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Original Research Article

Greener approach to the comprehensive utilization of algal biomass and oil using novel Clostridial fusants and bio-based solvents

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ABSTRACT

A greener method has been tested to utilize algal biomass as a feedstock to produce bio-oil in addition to acetone, butanol, and ethanol (ABE) products. Various hydrolysis treatments were used prior to fermentation including combination of thermal, chemical, and enzymatic, which resulted in maximum sugar release of 27.78 g/L. Bio-based terpenes was used instead of common toxic chemicals together with Clostridial fustants to produce bio-alcoholic fuels. Protoplast fusion technique were used to produce the novel *Clostridia* fusants (*C. beijernickii* + *C. thermocellum* and *C. acetobutylicum* + *C. thermocellocum*). Fused strains were then subjected to UV radiation for strain enhancement. Final fusansts showed clear improvement in thermal stability and resistance to biobutanol toxicity. Fermentation experiments showed maximum biobutanol final production of 7.98 g/L using *CbCt* versus 7.39 g/L using *CaCt*. Oil extraction from virgin algae was tested using a green, bio-based approach using terpenes with ultrasonication and green Bligh and Dyer method, separately. In preliminary study on algal biomass, the combinations of ultrasonication followed by the green Bligh and Dyer have resulted in oil yield of 46.27% (d-limonene) and 39.85% (p-cymene). Oil extraction from an algae sample following fermentation using the combined extraction method resulted in significantly higher oil yield of 65.04%.

1. Introduction

Pressing issues of climate change, energy security and resource depletion have urged the development of sustainable, greenhouse gas (GHG) reducing technologies. Renewable energies in the form of biofuels have manifested superior qualities in terms of climate mitigation and improving clean energy availability. Biofuels can be defined as fuels that are derived from naturally occurring resources such as plants, crops, and agricultural residues [1]. However, recent assessments have indicated that biofuels have fallen short due to the disadvantages associated with feedstock choices. First- and second-generation biofuels, derived from food and non-food crops, have resulted in minute GHG emission reductions, proven to be expensive investments, and have also resulted in minor environmental consequences. Consequently, the recent development of third-generation biofuels, derived from algal biomass, has received significant attention. Algal-based biofuels offer a superior set of characteristics whilst also eliminating the drawbacks associated with its predecessors. Algae can be classified as diverse set of photosynthetic organisms ranging from simple unicellular cyanobacteria to complex multicellular macroalgae [2]. Furthermore, algal biomass is rich in carbohydrates, proteins, and lipids which ultimately provide an array of biological products including biodiesel, bioethanol, biogas, and biobutanol [2,3]. The production of biologically based products, including bio-alcoholic fuels, is dependent upon a fermentation process known as the acetone-butanol-ethanol (ABE) fermentation process. During this chemical process, which relies upon bacteria fermentation, three different products are produced: acetone, butanol, and ethanol from the carbohydrate content in the algal biomass. The process depends upon bacteria from the Clostridia family and subsequently produces acetone, butanol, and ethanol in the following ratios: 3 parts acetone, 6 parts butanol, and 1-part ethanol [4]. *Clostridia* species such as *C. beijernickii* (Cb) and *C. acetobutylicum (Ca)* are the most studied biomass-metabolizing bacteria. Furthermore, members of the Clostridia family have the beneficial ability to use both hexose and pentose sugars found in biomass which can be converted to bio-alcoholic fuels [5–8]. Previous investigations [5–8] have successfully deployed the utilization of a fused bacteria to improve thermal stability and improved resistance to biobutanol toxicity. Fused bacteria strains used during the fermentation process have resulted in higher biobutanol yields for wheat straw and algal biomass.

Various oil extraction procedures have been developed for the removal of the lipid content found in different types of biomasses including algae. The effectiveness of utilizing a solvent for lipid extraction is dependent upon its volatility and chemical affinity towards the polar lipid [9]. When a microalgae cell is exposed to a non-polar solvent such as d-limonene or p-cymene, the goal of the solvent is to penetrate through the cell membrane into the cytoplasm and interact with the

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neutral lipids using Van der Waals forces [9]. A solvent-lipid complex is created that is subjected to a concentration gradient thus leading to the diffusion of the solvent-lipid complex outside of the cell membrane. The effectiveness of the penetration, strength of the Van der Waal forces and subsequent production of the solvent-lipid complex is subject to the chemical affinity of the solvent, volatility, and most importantly the Hansen Solubility Parameter (HSP) [9]. Although no standard procedure is followed at the industrial scale, the most commonly used method is chemical that is dependent upon toxic chemicals that pose significant environmental and human health risks. Consequently, there is a necessity to investigate the feasibility of alternative, green solvents. Replacing toxic chemicals with bio-based solvents can be a viable alternative and has the potential to improve oil extraction rates. Terpenes have been recognized a viable alternative and are naturally occurring solvents that are found in renewable feedstocks of citrus fruits and many other plants [10]. Furthermore, they are comprised of a variety of hydrocarbons that are optimal alternatives to petroleum solvents in various industrial applications. Relative to petroleum solvents, terpenes have a lower cost and lower toxicity [10]. Previous studies, investigating the use of terpenes in oil extraction of rapeseed and rice bran, have shown the promise of such bio-based solvents [10]. However, little research has been designated towards the examination of terpene-based oil extraction from algae.

The present study focuses on applying a greener approach to produce algal derived biofuels using novel Clostridal fusants and bio-based terpenes. Protoplast fusion technology was applied to develop novel fused bacteria strains of *Clostridia* sp. The fusants were exposed to UV radiation to produce desirable mutants used during the fermentation process to produce biobutanol using algal biomass. The algal biomass was subjected to various hydrolysis pre-treatments prior to the fermentation process. Additionally, this study also investigated the use of ultrasonication and a green Bligh and Dyer method using terpenes to extract oil from algal biomass. Oil extraction was achieved from virgin, untreated algal biomass and algal biomass recovered following the fermentation process. In the present study, fermentation was deployed prior to oil extraction because it has been recognized that fermentation processes do not affect the fatty acid profile and quality of the lipids [11,12]. Algal biomass showing the highest biobutanol production from fermentation was then examined for oil extraction process. Furthermore, the extraction conditions producing the highest oil extraction yield, when using virgin algal biomass, were applied to the recovered algal biomass.

