Fresh *Ulva Lactuca* Alcoholic Fermentation
Products and yields

DOINITA ROXANA CIOROIU[1,2], CLAUDIA IRINA KONCSAG[2]*, DRAGOS GUDOVAN[1], TANASE DOBRE[1]
[1]University Politehnica of Bucharest, Chemical and Biochemical Engineering Department, 1-3 Gheorghe Polizu Str., 011061, Bucharest, Romania
[2]Ovidius University of Constanta, 124 Mamaia Str., 900527, Constanta, Romania

The optimization of the ethanol yield obtained by alcoholic fermentation of fresh Ulva lactuca in the presence of cellulase and Saccharomyces cerevisiae yeast was performed. Three main factors affecting the process were considered: the solid-to-liquid ratio (S), cellulase-to-algal biomass ratio (R), expressed as cellulase units (U) per gram of dry matter (DM), and the alcoholic fermentation temperature (t). The optimal parameters were: S=1/24 g/g, R=16 U/ g DM and t=35°C, when alcohol yield was 0.34 g/100 g fresh alga, within the range of other authors' experimental data. Over 200 compounds were identified in the distillate from the fermentation broth, recommending Ulva lactuca as a sustainable source of drugs, intermediates for biosyntheses and biomaterials. The yield of these compounds was 5.92 g/100 g fresh alga, in the same conditions. The yields of ethanol (0.013-0.023 g/g DM) and volatile compounds (0.199-0.398 g/g DM) were predicted depending on the process factors using regression equations obtained by ANOVA analysis of experimental data.

Keywords: Ulva lactuca, alcoholic fermentation, process optimization, statistical model

The decrease of energy consumption derived from fossil fuels would slow down the global warming process and reduce the energy dependence of certain countries, in the context of international disagreements. The third generation of biofuels obtained from seaweeds is seen among the most sustainable, renewable and environmentally friendly response to climate change in the future [1].

Bioethanol via alcoholic fermentation of algae is an alternative source to fossil fuels. The International Energy Agency predicted in 2012 that the demand for bioethanol will increase at least three times by 2035. Due to their abundance, macrophytes are taken into consideration as feedstock for bioethanol production. As a consequence, studies were dedicated to macrophytes [2-5] and some targeted *Ulva* sp. [6,7].

Ulva sp. has an opportunistic character, standing for high variations in salinity and temperature of seawater, its growth depending on geographical location, season, development stage and environmental conditions [8]. It was found that, along with proteins, few lipids, minerals and vitamins counting for food and biomedical applications [9], *Ulva* sp. contains carbohydrates 45.0-61.5 % wt. of dry matter (DM) [6,8,10], a rich source for biofuels and other valuable chemicals.

Ulva lactuca from the Black Sea coast, commonly known as *sea lettuce*, can reach biomass production up to 1700 g/m^2 water surface and contains carbohydrates, 48.5-55% wt. of DM depending on season, as found by the Institute Grigore Antipa [11].

In this context, our work aims at demonstrating the potential of this local resource for bioethanol and other valuable chemicals and more, to perform the mathematical model related to main factors influencing the production.

Experimental part
Materials and methods

The fresh *Ulva lactuca* was collected in July 2016 at Pescarie Beach (N 44.2184° E 28.6490°), Black Sea shore and transported to the laboratory in sea water. Alga was washed with tap water, drained and dried with a wad, then used fresh.

The extraction of sugars from algae can be performed by hot water, acid, alkaline or enzymatic hydrolysis [6,7]. A hot water pretreatment followed by enzymatic hydrolysis can lead to total extraction of reducing sugars representing about 20% of dry weight [7]. This hydrolysate is prone to ethanolic, lactic [12] or acetobutylic fermentations [7].

In a preliminary study [13], the acid hydrolysis was compared with enzymatic treatment and the last proved to be superior, so we adopted this way further. The hydrolysis was followed by ethanolic fermentation with *Saccharomyces cerevisiae*.

For enzymatic hydrolysis process, cellulase from *Aspergillus niger* was used, powder off-white, 0.8 U/mg, produced by Sigma Aldrich. *Commercial Saccharomyces cerevisiae yeast provided by SC ROMPACK SRL Romania was used for the alcoholic fermentation.*

The content of dry matter (DM) was determined with the moisture analyzer OHAUS, model MB45, programmed for wet vegetables as follows: 7 min at 200 °C, 1 min at 150 °C and 12 min at 105 °C. Since only fresh algae were processed in this experiment, the analysis was performed just to report the alcohol production to DM.

The concentration of volatile compounds in the fermentation broth was determined by distillation using an oenologic apparatus Class-CHEM, Italy, model OH-1 (Romanian standard method SR 184-2).

