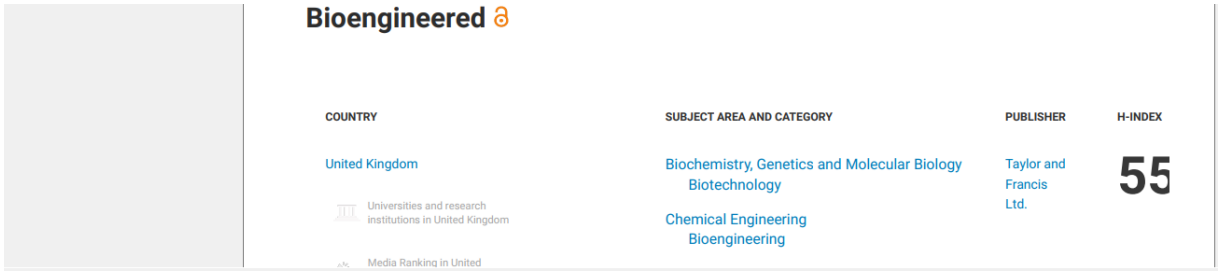
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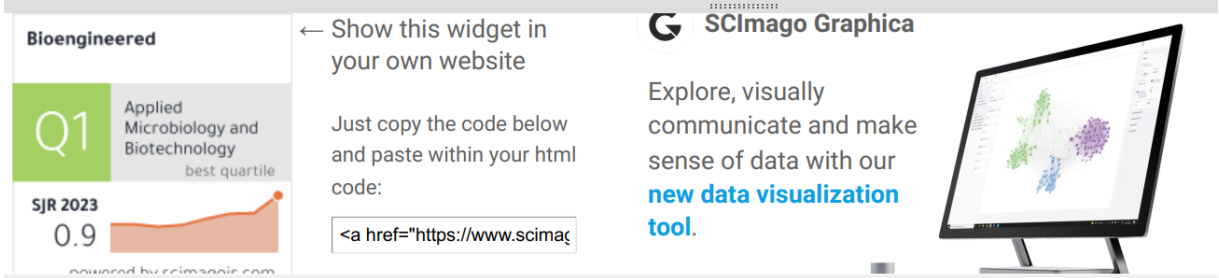
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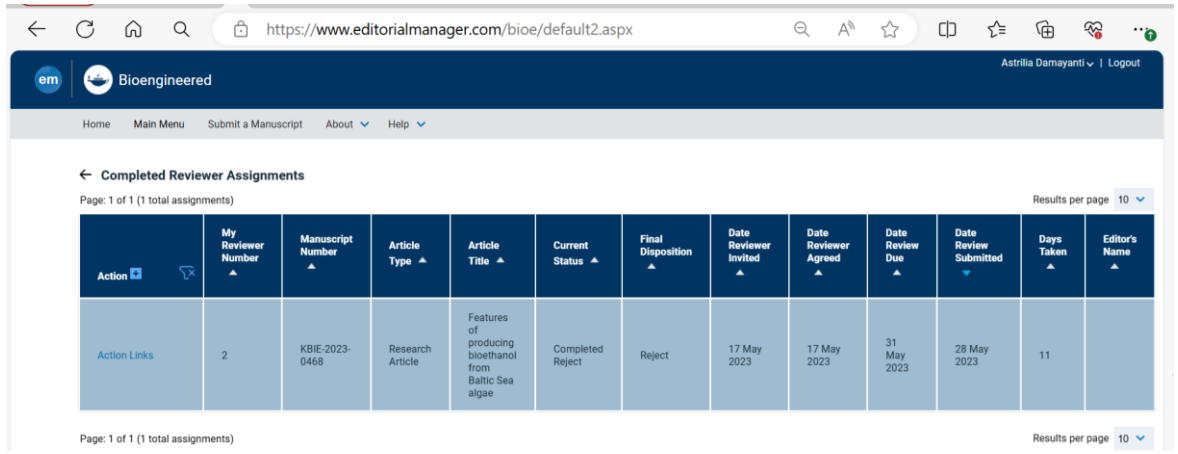
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2
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11
Page: 1 of 1 (1 total assignments)
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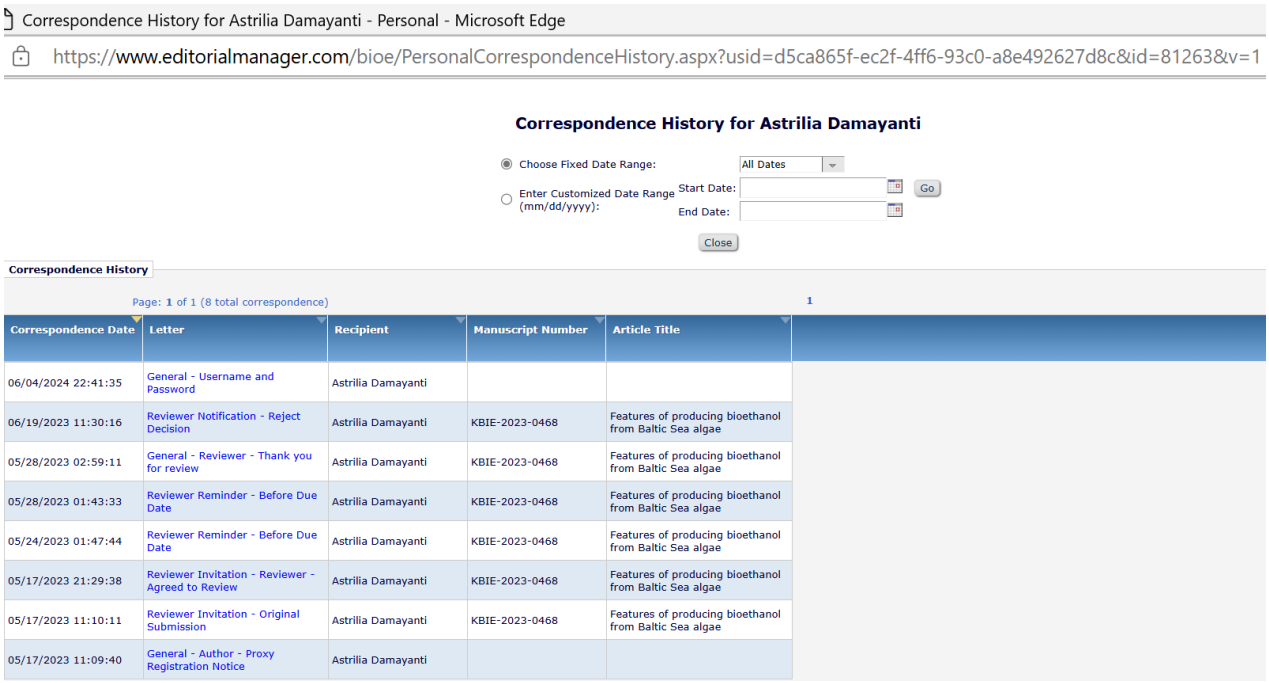
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Features of producing bioethanol from Baltic Sea algae
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Features of producing bioethanol from Baltic Sea algae
05/28/2023 01:43:33
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Features of producing bioethanol from Baltic Sea algae
05/24/2023 01:47:44
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Reviewer invitation-original submission