2. Materials and methods

2.1. Materials

The following chemicals were ordered from Sigma-Aldrich and used as received; 4-Aminobenzoic Acid, Agar, Ammonium Sulfate ($(NH_4)_2SO_4$), Arabinose, Asparagine, β-glucosidase, Calcium Chloride ($CaCl_2$), Casein Hydrolysate, Casamino Acids, Cellulase, Cysteine Hydrochloride, d-Biotin, d-Limonene, Dipotassium Phosphate (K_2HPO_4), Ferrous Sulfate Heptahydrate ($FeSO_4.7H_2O$), Galactose, Gelatine, Glucose, Glycerol, Lysozyme, Mannose, Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$), Manganese Sulfate Pentahydrate ($MnSO_4.5H_2O$), Monopotassium Phosphate (KH_2PO_4), p-Cymene, Peptone, Poly ethylene glycol (PEG), Sodium Acetate ($C_2H_3NaO_2$), Sodium Chloride (NaCl), Starch, Sucrose, Sulfuric Acid (H_2SO_4), Thiamine Hydrochloride, Yeast, and Xylose. Algal biomass, classified as the freshwater strain *Stichococcus* (in the family *Prasiolaceae*), that produces significant level of lipids that can be used in pharmaceutical or fuel industries, was generously provided by Pond Technologies, located in Markham, Ontario, Canada. Algae was grown in photobioreactors (red LED light source) with an optimal temperature of 30°C with varying amounts of nutrients (nitrogen and phosphorus) depending upon the specific batch. Three different bacteria were used in the present study: *Clostridium beijernickii* (ATCC BA101) (Cb), *Clostridium acetobutylicum* (ATCC 4259) (Ca), and *Clostridium thermocellum* (ATCC 27405) (Ct). All were purchased from the American Type Culture Collection (ATCC), Manassas, VA 20108, USA.

2.2. Experimental methods

2.2.1. Pre-treatment and hydrolysis of algae

Algae were subject to different pre-treatments including physical, chemical, thermal, and enzymatic. Initially, all biomass samples were subject to physical treatment to increase surface area. Total of 2 g of algae was ground into fine powder using a mortar and pestle. Chemical treatments were applied which included algae soaked in 1% H_2SO_4 or 2% H_2SO_4. Enzymatic treatments, which included cellulase and β-glucosidase, were incubated for a total of 3 days at a temperature of 40°C. A control treatment of algae soaked in 50 mL distilled H_2O was also applied. Each treatment was replicated with a thermal treatment that consisted of 121°C for 20 minutes. Table 1 summarizes the different pre-treatments applied.

2.2.2. Clostridia strain development and protoplast fusion process

As per the ATCC, Reinforced Clostridial Medium (RCM) was the predetermined cultural media for the reproduction of *Ca* and *Cb*. The parent strains (*Ca, Cb*, and *Ct*) were subjected to protoplast fusion to form *CaCt* (*C. acetobutylicum* + *C. thermocellocum*) and *CbCt* (*C. beijernickii* + *C. thermocellum*). Despite the higher ABE yield of fused strains as indicated by [5–8], a major issue which limits the total biobutanol production is the toxicity of the biobutanol towards the fusants. Current toxicity levels reported in literature are at 13 g/L [13]. Therefore, strain enhancement can assist in improving toxicity rates. Through mutation and selection, a clostridial strain becomes more resistant to biobutanol and consequently improves biobutanol productivity [13]. A mutagenesis study involving UV light was conducted on the fused strains of *CaCt* and *CbCt* [5,6,8]. Previous mutagenesis studies [5,6,8] have indicated that mutated fusants can withstand greater levels of biobutanol (15 g/L). More details on medium preparation, protoplast fusion, and mutagenesis were published earlier [5,6,8].

2.2.3. Biobutanol production using algal biomass

Three different hydrolysis treatments were applied for the fermentation process for each of the fusants and parent strains. Hydrolysis treatments included cellulase and β-glucosidase, 1% H_2SO_4 in addition to combining 1% H_2SO_4 and cellulase and β-glucosidase. Total of 2 g of algae was utilized in each fermentation experiment. Two different fermentation procedures were applied in this study. Treatments that applied the use of enzymes prior to fermentation were designated as separate hydrolysis and fermentation (SHF) and treatments that did not utilize the addition of enzymes in the fermentation procedure were designated as simultaneous hydrolysis and fermentation (SSF). Separate fermentation procedures were used in the present study due to the variation in optimal temperatures required for efficient enzymatic activity and fermentation. The optimal temperature for cellulase and β-glucosidase activity is 40°C whereas the optimal temperature for fermentation is 45°C [4]. Both fermentation procedures used samples that were inoculated with the mutated fusant exposed to UV radiation showing highest cell count in the presence of 15 g/L of biobutanol. The parent strains of *Clostridia* were utilized in the fermentation process without any further mutagenesis. A sample from each fermentation jar was taken initially and then every 24 h for up to 120 h. Samples were stored in a -82°C freezer until further analysis and were later analyzed using High Performance Liquid Chromatography (HPLC) to examine the yield of biobutanol, sugars, and other solvents.

2.2.4. Algae oil extraction

Two primary methods were utilized separately and combined to define best oil extraction from algae feedstock. This was done in comparison with oil extracted from algae recovered from the fermentation experiments above. These methods include ultrasonication and a greener

Table 1
Impact of different pretreatment and hydrolysis conditions on sugar hydrolysate composition.

Experiment	Conditions	glucose (g/L)	xylose (g/L)	galactose (g/L)	mannose (g/L)	arabinose (g/L)	Total Sugars (g/L)
1	H_2O	4.12	2.00	1.12	1.90	1.56	10.71
2	H_2O*	4.41	2.02	1.20	2.00	1.60	11.22
3	1% H_2SO_4	8.01	4.73	1.57	4.01	1.70	20.02
4	1% H_2SO_4*	8.96	4.86	1.64	4.06	1.72	21.24
5	EZ-1	8.01	5.41	1.72	4.05	1.76	20.96
6	EZ-2*	8.60	5.99	1.76	4.27	2.68	23.30
7	EZ-3	11.04	5.82	1.84	4.35	2.72	25.78
8	EZ-4*	11.97	6.63	1.92	4.46	2.81	27.78
9	2% H_2SO_4	3.96	1.96	1.05	1.98	1.57	10.51
10	2% H_2SO_4*	3.99	1.98	1.08	2.04	1.59	10.69

* Thermal application of 121°C for 20 min.EZ- enzymatic treatment w/ cellulase and β-glucosidase.

extraction medium (Bligh and Dyer) using two different biobased terpenes that are extracted from plants and citrus peels (d- limonene and p-cymene). Extraction process was according to experimental procedures in Ref. [14].

Ultrasonication assisted oil extraction was carried out using a UP200Ht Handheld Ultrasonic Homogenizer (Hielscher Ultrasonics; Germany). Ultrasonic homogenizer was set to standard parameters of max frequency 26 kHz, 100% amplitude and 200 W of power. The greener solvents were used in a modified version of the Bligh and Dyer instead of the conventional chemicals such as chloroform and methanol. A solution of terpene with water created the polar and non-polar solvent ratio necessary for oil separation.

