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SHORT COMMUNICATION

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ENHANCING LIPID EXTRACTION FROM GREEN MICROALGAE *Chlorella* SP. USING A DEEP EUTECTIC SOLVENT PRETREATMENT

Abstract

In recent years, many researchers have focused on microalgae as a potential source of lipids for various purposes. To improve the lipid yield, different biomass pretreatments have been investigated. The aim of this work was to determine the effect of an ultrasound and deep eutectic solvent (DES) pretreatment on lipid yield from green microalgae Chlorella sp. The chosen DESs were choline chloride:urea (ChCl:U), choline chloride:glycerol (ChCl:G) and choline chloride:acetic acid (ChCl:Aa), all in the mole ratio of 1:2. Lipids were extracted from the pretreated and untreated biomasses by Bligh and Dyer's method. The results showed that the lipid yields for the untreated and ultrasound-pretreated biomass were 39 and 48%, respectively. The pretreatments with ChCl:U, ChCl:G and ChCl:Aa resulted in lipid yields of 51, 46 and 40%, respectively. Therefore, the use of efficient and environmentally friendly DESs for the microalgae biomass pretreatment resulted in a higher lipid yield.

Keywords: Chlorella sp., deep eutectic solvents, lipid extraction.

Microorganisms such as yeasts, bacteria, and microalgae are often investigated as alternative sources of lipids due to their high productivity and rapid growth. Under specific conditions, some microalgae species can accumulate 30-70% of the lipids in dry biomass [1]. Among them, green microalgae *Chlorella* spp. and *Scenedesmus* spp. are considered potential feedstock for biodiesel production [2]. Also, due to its cell composition, this microalgae can be used for food and health products production. *Chlorella* spp. have the ability of fast growth on the commercial scale and great biomass and lipid accumulation. [3].

Classic extraction methods, which involve one or a mixture of different polar solvents, have been widely used for lipid extraction from microalgae biomass [4]. To improve lipid extraction efficiency, many studies have been directed towards the use of a combination of both mechanical and chemical extraction methods. Such processes involve the use of conventional solvents in combination with the various types of biomass pretreatment (ultrasound, microwave, enzymatic, ionic liquids, etc.) [5]. Methods used for cell disruption, including bead mill, microfluidization, microwave treatment, etc., are energy consuming, cannot be easily scaled up and often require extreme conditions of temperature and pressure. The small size of microalgae cells additionally aggravates the disruption process [6]. Thus, the use of new methods, including ultrasound, ionic liquids or integration of green solvents and mechanical treatments, is being extensively investigated [4].

The use of ultrasound is widely spread in the food industry. Originally, it was used as a control tool, but the interest increased as it has been proven to be an effective method for improving the extraction process due to the cell wall destruction by the cavitation mechanism [7]. The use of ultrasound as a biomass pretreatment in the process of lipid extraction from microalgae has been the subject of numerous studies [8,9,10]. Other pretreatments have also been used to obtain higher yields of microalgae lipids: microwave, bead milling, heat [9], chemical, and enzymatic pretreatments, as well as high-pressure homogenization [10]. Also, supercritical fluid extraction has gained much interest in the extraction of functional compounds from microalgae biomass [11].

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Some ionic liquids, being safe and environmentally friendly solvents, are also used for lipid extraction from microalgae biomass. The mode of the action of ionic liquids includes the interaction between the specific functional groups of the cell wall and the charged particles of ionic liquids. Deep eutectic solvents (DESs) represent a new generation of ionic liquids, usually formed by mixing organic salts (such as choline chloride, ChCl) and hydrogen bond donors (amides, amines, alcohols, and carboxylic acids), resulting in a mixture with a lower melting point [12,13]. DES can form hydrogen bonds with biopolymers like cellulose which are present in the microalgae cell walls. Cellulose microfibrils are connected with hydrogen bonds which are being disrupted in the presence of DES. Thus, the cell wall structure becomes disrupted allowing more efficient extraction of lipids [14]. Simple preparation, low price, low toxicity, and high biodegradability are some of the advantages of DESs compared to classical ionic liquids [15]. In line with this, DESs pretreatment has a high potential for the green algae lipid extraction [16]. In addition, DESs can be used to extract various functional compounds from different plant materials [17].