Date: 17 May 2023
To: "Astrilia Damayanti" astrilia.damayanti@mail.unnes.ac.id
From: "Bioengineered" kbie-peerreview@journals.tandf.co.uk
Subject: Bioengineered - invitation to review manuscript KBIE-2023-0468

Dear Astrilia Damayanti,

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Abstract:

Biofuel is a fuel derived from biodegradable, non-toxic, and environmentally friendly biological sources such as plants, animals, and microbes. Macroalgae require little energy to grow, are easy to cultivate, and produce large amounts of biomass. This study aimed to investigate the characteristics of biofuel production from Baltic Sea macroalgae. Capillary sequencing using amplicon cloning identified four species of macroalgae collected on the coast of the Baltic Sea: Ulva clathrata, Ulva prolifera, Cladophohora fracta, and Ulva intenstinalis. Cellulase and xylanase enzymes, as well as microorganisms Saccharomyces cerevisiae, Lachancea thermotolerans, and Pachysolen tannophilus were used to treat macroalgae biomass. The content of reducing substances was determined by spectrometry, the content of alcohols (including ethanol) was determined using gas chromatography. Before macroalgae fermentation, acid, alkaline, and enzymatic pretreatments were performed. The fermentation of macroalgae U. clathrata with the yeast S. cerevisiae produced the highest yield of ethyl alcohol (32%). The alkaline treatment of macroalgae biomass with sodium hydroxide (NaOH) in a volume of 35 mL has been evidenced to be the most effective method of pretreatment of macroalgae, demonstrated on the example of U. clathrata. The enzymatic method of processing microalgae biomass with cellulase and xylanase enzymes produced sufficiently good results. Acid treatment demonstrated less effectiveness compared to alkaline treatment. Based on the research, a strategy for producing bioethanol from marine macroalgae with the highest ethyl alcohol yield was developed. The experiment results indicate that macroalgae can be a potential source of bioethanol, biohydrogen, and biomethane, which are used as biofuels.