The combination oil extraction method used 2 g of algal biomass, suspended in distilled H_2O, and was subjected to 30 minutes ultrasonication. Following this, the algae suspension was centrifuged, and algae debris was removed. Remaining solution was then transferred to 50 mL d-limonene or p-cymene. The solution was allowed to sit for 90 min. Within this time, the oil and solvent were at the top while the water remained at the bottom of the test tube. A control sample was used with distilled H_2O with an exposure time of 40 min. Each experiment, involving d-limonene and p-cymene, was repeated to ensure validity. Following each experiment, d-limonene and p-cymene were removed from the oily phase using a rotary evaporator. The remaining solution was boiled at the respective solvent boiling point to ensure that all the solvent was remove. Solvent loss did not occur as the boiling point and thus evaporation of oil is significantly higher than that of both terpenes.

Following fermentation, the algal debris was recovered from the fermentation broth. The fermentation sample was centrifuged at 1000 rpm for 10 min and the algal debris was filtered and collected for drying prior to oil extraction. Algal debris was dried initially through water steam and then placed in an oven for 60 minutes at 60°C. Following this, the algal biomass was exposed to optimal conditions of ultrasonication (30 minutes, in distilled H_2O) followed by centrifugation and thus algae debris removal. After this, d-limonene was added to the remaining suspension and allowed to sit for 90 minutes. Next, the solvent was removed, and the oil quantity and oil yield were calculated as described in the next section.

2.2.5. Analytical procedures

Sugar and solvent composition were analyzed using pre-calibrated HPLC (Agilent 1260, USA) incorporated with an ion exchange pump, a pump series (Agilent 1290 Infinity II), an auto sampler (Agilent 1290 Infinity II) and refractive index detector (RID) (Agilent 1260 Infinity II). Two different columns were used: Shodex SP010 for measuring sugar concentration and Aminex HPX-87H for measuring solvent and acid concentrations. Each sample was centrifuged at 4,500 rpm for 25 minutes and double filtered through 0.2 μm PTFE- filters (Whatman, USA). A total of 10 μL from each dilute sample was injected into the column and circulated for 35 minutes at a flow rate of 0.6 mL/min. Concentrations were quantified from calibration curves that were developed from standard solutions of known concentrations.

Cell concentration was determined using a hemocytometer (Bright-Line, Hausser Scientific). The cell count method as described in [5,6,8] was used to determine the number of cells following each UV exposure and used to calculate the surviving fraction (SF) and the relative induced mutation frequency (RF). SF is the number of cells that survived the exposure to the UV radiation and RF is a proportion of the mutant strain in the cell population that had survived exposure to the UV radiation divided by the proportion of the mutant strain that was present but not exposed to the UV radiation. Cellulose and hemicellulose contents of the algal biomass were quantified as per the National Renewable Energy Laboratory (NREL) as described by [15]. Following oil extraction procedures, the cell disruption efficiency was calculated to determine the degree of cell rupture. It is calculated by the equation below as indicated by [16]:

$$n = \frac{(C_O - C_f)}{C_O}\ x\ 100$$

Where, Co is the initial cell count before the oil extraction method and C_f is the final cell count following the oil extraction method.

Oil quantification was determined after terpene removal [17]. The oil sample was removed using a spatula and placed in a pre-weighed Eppendorf tube (W1). The Eppendorf tube containing the oil was weighed on an analytical scale (W2). The final weight of the oil (W_O) was determined by the following equation:

$$W_O = W_2 - W_1$$

Based upon the weight of the oil, the oil yield was calculated as per the total amount of lipid content in the algae. The following equation was used to determine the oil yield:

$$Oil\ Yield\ \% = \left(\frac{W_o}{W_{AO}}\right) x\ 100$$

Where, W_O is the weight of the oil following oil extraction and solvent removal and W_{AO} is the actual weight of lipid in the algal biomass. Values of W_{AO} were calculated as 38.5% lipid content by weight of the total initial dry weight of *Stichococcus* algae [20].

2.2.6. Error analysis

All experiments were performed in triplicate, and reported data are the mean among these three readings. The standard deviation of biobutanol produced from algae was within the range of 0.1 to 0.4 and percent error within the range of 0.5% to 6.4%. The standard deviation of ultrasonication experiments was within the range of 0.01 and 0.2 and percent error within the range of 0.5% to 3.5%. The standard deviation of the modified Bligh and Dyer method was within the range of 0.05 to 0.025 and percent error within the range of 0.4% to 0.7%. The standard deviation from the combined extraction process 0.1 and percent error within the range of 0.4% to 0.5%. The standard deviation from the combined extraction process using the recovered algal biomass post-fermentation was 0.1 and the percent error was 0.4%.

3. Results and discussion

3.1. Pre-treatment and hydrolysis of algae

Table 1 indicates the individual and total sugar concentrations from algal biomass that were subject to different pre-treatment options. Treatment #8 resulted in the highest amount total reducing sugars (TRS) following chemical, thermal, and enzymatic applications (TRS = 27.78 g/L). Treatment #7 resulted in the second highest amount of TRS following chemical and enzymatic applications (TRS = 25.78 g/L). Thermal applications generally had a positive effect upon sugar release as total sugar concentrations were higher when exposed to heat when compared to no added heat.

Treatment #6 released the third highest amount of TRS following thermal, water and enzymatic application (TRS= 23.30 g/L). Thus, indicating that water and enzymes coupled with a thermal treatment can be rather efficient in breaking down the algal structure. Collectively, treatments #6, #7 and #8 suggest that enzymatic hydrolysis is integral for disintegration of the cellulosic structure and increasing the bioavailability of sugar monomers. Treatments #3 and #4 resulted in significantly higher TRS values when compared with treatments #9 and #10. This indicated that 1% acid treatment is far more efficient than 2% acid treatment [18]. This can be attributed to the less rigid structure of algae, which happens to be partially oxidized upon treatment with higher concentrations of sulfuric acid [19].

Physical components of biomass play an integral role in the effectiveness of the hydrolysis treatment as well as the availability of end products. Through hydrolysis, cellulose breaks down to glucose and hemicellulose breaks down to xylose, mannose, galactose, and arabinose which cumulatively yield the TRS potential. Lignin is also a defining characteristic of biomass. Unlike other agricultural feedstocks, algae contain little to low lignin and in this investigation, *Stichococcus* spp. of algae does not possess lignin. This significantly eliminates the recalcitrant component and allows for relatively easier disintegration of cellulose and hemicellulose to produce simple sugars necessary for fermentation. The crystalline nature of cellulose contributes to the overall recalcitrant nature of lignocellulosic material. Hemicellulose furthers this difficult characteristic because it acts as a physical barrier to the cellulose. Hemicellulose microfibers can be tightly bound and sheathed in a layer of pectin which subsequently act a strong physical barrier to the cellulose. Therefore, higher amounts of cellulose and hemicellulose, increase the recalcitrant nature and consequently require more energy for hydrolysis.

Algae are comprised of lipids that can also be extracted, in addition to sugars, to produce other biofuels such as biodiesel. Based on values indicated in literatures, the carbohydrate and lipid contents of *Stichococcus* are 38.9% and 38.5%; respectively [20]. This indicates that although the sugar potential of algae may be lower than other feedstocks [4, 21-23] it contains lipids that are not found in second-generation biomass. Lipids can be extracted to produce other energy containing fuels such as bio-oil and biodiesel. The addition of lipid and ability to produce different bio-based products increases the energy availability and overall economic potential of algae.

