ULTRASONIC PRETREATMENT OF MICROALGAL BIOMASS TO ENHANCE THE ENZYMATIC HYDROLYSIS FOR SUGAR RECOVERY

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Abstract

From the biotechnological point of view, microalgae are potential candidate of renewable and sustainable biomass resources for high-added value compounds such as bioethanol and enzymes due to their high levels of carbohydrates and low levels of lignin. There still remains significant room for improving the production of fermentable sugars from biomass in a cost-effective way that raises enzymatic saccharification efficiency. Although extraction of polysaccharides to convert fermentable sugar from microalga by acid or alkali pretreatments, and enzymatic hydrolysis are extensively studied, few studies exist that explore the use of ultrasonication (US) as emerging technology for the extraction of fermentable sugars (FSs) from microalgae biomass. To best of our knowledge no work has been reported on usage of ultrasonication as an extraction technique for the bioconversion of microalgae. The objective of the present study was to determine the effect of simultaneous ultrasonication and enzymatic hydrolysis on extraction of fermentable sugars from microalga biomass. C. vulgaris *cultivated with red light in photobioreactors is efficient in producing algal biomass and sugar. The ultrasonication pretreatments were carried out in a glass bottle containing 0.3 g of dry microalgae in 100 ml of dilute acid solution. Based on preliminary experiments, the ranges in the pretreatment were selected for the variables of sulphuric acid (H_2SO_4) concentration (1, 2 and 3% (v/v)), amplitude (60, 75 and 90), and pretreatment time (10, 20 and 30 min). Then, enzymatic saccharification (cellulose and Viscozyme L at 50℃ and 120 rpm for 48 h) were employed. Increased amplitude provided significant amount of sugar released. Among the variables studied, amplitude, acid concentration and time showed significant effects ($p<0.05$) on sugar released from alga biomass. The sugar released from alga pretreated biomass ranged at 158.14-447.09 mg/g dry weight microalga. The highest amount of biomass (0.38 g/L) and sugar released (447.09 mg/g) was obtained from simultaneous ultrasonication and enzymatic hydrolysis of microalga at a 75% amplitude, 2% H_2SO_4 and 20 min after 48 h. Thus, the ultrasonication appeared to be a successful alternative method when compared to the conventional pretreatment methods.*

Keywords: *microalgae, ultrasonication, enzymatic hydrolysis, reducing sugar*

1. INTRODUCTION

Microalgal carbohydrates have emerged as a promising alternative to traditional fermentation processes, contributing to the advancement of third-generation biofuels (Silva and Bertucco, 2016). The predominant sugars found in microalgal biomass are glucose polymers, such as cellulose, starch, or glycogen (in the case of cyanobacteria). However, for efficient fermentation, these sugars need to be hydrolyzed (Vitova et al., 2015). Among the various algal hydrolysis methods, dilute acid and enzymatic procedures have demonstrated superior efficiency. The acid pretreatment method has some drawbacks, including the requirement for a large amount of chemicals during the process, the need for pH adjustment before fermentation, and the formation of a high amount of inhibitory salts, which can hinder the action of yeasts (Silva et al., 2018). In contrast, enzymatic processes, while offering a more specific and mild treatment at moderate temperatures and pressures, come with the disadvantage of high enzyme costs. However, to employ enzymatic hydrolysis successfully, a pretreatment to enhance carbohydrate accessibility for enzymatic attack becomes necessary(Silva and Bertucco, 2016).

Several algal cell disruption methods have been evaluated, such as ultrasonication, bead beating, microwave treatment, osmotic shock (using NaCl), and autoclaving (at 121°C) (Kurokawa et al., 2016). Ultrasonication stands out due to its ability to disrupt cells at relatively low temperatures compared to microwave and autoclaving methods. It also offers faster extraction, suits all cell types, and does not require beads or chemicals, thus reducing production costs (Kurokawa et al., 2016; Byreddy et al., 2015). For example, researchers have found that applying ultrasonication at 30W and 20 kHz for 30 minutes to *Chlamydomonas fasciata* effectively extracted all intracellular starch (Asada et al., 2012). Similarly, using 130W and 40 kHz for 25 minutes, Harun and Danquah (2011) achieved a 62.8% glucose hydrolysis yield from *Chlorococcum*. Kim et al. (2014) reported 75% sugars recovery in *Chlorella vulgaris* after subjecting it to ultrasonication at 24 kHz and 40% amplitude for 15 minutes. In contrast, *Chlorella sp*. KR-1 showed only 27.4% hydrolyzed sugars (Lee et al., 2015), and *Chlorella homosphaera* (both unpretreated biomass) exhibited 47% of total glucose in biomass (Rodrigues et al., 2015), highlighting the importance of pretreatment. Moreover, research efforts are currently focused on optimizing parameters to identify the most energy-efficient pretreatment method for algal materials (Luo et al., 2014).

