowledge, zwitter-type ionic liquids were not yet employed in the lipid extraction of wet microalgae. First, different cultivation techniques, heterotrophic, autotrophic, and mixotrophic, were compared. The results showed that the algae diameter depended on the type of cultivation technique, but it had little to no significant influence on the extraction and transesterification yields (Figure 2). A reason for this could be the fact that the microalgae contained variable amounts of spores, which equally pose as a membrane. Therefore, the diameter of a microalgae was less important than the total membrane surface in an algae to extract its oil. Also,

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Table 3. In Situ Transesterification Using Wet Whole-Cell Microalgae at 60°C for 65 h[a]

entry	additive	extracted oil[b] [%]	yield biodiesel (based on total oil) [%]	yield biodiesel (based on extracted oil) [%]	free FA (based on extracted oil) [%]
1	no additive	74.8 ± 5.6%	7.5 ± 2.7%	10.3 ± 4.5%	17.4 ± 3.2%
2	coated enzyme[c]	69.8 ± 1.8%	5.2 ± 0.5%	7.5 ± 0.5%	0%
3	Oct-PrSO$_3$[d]	67.8 ± 11.1%	4.4 ± 1.0%	6.4 ± 0.5%	0%
4	Oct-PrSO$_3$[d,e]	60.4 ± 9.9%	9.6 ± 0.4%	16.2 ± 1.9%	0%

[a]300 mg of algal dry mass, additive, 8 mmol MeOH, 400 U Novozyme 435 (1.35 U/mg ADM) in 4.0 mL of H_2O at 60 °C for 65 h; all values are in triplicates. [b]The value is based on the total lipid content of the algae. [c]Consisted of Novozmye beads coated with Oct-PrSO$_3$.[22] [d]0.5 mmol. [e]115 h.

the average size of an algae mixture changes upon sporulation which was easily observable with larger microalgae cells and less easily observable with smaller cells. At this point it was thought to assess transesterification rates by turnover numbers (TON) as a means to normalize data and understand the kinetics to show eventual advantages for certain cell sizes. These TONs were calculated per active site in used lipase to determine activity variations in the transesterification depending on cell size and used reaction conditions. Here the heterotroph cultivation showed a TON = 17.3; for autotroph the TON was 8.4, and it was somewhat lower for mixotroph, TON = 5.2. These TON numbers were in line with the observation that heterotroph cultivation usually was the best oil per weight producer followed by autotroph cultivation and less for mixotroph. Still, the TONs were rather close together, which was also the case for the extraction efficiency as well as conversion. Conversely, with addition of the ionic liquid Oct-PrSO$_3$, the TON improved for all three types of cultivations reaching TONs in general above >20. Besides the positive influence from the ionic liquid, there again was no trend indicating an advantage for a certain cell size or cultivation technique. We therefore chose to study the autotrophic cultivation method as it is the most sustainable cultivation technology examined here.

The results from the biotransformation with whole-cell microalgae obtained for the blank reaction, in the absence of Oct-PrSO$_3$, were in accordance with the experiments with sunflower oil. Not only biodiesel but also free fatty acids were formed in 10.3 ± 4.5% and 17.4 ± 3.2% yields (Table 3, entry 1). When an ionic liquid coated enzyme[22] was employed, the selectivity increased (no free fatty acid was observed), but less biodiesel 7.5 ± 0.5% was formed (Table 3, entry 2). Surprisingly, when Oct-PrSO$_3$ was employed, the yield was reduced to 6.4 ± 0.5% (Table 3, entry 3). Longer reaction times of 115 h increased the yield to 16.2 ± 1.9% (Table 3, entry 4). The yields were in comparison to the previously screened transesterification with sunflower oil and pure microalgae oil clearly lower. These observations led to the conclusion that Novozyme 435 was partially inhibited in the presence of microalgae. A few factors were considered as possible reaction inhibitors: the presence of ions in solution from the cultivation medium, variations of the pH in comparison to the standardized system with sunflower oil, and organic contaminants that were equally extracted from the microalgae cells. These factors were closely examined in selective experiments. They consisted of ion adsorption/exchange trials first to understand if there is a destabilizing kosmotrop influence from inorganic cations contained in CZ-M1 cultivation medium.[24] Three samples containing *C. zofingiensis*, Oct-PrSO$_3$, and MeOH were prepared. The ion exchange resin Amberlite IR120 and Amberlite IRA420, cation and anion exchange resins, were added. The sample containing solely the cation exchange resin, Amberlite IR120, showed a slight increase of biodiesel production after 65 h at 60 °C with 8.0%. The samples containing the anion exchange resin enhanced the effect, Amberlite IRA420, and the mix of Amberlite IRA420 and Amberlite IR120 could increase the biodiesel yield to 13.5% and 13.9%, respectively. The presence of anions showed a positive chaotrop influence on the reactivity of Novozyme 435. As a result, the presence of anion exchange resin doubled the conversion to biodiesel. The yields were still somewhat lower compared to the reference experiment with pre-extracted algae oil (39.3% yield).

Also, the pH influence was examined as a determining factor for the low reaction rates. Performing the biotransformation in the presence of phosphate buffers at pH = 5, 7, and 9 resulted in 13.3%, 6.2%, and 5.6% yields. Only the use of a Tris buffer (50 mM Tris in 1 M $NaCl_{aq}$, pH 7.5) resulted in 30.7% conversion, but the oil extraction efficiency dropped to 16.8%. In comparison, 60−70% values were typically observed. As shown here, ions and pH show an influence of the efficiency of Novozyme 435 but also on the extraction. Algae derived organic compounds were discussed earlier to inhibit lipase activity.[39,40] An inhibition complex could be formed which is not easy to detect as a microalgae slurry matrix was used. The usually reported inhibitory effects in lipase catalyzed biodiesel generation are for high concentrations of methanol or glycerol, but here they were hardly the causation for the observed inhibition. A reference experiment with pre-extracted algal oil resulted in a notable oil conversion (39.3% biodiesel) and confirmed that there was an inhibitor in the algae slurry. These algae mixtures consisted mostly of whole cells, which were not broken after processing (Figure 1S, parts C and D). The isolation and characterization of the inhibitory compounds were determined to be beyond the scope of this work. Nevertheless, the characterizations of possible inhibiting factors by a broad screening of different lipase activity and/or efficacy of different microalgae, other than *C. zofingiensis*, are worth consideration in future research.

CONCLUSIONS

A one-pot microalgae to biodiesel conversion was examined. First, an enzymatic method was developed that directly converted oil suspended in aqueous media to the corresponding fatty acid methyl esters. The selectivity increased when the zwitter-type ionic liquid, such as Oct-PrSO$_3$, was used with yields up to 63.8%. These conditions were also applied to algal slurries where yields of up to 16.2% were reached. The direct whole-cell microalgae transesterification readily converted algal oil into the corresponding fatty acid methyl ester without the need to isolate and lyse the microalgae. This integrative methodology is thought to be a contribution to the quest to improve the economy of microalgae derived biodiesel production.

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■ ASSOCIATED CONTENT

Supporting Information

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Raw experimental data and microscopy images (PDF)

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Notes

The authors declare no competing financial interest.

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