3.2. Protoplast fusants mutagenesis and biobutanol production fermentation

Fig. 1 illustrates the cell count following the UV mutagenesis study for both fusants in the absence and presence of biobutanol. When subjected to UV radiation, in the absence of biobutanol, *CaCt* began with a cell concentration of 26×10^5 cells/mL and then experienced a decline in cell concentration after 5-min time intervals. Following the 25-minute exposure, the cell concentration dropped to 0.5×10^5 cells/mL. When examining the effect of UV mutagenesis on *CbCt*, the fusant also experienced a decline in cell concentration following each 5-min interval. After the 25-min exposure to UV radiation, the cell concentration of *CbCt* dropped to 1.0×10^5 cells/mL. Prolonged UV exposure has a positive correlation with cell count due to the impact of excessive DNA damage [24]. When subjected to UV radiation, in the presence of butanol, *CaCt* had an initial concentration of 1.5×10^5 cells/mL. Following this, *CaCt* experienced an increased cell count reaching a peak at 10 minutes of 11.25×10^5 cells/mL and subsequently experienced a decline. Thus, indicating highest butanol resistance at an exposure time of 10 minutes. A similar pattern can be seen for *CbCt* in the presence of butanol. Initial cell concentration for *CbCt* was 2.25×10^5 cells/mL. Following this, cell count increased and reached a peak at 10 minutes (16.75×10^5 cells/mL) and subsequently experienced a decline. Like *CaCt*, the highest butanol tolerance was achieved at an exposure time of 10 minutes.

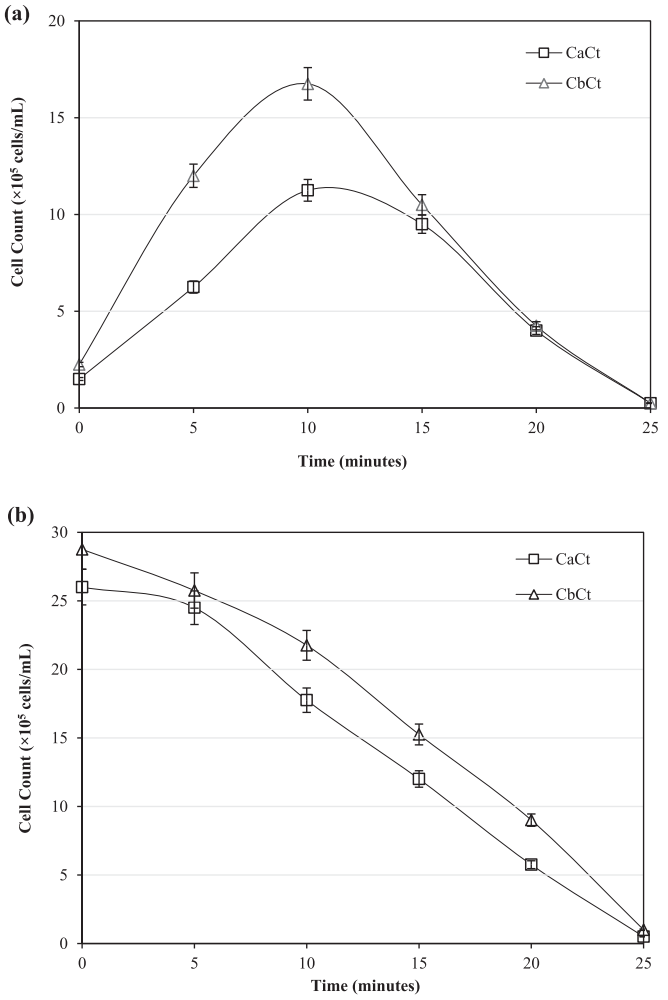

Fig. 1. Change in cell concentration following UV mutagenesis for fused strains *CaCt* and *CbCt*. (a) in the presence of butanol (b) in the absence of butanol.

Fig. 2 indicates the surviving fraction and relative induced mutation frequency of *CaCt* and *CbCt*, when exposed to UV mutagenesis in the presence of biobutanol. The surviving fraction decreased over the exposure time. Following the 15-min exposure time, the surviving fraction began to decrease significantly for both fusants indicating excessive DNA damage [24]. It is apparent that prolonged exposure to UV radiation implicated both fusants in a similar manner as the surviving fraction curves have similar shapes. However, it can be concluded that *CbCt* resulted in a higher surviving fraction following the full- time exposure of UV radiation. The surviving fraction after the 25-min exposure was 0.019 (equivalent of 0.5×10^5 cells/mL) and 0.035 (equivalent of 1×10^5 cells/mL) for *CaCt* and *CbCt*; respectively. The higher surviving fraction obtained with the CbCt demonstrates higher tolerance for UV radiation.

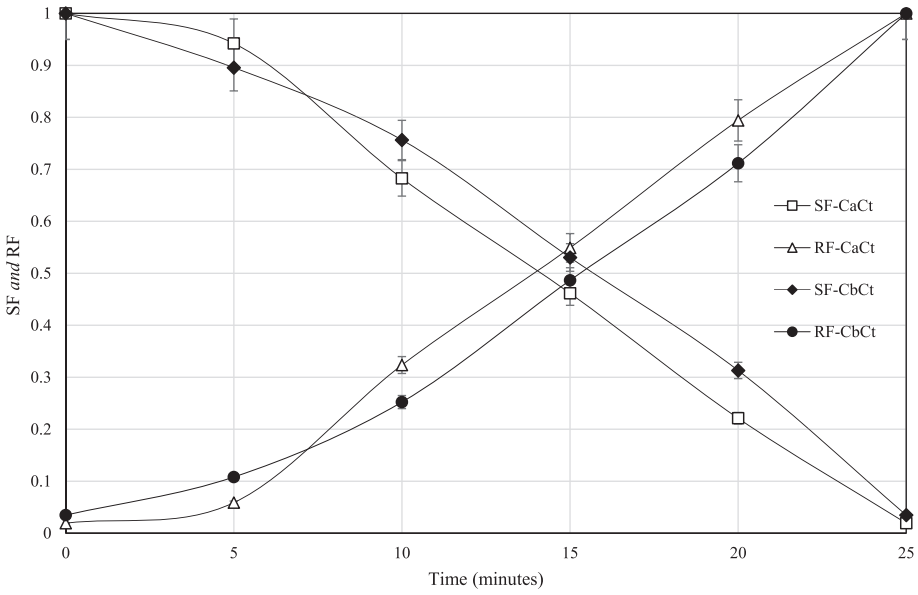

Fig. 2. Surviving fraction (SF) and relative frequency (RF) of induced mutation from UV mutagenesis of fusants *CaCt* and *CbCt* in the presence of biobutanol.

Table 2
Sugar concentrations and ABE concentrations following algal-based fermentation.