The objectives of this study included: (1) to explore the effects of ultrasonication process conditions (amplitude, acid concentration and treatment time) on maximum reducing sugar release, (2) to analyze potential of ultrasonication followed by enzymatic hydrolysis for reducing sugar release, (3) to apply BBD-RSM to quantify the effect of maximum reducing sugar release.

2. MATERIALS AND METHODS

2.1. Microalga cultivation

The microalga, *C. vulgaris,* was kindly provided by Dr. Muge Hosoglu from Department of Food Engineering, Canakkale 18 Mart University, Turkey. Based on the previous experiments, microalgae was cultivated in single beam photobioreactors (PBR) including modified BG-11 medium and set to 30 ºC with red light of 4000 lux under continuous illumination (12:12 light:dark cycle). CO_2 was supplied 11L/min daily by CO_2 gas tube installed with a filter (0.45 µm). All media were prepared and autoclaved at 121ºC for 20 min. The cultivation time for experiment was 7 days with an initial biomass of *C. vulgaris* of approximately 0.14 g/L. The fans were positioned 2 cm away from the PBR to avoid damage to microalgae by overheating. Light intensity was measured with a lux meter. The PBR was placed in cabinet covered with an aluminum foil to minimize the effect of light disturbance.

2.2. Ultrasonication pretreatment method

The Optic Ivymen Systems CY-500 homogenizer (Hielscher, Germany) with the standard probe of 5.6 mm diameter, a 500 W power and 20 kHz frequency was used to leach reducing sugar. Extraction temperature was kept constant using a water bath. The sample was placed into a 100 ml beaker, and an ultrasonic probe was dipped at most 1.5 cm depth into the extraction media. The grounded microalgae biomass (0.3 g) was pretreated with 87 ml H_2SO_4 solution at the concentrations of 1, 2 and 3% (w/v), the amplitudes of 60, 75 and 90%, and the pretreatment times of 10, 20 and 30 min according to the experimental conditions.

2.3.Enzymatic hydrolysis

Recovered dried algal residues after ultrasonication pretreatment method were hydrolyzed using cellulase and Viscozyme L enzyme cocktail (Sigma-Aldrich, Denmark) containing cellulase and xylanase enzymes. Enzymatic hydrolysis step was carried out at 50 ºC, 130 rpm and pH 5.0 for 48 h. After enzymatic hydrolysis, the samples were centrifuged at 10.000 rpm for 10 min, and the supernatant was used to estimate reducing sugar by DNS method.

2.4.Analysis

2.4.1. Determination of reducing sugar content-DNS method

Reducing sugar content of the dried microalgal pellet was estimated using DNS method (Miller, 1959). A calibration curve for this method was prepared using analytical grade D-glucose (Merck Chemical companies, Deisenhofen, Germany) solutions at 0.15-1.00 g/L concentrations.

2.5. Experimental design and optimization

Statistical analyses were conducted to test the mean significant differences in reducing sugar release using US conditions in terms of RS as a function of H_2SO_4 concentration, pretreatment time, and amplitude. Optimization was carried out using the Box-Behnken Design (BBD) as the response surface methodology with a quadratic model. The chosen ranges of factors for RS production using US were selected as amplitudes of 60, 75 and 90%, H_2SO_4 concentrations of 1, 2 and 3%, and pretreatment times of 10, 20 and 30 min (Table 1). All the statistical analyses were performed using MINITAB 17.0 (Minitab Inc. State College, PA, USA). To validate the models, additional experiments were conducted in triplicate under the optimal conditions of RS using US. Analysis of variance (ANOVA) and regression models were performed at 95% confidence interval ($p < 0.05$) to define the significant terms of the predictive model. ANOVA was performed to determine the statistically significant effects of the three predictors ($p < 0.05$). Multiple comparisons were made using Tukey's test. The coefficient of variation (CV) value was computed to verify the predicted model as follows:

$$CV = \frac{\sigma}{\overline{X}} 100 \quad (1)$$

where σ is sample standard deviation, and $\overline{X}$ is sample mean.