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a. The title is out of sync with the abstract. The title says "bioethanol", while the abstract says "...macroalgae can be a potential source of bioethanol, biohydrogen, and biomethane, which are used as biofuels"
b. There is no relation between raw material and bioethanol from various microalgae, enzymes and microbes.
3. Introduction
a. Page 2 line11. Similar to microalgae, macroalgae can also…. What does it mean?
b. Page 2 lines 28-29. Algae does not contain lignin, so lignin removal is not required. The removal of lignin is….These sentences are contradictory. Explain
c. Page 52. "…Baltic sea". why?
4. Results
a. Line 21-22 : The macroalgae samples were identified as Ulva clathrata, Ulva prolifera, Cladophohora fracta, and Ulva intensinalis. However, their explanation is incomplete.
b. Table 5 and Table 6 have no prologue and explanation
5. Discussions
a. All discussions do not explain the results from either tables or graphs, but tend to be about the background.
6. Conclusion
a. Conclusion does not explain the results of the discussion.

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5. Discussions
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6. Conclusion
a. Conclusion does not explain the results of the discussion.

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Date: 19 Jun 2023
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Subject: Bioengineered - Decision made on manuscript you reviewed

Ref.: Ms. No. KBIE-2023-0468
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Thank you for your review of this manuscript for Bioengineered. The Editor has now made a decision of Reject on this manuscript.

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Features of producing bioethanol from Baltic Sea algae

--Manuscript Draft--

Manuscript Number:	KBIE-2023-0468
Full Title:	Features of producing bioethanol from Baltic Sea algae
Article Type:	Research Article
Order of Authors:	Stanislav Sukhikh
	Svetlana Ivanova
	Olga Babich
	Anastasia Davydova
	Ashok Pandey
	Philippe Michaud
	Vyacheslav Dolganyuk
	Olga Krigera
Abstract:	Biofuel is a fuel derived from biodegradable, non-toxic, and environmentally friendly biological sources such as plants, animals, and microbes. Macroalgae require little energy to grow, are easy to cultivate, and produce large amounts of biomass. This study aimed to investigate the characteristics of biofuel production from Baltic Sea macroalgae. Capillary sequencing using amplicon cloning identified four species of macroalgae collected on the coast of the Baltic Sea: Ulva clathrata, Ulva prolifera, Cladophohora fracta, and Ulva intenstinalis. Cellulase and xylanase enzymes, as well as microorganisms Saccharomyces cerevisiae, Lachancea thermotolerans, and Pachysolen tannophilus were used to treat macroalgae biomass. The content of reducing substances was determined by spectrometry, the content of alcohols (including ethanol) was determined using gas chromatography. Before macroalgae fermentation, acid, alkaline, and enzymatic pretreatments were performed. The fermentation of macroalgae U. clathrata with the yeast S. cerevisiae produced the highest yield of ethyl alcohol (32%). The alkaline treatment of macroalgae biomass with sodium hydroxide (NaOH) in a volume of 35 mL has been evidenced to be the most effective method of pretreatment of macroalgae, demonstrated on the example of U. clathrata. The enzymatic method of processing microalgae biomass with cellulase and xylanase enzymes produced sufficiently good results. Acid treatment demonstrated less effectiveness compared to alkaline treatment. Based on the research, a strategy for producing bioethanol from marine macroalgae with the highest ethyl alcohol yield was developed. The experiment results indicate that macroalgae can be a potential source of bioethanol, biohydrogen, and biomethane, which are used as biofuels.