Experiment	Strain Used	Algae Pre-treatment/ Hydrolysis Conditions*	Sugar Compositions of Algal Hydrolysate Prior to Fermentation (g/L)						Sugar Consumed (%)	Concentrations of Fermentation Products (g/L)					Total ABE (g/L)
			Glucose	Xylose	Galactose	Mannose	Arabinose	Total Sugar		Acetone	Butanol	Ethanol	Acetic Acid	Butyric Acid	
1	*CaCt*	H_2O + EZ**	5.21	2.10	0.53	0.60	0.75	9.19	77.49	3.41	7.06	1.01	0.64	0.61	11.48
2	*CaCt*	1% H_2SO_4	4.80	2.03	0.40	0.50	0.56	8.29	75.31	3.61	7.39	1.10	0.73	0.66	12.1
3	*CaCt*	1% H_2SO_4+EZ	3.64	1.92	0.39	0.45	0.40	6.80	79.42	4.02	7.39	1.13	0.76	0.69	12.54
4	*CbCt*	H_2O + EZ	4.00	1.77	0.36	0.60	0.60	7.33	85.21	3.84	7.42	1.02	0.66	0.64	12.28
5	*CbCt*	1% H_2SO_4	3.61	1.66	0.35	0.46	0.49	6.57	79.29	4.41	7.84	1.17	0.78	0.74	13.42
6	*CbCt*	1% H_2SO_4+EZ	3.23	1.53	0.33	0.47	0.40	5.96	87.56	4.85	7.98	1.19	0.79	0.76	14.02
7	*Ca****	1% H_2SO_4+EZ	11.35	2.05	0.75	0.48	1.21	15.84	74.15	3.18	5.94	0.83	0.59	0.42	9.95
8	*Cb****	1% H_2SO_4+EZ	10.89	1.98	0.74	0.48	1.20	15.29	75.92	3.57	5.97	0.88	0.60	0.48	10.42
9	*Ct****	1% H_2SO_4+EZ	10.81	1.92	0.71	0.45	1.19	15.08	76.59	3.96	6.04	0.99	0.70	0.60	10.99

* At 121°C for 20 minutes.
** Enzymatic treatments were conducted after pretreatment w/ cellulase and β-glucosidase.
*** Used without mutagenesis as a control.

According to Table 2, fermentation experiments #3, #5 and #6 resulted in the lowest remaining sugar concentrations. This indicates that these three experiments were the most successful in releasing sugars necessary for ABE production. Experiment #6 resulted in the least amount of total sugars thus indicating that fusant *CbCt* with hydrolysis of algae (chemical, thermal, and enzymatic) was most successful in breaking down and utilizing majority of available sugars for ABE production.

Table 2 also shows that hydrolysis treatments incorporating thermal, chemical, and enzymatic applications and fusant *CbCt* resulted in the highest biobutanol concentration of 7.98 g/L. *CaCt*, coupled with the same hydrolysis treatments resulted in a lower biobutanol concentration of 7.39 g/L. Fusants resulted in a higher ABE concentration compared to single strains (experiments #7, #8 and #9). Thus, this can indicate the successful application of protoplast fusion and development of viable fusants for biobutanol production. Moreover, this can also elucidate that the combined properties of the two *Clostridia* (in the form of a hybrid) have a greater ability to degrade algal biomass and release the necessary sugars for the ABE fermentation pathway.

Parent strains achieved similar values of biobutanol production. However, *Ct* achieved the highest concentration relative to *Ca* and *Cb*. As per the resultant data, *Ct* has the highest cellulolytic rate as perceived from the biobutanol concentration and the total sugar consumption of 61.21%. Various studies have concluded that out of all cellulose degrading microorganisms, *Ct* exhibits the highest rate of cellulose degradation [25–27]. Therefore, utilizing its promising characteristics alongside advantageous qualities of *Ca* and *Cb*, the biobutanol production and sugar consumption of the fusants are superior.

Variety of factors influence the final biobutanol concentration which can explain differences noted in literature. Hydrolysis treatments, bacteria used in the fermentation process, and physical structures of the feedstock can all affect the resultant biobutanol concentration. [28] obtained a biobutanol concentration of 4 g/L using *Cb* which is 1.97 g/L less than the biobutanol concentration obtained in the present study, also using *Cb*. This variation in biobutanol concentration can be accounted for by different algae species and the specificity of the Clostridia strain. Although *Ulva Lactuca* has a generally higher carbohydrate range (50-60%) when compared to *Stichococcus* spp. (38.9%), the lower biobutanol concentration can be accounted for by the utilization of a mutated strain of *C. beijernickii* (BA101) used in the present study. This mutated strain has enhanced capabilities to break down starch and also has a higher butanol tolerance (0.017-0.021 kg butanol/L of fermentation broth) [29].

When compared with other feedstock, such as rice straw and seepweed, the present study was able to achieve higher biobutanol concentration when utilizing *Ca* during fermentation [4,29]. Although the carbohydrate concentration of rice straw (35-40%) is similar to that of *Stichococcus* spp., the lower biobutanol concentration can be a result of experimental differences including the lack of additional enzymes and variations in chemical treatments [29]. Similarly, the seepweed-based biobutanol concentration is lower than the biobutanol concentration ob-

Table 3
All oil extraction procedures and resultant oil concentration (g), oil yield (%), and cell disruption (%).

Experiment	Method	Condition	Time (minutes)	Oil Amount (g)	Oil Yield (%)	Cell Disruption (%)
1	Ultrasonication	in 50 mL d-limonene	10	0.04	5.14	23.46
2			20	0.24	30.85	49.38
3			30	0.30	38.56	62.96
4			40	0.225	28.92	37.04
5		in 50 mL p-cymene	10	0.035	4.50	22.22
6			20	0.14	17.99	37.04
7			30	0.27	34.70	55.56
8			40	0.20	25.71	50.62
9		in 50 mL water (control)	40	0.01	1.21	—
10	Green Solvent Bligh & Dyer	Direct solvent in d-limonene (45 mL d-limonene w/ 5 mL H_2O)	90	0.21	26.99	33.33
11		Direct solvent in p-cymene (45 mL p-cymene w/ 5 mL H_2O)	90	0.197	25.06	28.40
12	Combination	Ultrasonication 50 mL distilled H_2O followed by Green Solvent Bligh & Dyer (50 mL d-limonene)	30	0.36	46.27	77.78
13		Ultrasonication 50 mL distilled H_2O followed by Green Solvent Bligh & Dyer (50 mL p-cymene)	30	0.31	38.85	69.14
14	Combination Post-Fermentation	Ultrasonication 50 mL distilled H_2O followed by Green Solvent Bligh & Dyer (50 mL d-limonene)	30	0.506	65.04	—

tained in the present study. This can be accounted for by the lower carbohydrate concentration of seepweed (25%) [29]. However, when comparing the two parent strains *Ca* and *Cb, Cb* was more successful in breaking down wheat straw to yield a higher biobutanol concentration of 7.6 g/L [27]. When examining the effectiveness of *Cb* on biobutanol yield, it can be seen that wheat straw faired better as it resulted in a higher biobutanol concentration of 7.4 g/L when compared to algae (5.97 g/L). This can be explained by the higher cellulose and hemicellulose contents of wheat straw. Additionally, it can be assessed that the lower biobutanol yield from algae as compared with wheat straw can be explained by presence of inhibitors [26,29]. Inhibitors limit growth and also reduce the amount of available sugars necessary for ABE production [27,30]. Despite lower biobutanol concentrations, relative to other feedstock, such as wheat straw, the productivity of algae is significantly greater than wheat straw.