Code	Amplitude (%) (X_1)	Acid conc.(%) (X_2)	Time (min) (X_3)
US-1	75(0)	2(0)	20(0)
US-2	60(-1)	3(+1)	20(0)
US-3	75(0)	3(+1)	30(+1)
US-4	60(-1)	1(-1)	20(0)
US-5	75(0)	3(+1)	10(-1)
US-6	75(0)	1(-1)	30(+1)
US-7	75(0)	1(-1)	10(-1)
US-8	60(-1)	2(0)	30(+1)
US-9	90(+1)	3(+1)	20(0)
US-10	90(+1)	1(-1)	20(0)
US-11	90(+1)	2(0)	10(-1)
US-12	90(+1)	2(0)	30(+1)
US-13	60(-1)	2(0)	10(-1)

Table 1. (Un)coded variables of Box-Behnken design of ultrasonication pretreatment conditions

3. RESULTS

The experimental values for RS under different conditions are represented in Fig.1. The RS in this study ranged between 158.14-477.49 mg/g irrespective of the amplitude, acid concentration and treatment time. The maximum RS (4.77.49±5.18 mg/g) value was observed at 90% amplitude with 1% of acid concentration for 20 min, whereas the minimum RS (158.14±7.34 mg/g) was obtained at 60% amplitude with 2% acid concentration for 30 min (Fig.1).

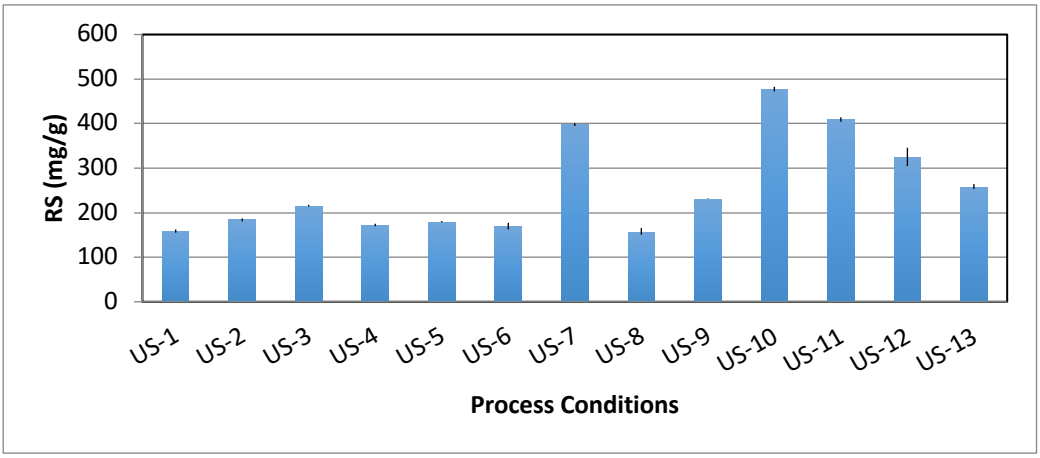

Fig. 1. Reducing sugar (mg/g) release under the different process conditions

Effects of amplitude, acid concentration, and treatment time were investigated in terms of reducing sugar (RS). In order to select the suitability of the model, R^2 must be closer to 1, VIF must be 1, and lack-of-fit (LOF) value must be p>0.05, respectively. A quadratic model was chosen based on these values for the RS. The regression coefficients for the second-order polynomial equation and linear, quadratic and interaction terms are represented in Table 2.

Terms	**RS (mg/g)**		
	Coeff	**SE Coeff**	***p*-value**
X_1	83.83	2.63	0.000
X_2	-50.79	2.63	0.000
X_3	-47.08	2.63	0.000
X_1* X_1	74.72	3.87	0.000
X_2* X_2	27.78	3.87	0.000
X_3* X_3	49.23	3.87	0.000
X_1* X_2	-64.26	3.72	0.000
X_2* X_3	66.03	3.72	0.000
Lack-of-fit	0.085		
R^2	0.99		
R^2(adj)	0.99		
R^2(pred)	0.98		

Table 2. Revised ANOVA results and estimated regression coefficients for the coded RS

The RS significantly increased with increase in amplitude ($p<0.05$), whereas it decreased with an increase in acid concentration and treatment time (Table 2). Among the variables, amplitude, acid concentration, and treatment time showed significant effects ($p<0.05$) on RS. It showed a significant main effect of amplitude with a positive effect on RS, whereas the main effect of acid concentration and treatment time had a negative effect on the RS (Table 2). High amplitude promoted algal sugars production. The most important factor was the amplitude with the highest coefficient (83.83), showing that it is the most dominant factor influencing the ultrasonication pretreatment method of microalga (Table 2).