In order to identify the volatile compounds, the LC-MS analysis was carried out on an ultra precision liquid chromatograph coupled with a liquid-gas (nitrogen) mass spectrometer- Agilent Technologies 6540 UHD Accurate-Mass Q-TOF LC/MS. The mobile phase was 0.1% (v/v) trifluoroacetic acid in acetonitril (Sigma-Aldrich) with water 50:50. The analysis was performed at 30 °C with positive and negative ionization.

Enzymatic hydrolysis of fresh Ulva lactuca

For the enzymatic hydrolysis, samples of 320 g fresh *Ulva lactuca* sp. (approx. 48 g DM) were randomly fragmented with a mixer and boiled in a certain quantity of

* email: ckoncsag@yahoo.com

distilled water (580 or 1160 mL) for 20 min. After cooling to 40 °C, cellulase was added (800 or 400 U). The mixture was kept at this temperature on an orbital shaker for 24 h in order to ensure the polysaccharides conversion in reducing sugars. Then the insoluble matter was separated and the hydrolysate was processed further. The *p*H of supernatant was 6-6.5.

Alcoholic fermentation

To start the process of alcoholic fermentation, 1 g of *Saccharomyces cerevisiae was* added to the hydrolysate. The fermentation was performed at 25 or 35 °C for 24 hr, in anaerobic conditions, under agitation on an orbital shaker at 40 rpm. The production of CO_2 proceeding from sugars fermentation was measured and related to the ethanol production. Then, the concentration of volatile compounds in the broth was determined by distillation.

Experiment design

8 experiments of enzymatic hydrolysis followed by ethanolic fermentation were conducted according to a 2^3 factorial plan (3 process factors and 2 levels of each factor) [14-19]. Solid-to-liquid ratio (S=1/12, 1/24 g/g), cellulase-to-algal biomass ratio (R=8, 16 U/g DM), and fermentation temperature (t=25, 35 °C) were selected as process factors (independent variables), whereas the yields of ethanol and volatile compounds were considered as process responses (dependent variables). Every sample was processed in triplicate.

Results and discussions

At the experiment time, the moisture content in algae was 85.12±0.5% wt. and polysaccharides content was 59.1±0.3 % wt. of DM. The yields of volatiles (V) and ethanol (E), expressed as g/g DM, are shown in table 1.

As seen in table 1, the volatile compounds yield and also the ethanol yield are favoured by the higher temperature (35 °C), the higher number of cellulase units (16 U/g DM) and lower solid-to-liquid ratio (1/24 g/g). The maximum volatiles yield in these conditions was of 0.398 g/g DM, corresponding to 5.92 g/100 g fresh weight. In the same conditions the ethanol yield was of 0.0234 g/g DM, corresponding to 0.34 g/100 g fresh weight. This is comparable with data in literature on other algae, from 0.23 g/100 g fresh weight *Kappaphycus alvarezii* [3] to 0.38 g/100 g fresh *Gracilaria verrucosa* [4] and 0.93 g/100 g fresh *Ulva fasciata* [6].

Data analysis and statistics were performed with Microsoft Excel facilities. The polynomial model for product yield obtained by regression is described by eqs. 1 and 2.

$$V = 0.076375 + 2.771084\,S + 0.123159\,tS + 0.00015\,tR - 0.55689\,SR + 0.000132\,t^2 + 0.000978\,R^2 \quad (1)$$

$$E = 0.003775 + 0.108434\,S + 0.00589\,tS + 0.000084\,tR - 0.01764\,SR - 0.000007\,t^2 + 0.0006\,R^2 \quad (2)$$

For eq. 1, the correlation coefficient r^2 was 0.9868, adjusted r^2 was 0.6446 and the standard error was 0.009821. Analysis of variance ANOVA revealed the null hypothesis rejection (p<0.05) for all coefficients except the fourth: 0.00015 (p=0.34). For eq. 2., r^2=0.9920, adjusted r^2=0.6533 and standard error was 0.000403. ANOVA analysis revealed that all coefficients in eq. 2 are relevant. Predicted yields according to the statistical model, which are presented in table 1, show a good fitting with the experimental values.

It was suggested that *Ulva lactuca* contains numerous compounds with antimicrobial, antioxidant, antiviral, antihyperlipidemic, anti-inflammatory and antitumor activity [8]. The water extractible compounds during hydrolysis and fermentation operations were confirmed in our study by the high volatile compound yields in the distilled samples (table 1).

An LC-MS chromatogram was performed (fig. 1) and the main peak was at RT: 0.383-0.427 min. From MS spectra, about 200 compounds were identified in the apparatus library, with possible formulae and ions.