The use of DESs for biomass pretreatment is an environmentally acceptable and safe process, which involves cheap, biodegradable, and non-toxic substances. Quaternary ammonium salt ChCl is included in the majority of DESs. The combination of ChCl with other compounds (carbohydrates, carboxylic and amino acids) is almost unlimited [18].

This work dealt with the use of several ChCl-based DESs, such as ChCl:urea (ChCl:U), ChCl:glycerol (ChCl:G), and ChCl:acetic acid (ChCl:Aa), and ultrasound for pretreating a freshwater green microalgae (*Chlorella* sp.) biomass to enhance lipid extraction The main goal was to compare the effect of the applied pretreatment methods of the lipid extraction efficiency

MATERIAL AND METHODS

Chemicals

ChCl (98.0%) was provided from Sigma Aldrich (St. Louis, USA), while glycerol (Ph. Eur. grade) was purchased from MeiLab (Belgrade, Serbia). Both urea (99.5%) and glacial acetic acid (>99.8%) were obtained from Zorka (Šabac, Serbia). All chemicals were used as received, without any purification.

Microalgae cultivation

Microalgae *Chlorella* sp. was isolated from standing water from Leskovac and identified [19]. The microalgae was cultivated in the BBM medium (g/dm^3): $NaNO_3$ 0.249; $CaCl_2{\cdot}2H_2O$ 0.0250; $MgSO_4{\cdot}7H_2O$ 0.075; K_2HPO_4 0.072; KH_2PO_4 0.175; NaCl 0.025; EDTA 0.16; KOH 0.077; $FeSO_4{\cdot}7H_2O$ 0.012; H_3BO_3 0.028; $ZnSO_4{\cdot}7H_2O$ 0.019; $MnCl_2{\cdot}4H_2O$ 0.004; MoO_3 0.002; $CuSO_4{\cdot}5H_2O$ 0.004; $Co(NO_3)_2{\cdot}6H_2O$ 0.001. Inoculum was prepared in 0.5 dm^3 BBM for 28 days at 27 °C. An appropriate volume of the inoculum was transferred to the 2 dm^3 sterile BBM medium (autoclaved for 15 min at 121 °C), to obtain an initial optical density of 0.05 at 680 nm. The microalgae cultivation flasks were fixed on a rotary shaker (PSU-20i, Biosan, Latvia, EU), 150 min^{-1}, at constant illumination (2000 lux) and 27 °C for 40 days.

Preparation of DESs

DESs were prepared as described elsewhere [20]. ChCl was mixed with the selected donors of hydrogen bonds (glycerol, urea, or acetic acid) in a 1:2 mole ratio in a round-bottomed flask placed on a rotary evaporator and held at 75 °C within 2 h (until the homogeneous, transparent liquid was formed). After preparation, the obtained DESs were kept in the well-closed glass bottles in a $CaCl_2$-containing desiccator. All DESs were viscous, homogeneous, and colorless. Only ChCl:U DES turned into a white semi-solid upon cooling.

Pretreatments

Algal biomass was first centrifuged (2-6E, Sigma, Germany) and then dried under vacuum at 40 °C (V50, Kambič Anton, Semič, Slovenia). The dried biomass (100 mg) was ultrasonically pretreated in an ultrasound water bath (ViMS electric, Sonic 4 GT, 250 W, 50 Hz) for 10 min at room temperature. The DES pretreatment was performed according to the method described by Tommasi *et al.* [15]. The DES (1 cm^3) was added to the pretreated dried biomass and stirred by a magnetic stirrer (MS-H280-Pro, China) for 24 h at room temperature. Then, distilled water (3 cm^3) was added and the biomass was separated by centrifugation at 3900 rpm for 12 min. The biomass was washed with distilled water three times and the collected aqua phase containing the DES (aDES phase) was collected to determine the lipid content.

Lipid extraction

Lipids were extracted from the untreated and pretreated biomass, as well as from the aDES phase. The extraction was performed in duplicate according to Bligh and Dyer's method [21] with a mixture of chloroform and methanol (2:1 volume ratio). After adding the solvent mixture (6 cm^3), the suspension of the biomass was stirred (MS-H280-Pro, Biobase,

China) for 2 h at room temperature. The lipid content in the aDES phase was determined in a similar way with the addition of 12 cm^3 of the solvent mixture. Then, the resulting suspension was transferred to a separation funnel and distilled water (6 cm^3) was added. The chloroform layer containing lipids was transferred to a pre-weighed flask and dried under a nitrogen stream. The lipid content was determined gravimetrically.