3.3. Algae oil yield

Table 3 shows results for the oil yield from all extraction experiments. Optimal time for maximum oil extraction through ultrasonication was 30 min for both terpenes examined. As shown in Table 3, ultrasonication after the 40-min exposure negatively impacted the oil yield as the yield decreased by 9.64% and 8.99% for d-limonene and p-cymene, respectively. Apparently, prolonged ultrasonication time can impede oil yield and quality through the formation of radicals [30]. Application of chemicals alongside with ultrasonication has been known to improve cell disruption efficiency and subsequent oil recovery [32,33]. Generally, higher sonication power that requires more energy can result in a higher oil recovery rate. However, the utilization of organic solvents reduces the amount of energy required for the process [9,31–33]. Furthermore, the physical properties of added chemicals can also influence the effect of ultrasonication upon cell disruption and subsequent lipid recovery. Physical properties such as surface tension and viscosity play a critical role in the severity of cavitation development and collapse. During the ultrasonication process, the hydrophobic solvents, d-limonene and p-cymene, gather at the bubble interface and can be either radical scavengers in a hot region surrounding the bubble or reduce the maximum temperature reached during the bubble's collapse due to the evaporation of the solute. Also, oscillations in the bubble allow for solvent evaporation and consequently degradation reactions of the cell wall. The more volatile a solvent, the more it is able to squeeze the bubble through the ability of increased evaporation [9,31–33]. D-limonene and p-cymene have fairly comparable physical properties [10]. However, the slightly more volatile state of d-limonene can account for the 3.86% increase in oil yield. The lower surface tension of d-limonene (25.8 dyne·cm^{-1}) relative to p-cymene (28.5 dyne·cm^{-1}) can result in enhanced ultrasonication effects [10,35].

Achard et al. [36] utilized similar sonication parameters and solvent combination on Chlorella spp. resulted in a significantly higher oil yield of 67.4%. The significant increase in oil yield can be attributed to the use of chloroform and methanol that allows for the usage of residual endogenous water in the microalgae cell that can subsequently allow for the complete extraction of both neutral and polar lipids [36]. Such solvents are highly toxic. The addition of ethanol in a solvent system can be permissible as a greener solvent. However, when comparing the oil recovery of terpenes in this study with that in the literature [37] that utilized a chloroform and ethanol system with 28.33% oil recovery for algal oil extraction, the present study achieved higher oil recovery of 38.66% and 34.70% for d-limonene and p-cymene; respectively. This can indicate the greater suitable of a single solvent system compared to a chloroform-ethanol system when combined with ultrasonication. Furthermore, this also indicates that combining ethanol with chloroform reduces extraction efficiencies of chloroform. Lastly, it can be concluded that perhaps terpenes fair better in terms of oil extraction efficiencies when compared with ethanol. According to Table 3, the green Bligh and Dyer method resulted in a higher oil extraction yield of 26.99% with the application of d-limonene relative to an oil extraction yield of 25.06% achieved through p-cymene. Further examination of the 1.93% increase in oil yield, accounted by d-limonene, can be attributed to its higher chemical affinity to the neutral lipids and consequently its lower HSP value [9].

Chloroform and methanol obtained an oil yield of 70.20% from *C. vulgaris* when using a direct solvent method [38]. However, when comparing direct solvent methods utilizing other greener solvents, the present study resulted in some variation compared with previous studies. Ionic liquid mixtures used with microalgae resulted in a lower oil extraction of 25.27% [39]. Greener solvent, cyclopentyl/methyl Ether (CPME), mixed with ethanol, resulted in a higher microalgae oil yield of 39.4% [40]. Consequently, this can specify that perhaps CPME, even combined with ethanol, in a direct solvent method, has a higher oil extraction efficiency than terpenes. However, the single terpene system in this study achieved higher oil extractions yields when compared to a hexane-methanol-water system that achieved a lower oil yield of 10.46% [41].

In the present study, the two-step oil extraction process in the preliminary study on algae resulted in a higher oil yield relative to the two separate methods. Combination processes, with d-limonene, resulted in an oil yield of 46.27%. Combining different extraction methods can result in a great efficiency of oil recovery [31–34]. As shown in this study, the combination of ultrasonication and a green Bligh and Dyer method increased the oil yield for the respective microalgae. Combination extraction have proven to an effective option for certain algal species that have more rigid cell walls and that require more energy for cell disruption. Farias et al. [42] evaluated the effects of five different extraction methods coupled with the Bligh and Dyer method. It was concluded that the ultrasound assisted Bligh and Dyer method applied to *C. vulgaris* resulted in the highest oil extraction of 52% [42]. The present study obtained oil extraction of 46.27% with d-limonene. This is only 5.73% lower than that of what has been reported in literature thus indicating the viability of using terpenes in conjunction with ultrasonication. Cell disruption efficiency of the combined extraction improved by 7.71% and 19.28%, respectively from ultrasonication and the green Bligh and Dyer single extraction; with application of d-limonene. Cell disruption efficiency of the combined extraction improved by 4.25% and 14.79%; respectively from ultrasonication and green Bligh and Dyer single extraction; with application of p-cymene.

Out of all oil extraction experiments, using algae recovered from the fermentation experiments resulted in the highest yield of 65.05% using d-limonene. The higher oil extraction yield, relative to using virgin algae in the preliminary study, can be accounted for by the exposure of hydrolysis treatments applied to the recovered algal biomass. This can be advantageous as it allows for the disintegration of lignocellulose biomass to expose sugars necessary for bio-alcoholic fuel production and provides accesses to the lipids in the interior of the cells [34]. However, destruction of the microalgae cell is still not sufficient to access lipid. Thus, a solvent, in this case a terpene, is still required to create a solvent-lipid complex to sufficiently release lipids. The recovered algal biomass following fermentation has also been exposed to the enzyme-secreting *Clostridia* fusant *CbCt* during the fermentation process. Enzymatic cell disruption is an advantageous approach because it incorporates highly selective disruption that permits the extraction of the desired products, mild reactions, and the absence of energy intensive drying steps [34]. Additionally, enzymatic pre-treatments are used in combination with solvents to breakdown the cell wall, release lipid bodies from the cellular structure and separate the lipids from the protein/lipid matrix [33]. Due to the high costs of enzymes, it has been recognized that improving enzymatic hydrolysis treatments is necessary in order to promote efficiency [33].