A spectrum example is presented in figure 2. Most of compounds have a mass-to-charge under 200 Da but a few are obviously macromolecules (m/z=622; 922; 1222; 1522). Some can be listed for their medical use: hemagglutinin (m/z=108.12) is a protein, the key antigen for preparing influenza vaccines (hemagglutinin was also found in the red alga *G. verrucosa* [4], carbapenam (m/z=111.14) a biosynthetic intermediary for carbapenem antibiotics, isovaleramide (m/z=101.15) an anxiolytic and a sedative, conhydrine (m/z=143.23) an alkaloid used in drug syntheses. Other compounds have correspondents in petrochemical synthesis: caprolactam (m/z=113.16), benzylamine (m/z=107.16), 2-(dimethylamino)

Exp.	t (°C)	S (g/g)	R (U/g DM)	V (g/g DM)		E (g/g DM)	
				Experimental	Predicted (Eq. 1)	Experimental	Predicted (Eq. 2)
1	25	0.0830	16	0.214±0.005	0.214	0.0153±0.0003	0.0153
2	25	0.0830	8	0.199±0.006	0.193	0.0134±0.0002	0.0135
3	25	0.0415	16	0.295±0.008	0.295	0.0142±0.0002	0.0142
4	25	0.0415	8	0.216±0.004	0.222	0.0130±0.0002	0.0129
5	35	0.0830	16	0.225±0.002	0.225	0.0201±0.0004	0.0201
6	35	0.0830	8	0.278±0.006	0.284	0.0161±0.0004	0.0160
7	35	0.0415	16	0.398±0.010	0.398	0.0234±0.0004	0.0234
8	35	0.0415	8	0.319±0.003	0.313	0.0153±0.0002	0.0154

Table 1
YELDS OF VOLATILE COMPOUNDS AND ETHANOL OBTAINED IN THE ALCOHOLIC FERMENTATION FROM *ULVA LACTUCA*

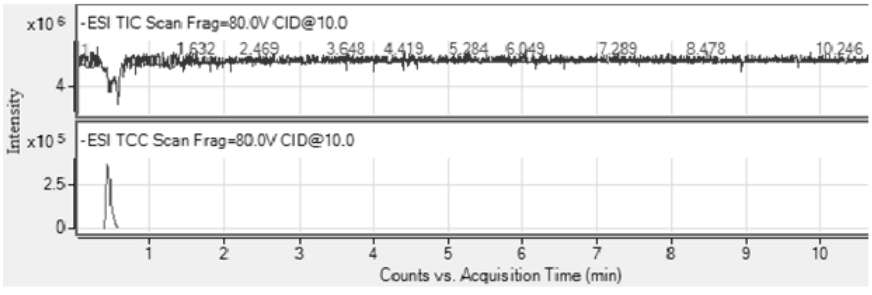

Fig. 1. LC-MS chromatogram of distilled sample

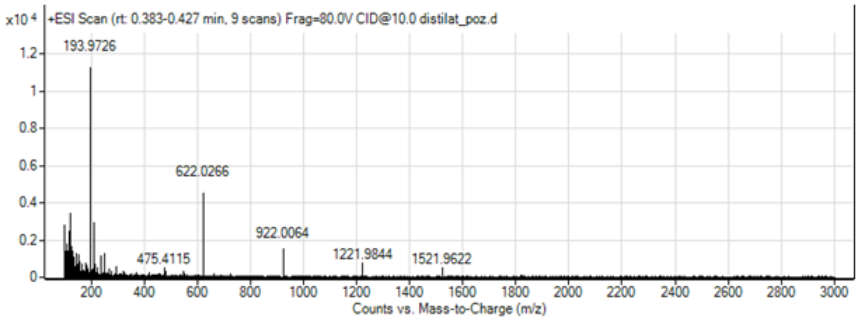

Fig. 2. MS spectrum related to chromatogram in figure 1

ethanethiol (m/z=105.19), cyclohexanecarbonitrile (m/z=119.17). Another ion with high abundance is m/z= 193.97, still unidentified, with possible formula $[C_4H_3ClN_2O_5)]^+$.

Separation of certain compounds or fractions can be a difficult task but an economic analysis could be performed after the quantification of these compounds.

Conclusions

A study was performed to optimize the process of ethanol yield obtained by alcoholic fermentation of fresh Ulva lactuca in the presence of cellulase and *Saccharomyces cerevisiae yeast.* Three main factors were taken into account, *i.e.*, the solid-to-liquid ratio (S=1/12, 1/24 g/g), cellulase-to-algal biomass ratio (R=8, 16 U/g DM), and fermentation temperature (t=25, 35°C). The process performances in terms of yields of ethanol (0.013-0.023 g/g DM) and volatile compounds (0.199-0.398 g/g DM) were predicted using regression equations obtained by ANOVA analysis of experimental data.

The diversity of compounds found in the fermentation broth recommends *Ulva lactuca* as a sustainable source of drugs, intermediaries for biosyntheses and biomaterials.

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