Statistical analysis

Statistical comparisons were carried out by one-way ANOVA followed by Tukey's multiple comparison test using the SPSS 26.0 (IBM, USA). Differences were considered significant when the *p*-value was lower than 0.05.

RESULTS AND DISCUSSION

The biomass of *Chlorella* sp. was pretreated by the ChCl-based DESs or ultrasound to enhance lipid yield. The lipid yields achieved from the treated and untreated biomass are compared in Figure 1. The total lipid yield in the samples treated with the DESs includes the sum of the lipid yields from both the biomass and the aDES phase.

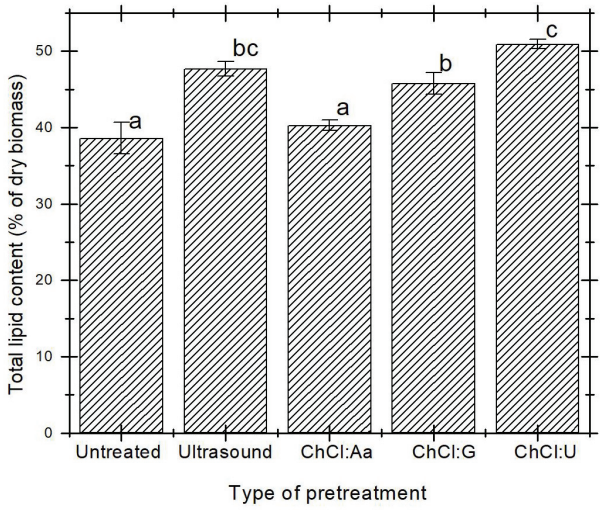

Figure 1. Total lipid yield from the untreated and Chlorella sp. biomass treated with ultrasound, ChCl:U, ChCl:G, and ChCl:Aa. Error bars represent standard deviation. Average values marked with different letters are significantly different according to one-way ANOVA ($p < 0.05$).

Statistical analysis of the results indicated significant difference between the untreated sample and samples treated with ChCl:U, ChCl:G and ultrasound. On the other hand, the lipid yield of the sample treated with ChCl:Aa was not significantly different compared to the untreated biomass. Based on the statistical analysis, the lipid yield of the samples treated with different DES was significantly different ($p < 0.05$) indicating the potential of the use of different DES pretreatments.

The extraction of lipids from the untreated biomass of *Chlorella* sp. resulted in a yield of 39%, which agreed with the results of Danilović *et al.* [19]. Under similar extraction conditions, a lower lipid yield of 26% was obtained from microalgae *C. vulgaris* [21]. A higher lipid yield from *Chlorella* sp. biomass (46%) was achieved with a mixture of chloroform and methanol (1:2) [22], while the same extracting agent applied to the *C. sorokiniana* biomass resulted in a lipid yield of only 19% [23].

The ultrasound pretreatment of the biomass of *Chlorella* sp. significantly increased lipid yield compared to the untreated biomass, with the final average value of 48%. Similar results were obtained by the ultrasound-assisted extraction of the wet *C. vulgaris* biomass (41%) [24] and a mixed microalgae culture (46%) [8]. In contrast, Lee *et al.* [25] obtained a lower lipid yield of only 6% from the phototrophically cultivated *C. vulgaris* in the BG11 medium despite the use of the ultrasound pretreatment. Another study has shown a lower efficiency of the combination of the ultrasound pretreatment and the Soxhlet method, applied to the microalgae *Nannochloropsis oculata*, with the lipid yield of 8% [26]. In the same research, a combination of ultrasound and the Folch method gave a higher lipid yield (24%). The use of ultrasound for pretreating of microalgae biomass increases the extracted lipid yield. However, the disadvantage of this mechanical pretreatment is reflected in their aggressive action to the cell wall, which accelerates the extraction of undesirable compounds (carbohydrates, protein, pigments) and reduces the purity of the final product [13].