4. Conclusions

A protoplast fusion technique was applied to develop two fusants; *CaCt* and *CbCt*. The fusant strains were subjected to UV radiation to improve butanol tolerance and thermal stability. The fused strains and single Clostridia sp. (*Ca, Cb*, and *Ct*) were used in SSF and SHF processes of algal biomass. It was indicated that the fused strains produced significantly higher biobutanol concentrations when compared to the single stains. The biobutanol concentrations achieved by *CaCt* and *CbCt* were 7.39 g/L and 7.98 g/L, respectively. Ultrasonication in terpene solvent and the green Bligh and Dyer with terpenes were utilized separately for oil extraction. A combination of these two steps were applied to determine the effect of combining these extraction methods. Ultrasonication with terpenes resulted in an oil yield of 38.56% (d-limonene) and 34.70% (p-cymene). When compared with another green solvent system, comprised of chloroform and ethanol used with ultrasonication for algae oil extraction (28.33%), the single terpene system in the present study resulted in a higher extraction yield. The green Bligh and Dyer method with terpenes resulted in an oil yield of 26.99% (d-limonene) and 25.06% (p-cymene). Compared with literature, CPME/EtOH green solvent resulted in a higher oil extraction yield of 39.4% . However, compared to a mixture of ionic liquids, the present study obtained a higher extraction yield. The combination method achieved a higher oil yield of 46.27% using d-limonene. The combination extraction method of ultrasonication followed by a direct bio-based solvent also greatly influenced the cell disruption efficiency. Algae recovered from fermentation was subjected to the optimal two-step oil extraction procedure (ultrasonication=30 minutes, and green Bligh and Dyer method with d-limonene) and resulted in the highest oil yield of 65.04%. It was assessed that the algal biomass used in the fermentation process was exposed to various pre-treatments to breakdown the cell wall to expose the necessary sugars for bio-alcoholic fuel production. The chemical, thermal, and enzymatic pre-treatments coupled with the exposure to the enzyme-secreting *Clostridia* fusant, *CbCt,* resulted in a disintegrated cell wall and exposed lipid structure at a higher degree. The recovered algae post-fermentation resulted in higher oil extraction yield when compared with literature work that utilized chloroform and methanol. This shows the promise of bio-based terpenes as a viable alternative and the usage of the enzyme-secreting fusant *CbCt*.

Declaration of Competing Interest

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

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References

[1] L. Gouveia, Microalgae As a Feedstock for Biofuels, Springer, Heidelberg, Heidelberg, 2011 (accessed 2022), doi:10.1007/978-3-642-17997-6_1.
[2] International Energy AgencyState of Technology Review-Algae Bioenergy, International Energy Agency, Paris, France, 2017 https://advancedbiofuelsusa.info/state-of-technology-review-algae-bioenergy/.
[3] M. El-Dalatony, E.-S. Salama, M. Kurade, S. Hassan, S.-E. Oh, S. Kim, et al., Utilization of microalgal biofractions for bioethanol, higher alcohols, and biodiesel production: a review, Energies. 10 (2017) 2110, doi:10.3390/en10122110.
[4] N. Qureshi, B.C. Saha, R.E. Hector, S.R. Hughes, M.A. Cotta, Butanol production from wheat straw by simultaneous saccharification and fermentation using clostridium beijerinckii: part I—batch fermentation, Biomass Bioenergy 32 (2008) 168–175, doi:10.1016/j.biombioe.2007.07.004.
[5] S. Begum, Y. Dahman, Enhanced Biobutanol production using novel *Clostridial Fusants* in simultaneous saccharification and fermentation of green renewable agriculture residues, Biofuels Bioprod. Biorefin. 9 (2015) 529–544, doi:10.1002/bbb.1564.
[6] K. Syed, Y. Dahman, Novel *Clostridial Fusants* in comparison with co-cultured counterpart species for enhanced production of biobutanol using green renewable and sustainable feedstock, Bioprocess. Biosyst. Eng. 38 (2015) 2249–2262, doi:10.1007/s00449-015-1462-z.
[7] M. Ferhan, Y. Dahman, Novel thermostable *clostridial* strains through protoplast fusion for enhanced biobutanol production at higher temperature—preliminary study, AIMS Energy 4 (2016) 22–36, doi:10.3934/energy.2016.1.22.
[8] B. Mohtasebi, M. Maki, W. Qin, Y. Dahman, Novel fusants of two and three *Clostridia* for enhanced green production of biobutanol, Biofuels 12 (2019) 1017–1027, doi:10.1080/17597269.2019.1573605.
[9] R. Halim, M.K. Danquah, P.A. Webley, Extraction of oil from microalgae for biodiesel production: a review, Biotechnol. Adv. 30 (2012) 709–732, doi:10.1016/j.biotechadv.2012.01.001.
[10] C. Dejoye Tanzi, M. Abert Vian, C. Ginies, M. Elmaataoui, F. Chemat, Terpenes as green solvents for extraction of oil from microalgae, Molecules 17 (2012) 8196–8205, doi:10.3390/molecules17078196.
[11] A.K. Rai, R. Jini, H.C. Swapna, N.M. Sachindra, N. Bhaskar, V. Baskaran, Application of native lactic acid bacteria (LAB) for fermentative recovery of lipids and proteins from fish processing wastes: bioactivities of fermentation products, J. Aquat. Food Prod. Technol. 20 (2011) 32–44, doi:10.1080/10498850.2010.528174.
[12] P.S.M. Ruthu, A.K. Rai, N. Bhaskar, Fermentative recovery of lipids and proteins from freshwater fish head waste with reference to antimicrobial and antioxidant properties of protein hydrolysate, J. Food Sci. Technol. 51 (2012) 1884–1892, doi:10.1007/s13197-012-0730-z.
[13] T. Lütke-Eversloh, H. Bahl, Metabolic engineering of *Clostridium* acetobutylicum: recent advances to improve butanol production, Curr. Opin. Biotechnol. 22 (2011) 634–647, doi:10.1016/j.copbio.2011.01.011.