The contour plots were used to show how the operational settings simultaneously influenced the response of the ultrasonication followed by enzymatic hydrolysis conditions (Fig.2). Fig.2a showed the positive interaction between acid concentration and time on RS. The contour plot is elliptical and the interaction effect is statistically significant (Figure 2a and 2c). In Fig. 2b, the contour plot is not elliptical, indicating that there are fewer interactions between amplitude and time. RS decreased with increased acid concentration under the center of amplitude (75%) for 25 min of treatment time (Fig. 2a). When the amplitude was increased from 80 to 90%, the RS increased from 350 to 400 mg/g (Fig.2b).

The optimum conditions for ultrasonication followed by enzymatic hydrolysis predicted by the model was 90% amplitude, 1% acid concentration for 10 min, which gave about 627.58 mg/g for RS. The smaller value of the coefficient of variation (CV) gives the best reproducibility. CV value was 2.4% for RS.

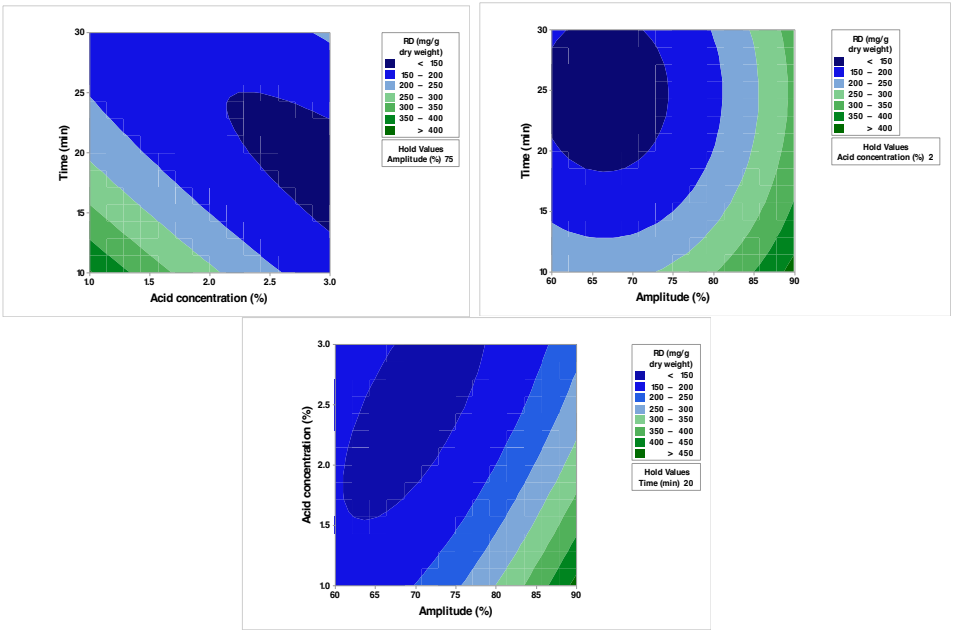

Fig. 2. Response contour plots showing two-way interaction effects of (a) acid concentration and time, (b) amplitude and time, (c) amplitude and acid concentration on RS during ultrasonication pretreatment

4. DISCUSSION

Some pretreatment methods and enzymatic hydrolysis should be needed to release these carbohydrates from the cell. Zhao et al. (2013) reported that ultrasonic power had a positive effect on microalgae hydrolysis for glucose yield during ultrasound assisted extraction. Park and Jeong (2021) investigated production of reducing sugar from *Gracilaria verrucosa* using US assisted acid catalyst and subsequent enzymatic hydrolysis. Park and Jeong (2021) reported that raising the ultrasonic amplitude

resulted in an increase in glucose production. The reducing sugar yield was obtained as 60.38% with 100 mM sulfuric acid and 60% amplitude for 60 min. According to study of Barajas-Solana et al. (2014), Up to 0.17 g sugars/g biomass and 0.14 g sugars/g biomass were found at 75 ºC after organosolv and acid hydrolysis treatment of *C. vulgaris* biomass, respectively. The RS of this study (627.58 mg/g) was found to be higher than previous study of Barajas-Solana et al. (2014) carried out with acid hydrolysis treatment.

5. CONCLUSIONS

The results of this investigation demonstrate that the US pretreatment with enzymatic hydrolysis can be used as an appropriate strategy for reducing sugar release from microalga biomass. The results also show that microalgal biomass could provide a cheap and sustainable feedstock for high-value added products. Furthermore, since US with enzymatic hydrolysis was effective for the cell wall degradation of microalga, this might be applied on an industrial scale to increase extraction efficacy of cellulose and hemicellulose present in microalga.

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