Total lipid yields from the biomass of *Chlorella* sp. treated with ChCl:U, ChCl:G and ChCl:Aa were 51, 46 and 40%, respectively. The total lipid yield involved the sum of the lipid yields from the biomass and the aDES phase. The application of ChCl:U and ChCl:G resulted in a significantly higher yield compared to ChCl:Aa. Other studies showed that the combination of *Chlorella* sp. pretreatment with the ChCl:oxalic acid (ChCl:O) (mole ratio 3:2), ChCl:ethylene glycol (ChCl:EG) (mole ratio 6.5:2), and urea-acetamide (U>A) (mole ratio 5:1) DESs and the Bligh-Dyer extraction method (ethyl acetate:ethanol, molar ratio 1:1) gave total lipid yields of 15, 14 and 14%, respectively [16]. A significantly lower lipid yield of 0.9% was reported for the lipid extraction from the microalgae *Phaeodactylum tricornutum* treated with

ChCl:U (mole ratio 1:2). The same microalgae was treated with several other DESs, such as ChCl-O (mole ratio 1:2), ChCl:levulinic acid (ChCl:L, mole ratio 1:2), ChCl:EG (mole ratio 1:2), and ChCl:sorbitol (ChCl:S, mole ratio 1:1), where the lipid yields obtained by the dimethyl carbonate extraction were 14, 13.5, 12 and 11.5%, respectively [15].

A comparison of the lipid yields from the biomass treated with DESs and the aDES phase indicated that a small amount of the lipids remained in the aDES phase. The lipid content of the aDES phase after the application of ChCl:U was 1.6% of the total isolated lipid. When the ChCl:G and ChCl:Aa DESs were used, the lipid content in the aDES phase was 1.0 and 0.6% of the total lipids, respectively which agreed with the lipid yields from the aDES phase in the case of the microalgae *Phaeodactylum tricornutum* pretreated by the ChCl-O (3:2), ChCl-EG (6.5:2) and U-A (5:1) DESs (0.5, 1.75 and 1.0%, respectively) [15]. Therefore, the lipid content in the aDES phase had no significant effect on the total lipid yield of microalgae biomass treated with the DESs.

CONCLUSIONS

The lipid yield from the untreated biomass of *Chlorella* sp. was 39%. Compared to the untreated biomass, the use of ultrasound and ChCl:U and ChCl:G DES resulted in a significantly higher lipid yield. The highest lipid yield (51%) was obtained using a ChCl:U DES. The lipid yields obtained from the aDES phases made less than 2% of the total extracted lipids, thus not affecting the total lipid yield significantly. Although the application of ultrasound gave similar results, the use of DESs is more appropriate due to the possibility of high-purity lipids production, which can be further used for biodiesel production or animal feeding. Due to their low price and easy preparation, DESs have a promising commercial application for pretreating the microalgal biomass and obtaining a high yield of functional compounds from them. Due to the great variety of deep eutectic mixtures, their impact on lipid yield from different oleaginous microalgae should be further investigated.

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NAUČNI RAD

POBOLJŠANJE EKTRAKCIJE LIPIDA IZ ZELENIH MIKROALGI *Chlorella* SP. PREDTRETMANOM DUBOKIM EUTEKTIČKIM RASTVARAČIMA

Poslednjih godina, mnoga istraživanja su fokusirana na upotrebu mikroalgi kao potencijalnog izvora lipida za različite namene. Različiti predtretmani biomase se primenjuju kako bi se poboljšao prinos. Cilj ovog rada je utvrđivanje uticaja predtretmana ultrazvukom i dubokim eutektičkim rastvaračima na prinos lipida iz zelenih mikroalgi Chlorella sp. U tu svrhu korišćeni su eutektički rastvarači holin-hlorid: urea (ChCl:U), holin-hlorid:glicerol (ChCl:G) i holin-hlorid: sirćetna kiselina (ChCl: Aa) u molskom odnosu 1:2. Lipidi su ekstrahovani iz prethodno obrađene i neobrađene biomase Bligh-Dyer metodom. Rezultati su pokazali da je prinos lipida za netretiranu biomasu 39%, a za ultrazvučno tretiranu biomasu 48%. Predtretmani biomase sa ChCl:U, ChCl:G i ChCl:Aa dali su prinosa lipida od 51, 46 i 40%, redom. Može se zaključiti da upotreba efikasnih i ekološki prihvatljivih dubokih eutektičkih rastvarača u predtretmanu biomase mikroalgi rezultira većim prinosom lipida.

Ključne reči: Chlorella sp., duboki eutektički rastvarači, ekstrakcija lipida.