[14] B. Yang, Z. Dai, S.-Y. Ding, C.E. Wyman, Enzymatic hydrolysis of cellulosic biomass, Biofuels 2 (2011) 421–449, doi:10.4155/bfs.11.116.
[15] C. Ververis, K. Georghiou, D. Danielidis, D.G. Hatzinikolaou, P. Santas, R. Santas, et al., Cellulose, hemicelluloses, lignin and ash content of some organic materials and their suitability for use as paper pulp supplements, Bioresour. Technol. 98 (2007) 296–301, doi:10.1016/j.biortech.2006.01.007.
[16] R. Natarajan, W.M. Ang, X. Chen, M. Voigtmann, R. Lau, Lipid releasing characteristics of microalgae species through continuous ultrasonication, Bioresour. Technol. 158 (2014) 7–11, doi:10.1016/j.biortech.2014.01.146.
[17] P. Prabakaran, A.D. Ravindran, A comparative study on effective cell disruption methods for lipid extraction from microalgae, Lett. Appl. Microbiol. 53 (2011) 150–154, doi:10.1111/j.1472-765x.2011.03082.x.
[18] S. Kumar, R. Gupta, G. Kumar, D. Sahoo, R.C. Kuhad, Bioethanol production from gracilaria verrucosa, a red alga, in a biorefinery approach, Bioresour. Technol. 135 (2013) 150–156, doi:10.1016/j.biortech.2012.10.120.
[19] M.T. Nguyen, S.J. Sim, J.H. Lee, J. Lee, S.P. Choi, Hydrothermal acid pretreatment of Chlamydomonas reinhardtii biomass for ethanol production, J. Microbiol. Biotechnol. 19 (2009) 161–166, doi:10.4014/jmb.0810.578.
[20] edited by J.S. Burlew, J.S. Burlew, Algal Culture: From Laboratory to Pilot Plant Carnegie Institution of Washington, Washington, D. C., 1976. edited by.
[21] N. Stevulova, J. Cigasova, A. Estokova, E. Terpakova, A. Geffert, F. Kacik, et al., Properties characterization of chemically modified hemp hurds, Materials 7 (2014) 8131–8150, doi:10.3390/ma7128131.
[22] C. Wilén, A. Moilanen, E. Kurkula, Biomass Feedstock Analyses, 282, VTT Publications, Espoo, 1996.
[23] S.Q. Turn, B.M. Jenkins, L.A. Jakeway, L.G. Blevins, et al., Test results from sugar cane bagasse and high fiber cane co-fired with fossil fuels, Biomass Bioenergy 30 (2006) 565–574, doi:10.1016/j.biombioe.2005.12.008.
[24] H. Ikehata, T. Ono, The mechanisms of UV mutagenesis, J. Radiat. Res. 52 (2011) 115–125, doi:10.1269/jrr.10175.
[25] L.R. Lynd, H.E. Grethlein, Hydrolysis of dilute acid pretreated mixed hardwood and purified microcrystalline cellulose by cell-free broth fromclostridium thermocellum, Biotechnol. Bioeng. 29 (1987) 92–100, doi:10.1002/bit.260290114.
[26] D.B. Levin, R. Islam, N. Cicek, R. Sparling, Hydrogen production by clostridium thermocellum 27405 from cellulosic biomass substrates, Int. J. Hydrog. Energy 31 (2006) 1496–1503, doi:10.1016/j.ijhydene.2006.06.015.
[27] L. Lynd, W. Zyl, J. McBride, M. Laser, Consolidated bioprocessing of cellulosic biomass: an update, Curr. Opin. Biotechnol. 16 (2005) 577–583, doi:10.1016/j.copbio.2005.08.009.
[28] T. Potts, J. Du, M. Paul, P. May, R. Beitle, J. Hestekin, The production of butanol from Jamaica Bay Macro Algae, Environ. Prog. Sustain. Energy 31 (2011) 29–36, doi:10.1002/ep.10606.
[29] S.H. Zhao, T.S. Ma, H.B. Zhang, Butanol Production from Halophyte Seepweed Suaeda salsa by Simultaneous Saccharification and Fermentation, Asian J. Chem. 23 (2011) 5285–5528, doi:10.1186/s13068-015-0266-3.
[30] T. Ezeji, N. Qureshi, H.P. Blaschek, Production of acetone–butanol–ethanol (ABE) in a continuous flow bioreactor using degermed corn and clostridium beijerinckii, Process Biochem. 42 (2007) 34–39, doi:10.1016/j.procbio.2006.07.020.
[31] S.P.J. Kumar, V.G. Kumar, A. Dash, P. Scholz, R. Banerjee, Sustainable Green solvents and techniques for lipid extraction from microalgae: A Review, Algal Res. 21 (2017) 138–147, doi:10.1016/j.algal.2016.11.014.
[32] J. Harris, K. Viner, P. Champagne, P.G. Jessop, Advances in microalgal lipid extraction for biofuel production: a review, Biofuels Bioprod. Biorefin. 12 (2018) 1118–1135, doi:10.1002/bbb.1923.
[33] M. Mubarak, A. Shaija, T.V. Suchithra, A review on the extraction of lipid from microalgae for biodiesel production, Algal Res. 7 (2015) 117–123, doi:10.1016/j.algal.2014.10.008.
[34] H. Sati, M. Mitra, S. Mishra, P. Baredar, Microalgal lipid extraction strategies for biodiesel production: a review, Algal Res. 38 (2019) 101413, doi:10.1016/j.algal.2019.101413.
[35] R. Ciriminna, M. Lomeli-Rodriguez, P. Demma Carà, J.A. Lopez-Sanchez, M. Pagliaro, Limonene: a versatile chemical of the bioeconomy, Chem. Commun. 50 (2014) 15288–15296, doi:10.1039/c4cc06147k.
[36] J.M. Roux, H. Lamotte, J.L. Achard, An overview of microalgae lipid extraction in a biorefinery framework, Energy Procedia 112 (2017) 680–688, doi:10.1016/j.egypro.2017.03.1137.
[37] F.G. Naghdi, L.M. González, W. Chan, P.M. Schenk, Progress on lipid extraction from wet algal biomass for biodiesel production, Microb. Biotechnol. 9 (2016) 718–726, doi:10.1111/1751-7915.12360.
[38] S.A. Choi, J.S. Lee, Y.K. Oh, M.J. Jeong, S.W. Kim, J.Y. Park, Lipid extraction from Chlorella vulgaris by molten-salt/ionic-liquid mixtures, Algal Res. 3 (2014) 44–48, doi:10.1016/j.algal.2013.11.013.
[39] S.A. Choi, Y.K. Oh, M.J. Jeong, S.W. Kim, J.S. Lee, J.-Y. Park, Effects of ionic liquid mixtures on lipid extraction from Chlorella vulgaris, Renew. Energ. 65 (2014) 169–174, doi:10.1016/j.renene.2013.08.015.
[40] I. Santoro, M. Nardi, C. Benincasa, P. Costanzo, G. Giordano, A. Procopio, et al., Sustainable and selective extraction of lipids and bioactive compounds from microalgae, Molecules 24 (2019) 4347, doi:10.3390/molecules24234347.
[41] E. Ryckebosch, K. Muylaert, I. Foubert, Optimization of an analytical procedure for extraction of lipids from microalgae, J. Am. Oil Chem. Soc. 89 (2011) 189–198, doi:10.1007/s11746-011-1903-z.
[42] G.S. Araujo, L.J.B.L. Matos, J.O. Fernandes, S.J.M. Cartaxo, L.R.B. Gonçalves, F.A.N. Fernandes, W.R.L. Farias, Extraction of lipids from microalgae by ultrasound application: Prospection of the optimal extraction method, Ultrason. Sonochem. 20 (2013) 95–98, doi:10.1016/j.ultsonch.2012.07.027.