Word count - 6983

RESEARCH PAPER

Features of producing bioethanol from Baltic Sea algae

Stanislav Sukhikh[a] , Svetlana Ivanova[b], Olga Babich[a], Anastasia Davydova[a], Ranjna Sirohi[c], Philippe Michaud[d], Vyacheslav Dolganyuk [a,e], and Olga Kriger[a,f]

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ABSTRACT

Biofuel is a fuel derived from biodegradable, non-toxic, and environmentally friendly biological sources such as plants, animals, and microbes. Macroalgae require little energy to grow, are easy to cultivate, and produce large amounts of biomass. This study aimed to investigate the characteristics of biofuel production from Baltic Sea macroalgae. Capillary sequencing using amplicon cloning identified four species of macroalgae collected on the coast of the Baltic Sea: *Ulva clathrata, Ulva prolifera, Cladophohora fracta,* and *Ulva intenstinalis*. Cellulase and xylanase enzymes, as well as microorganisms *Saccharomyces cerevisiae*, *Lachancea thermotolerans*, and *Pachysolen tannophilus* were used to treat macroalgae biomass. The content of reducing substances was determined by spectrometry, the content of alcohols (including ethanol) was determined using gas chromatography. Before macroalgae fermentation, acid, alkaline, and enzymatic pretreatments were performed. The fermentation of macroalgae *U. clathrata* with the yeast *S. cerevisiae* produced the highest yield of ethyl alcohol (32%). The alkaline treatment of macroalgae biomass with sodium hydroxide (NaOH) in a volume of 35 mL

has been evidenced to be the most effective method of pretreatment of macroalgae, demonstrated on the example of *U. clathrata*. The enzymatic method of processing microalgae biomass with cellulase and xylanase enzymes produced sufficiently good results. Acid treatment demonstrated less effectiveness compared to alkaline treatment. Based on the research, a strategy for producing bioethanol from marine macroalgae with the highest ethyl alcohol yield was developed. The experiment results indicate that macroalgae can be a potential source of bioethanol, biohydrogen, and biomethane, which are used as biofuels.

Keywords

Biofuel; algae; *Ulva clathrata, Ulva prolifera, Cladophohora fracta, Ulva intenstinalis;* yeast; enzymatic hydrolysis; reducing sugars.

Highlights

- 1st and 2nd generation biofuels are produced from crops and crop residue biomass;
- Macroalgae is one of the best raw materials for bioethanol production;
- The algae used belonged to the species *Ulva clathrata, Ulva prolifera, Cladophohora fracta*, and *Ulva intenstinalis*;
- Rational fermentation parameters are similar for all studied macroalgae;
- Cascade extraction of valuable algae components implements the Zero Waste approach.

Introduction

Biofuels are produced from biological raw materials such as plants, animals, and microorganisms [1]. Depending on the nature of the feedstock, biofuel production can be divided into three groups (generations): first, second, and third generation biofuels.

First-generation biofuels (biodiesel, biooil, or bioethanol) are produced from crops such as rapeseed, wheat, sugar beet and corn [2], which is a significant environmental concern, because mass production of such biofuels requires a significant amount of arable land and leads to a reduction in the production of food for people.

Second generation biofuels have been developed to overcome some of the important limitations of first generation biofuels. Biomass from trees, cake, agricultural waste, straw, grass, etc. are used as raw materials for biofuels of the second generation [3].

The limitations of first and second generation biofuels, such as low return on investment, complex production process methods, laid the foundation for the development of third generation biofuels, which is now considered an alternative energy source. The raw materials for third generation biofuels are photosynthetic microbes such as microalgae, cyanobacteria, and algae [4].

Third generation bioethanol produced from microalgae biomass is an environmentally friendly fuel. It has many advantages over first and second generation biofuels produced from higher plants, mainly due to the high rate of biomass generation, as microalgae cells have a short doubling time and can achieve high yields in about 1–10 days. Furthermore, the advantages include a low need for space and the ability of algae to grow in almost any water, including fresh water, sea water, and even industrial wastewater [5-7]. They can reduce carbon dioxide emissions emitted by power plants or other industrial sources, and, in turn, can reduce greenhouse gas emissions [8].

Similar to microalgae, macroalgae also can grow rapidly and produce huge amounts of biomass. High yields are possible because macroalgae require less energy to produce supporting tissue than terrestrial plants and can absorb nutrients across their entire surface [9]. In fact, the amount of bioenergy produced by red algae biomass is greater than that of any other biomass source [10].

Direct fermentation of algae biomass from complex carbohydrates to bioethanol consists of four successive stages [11]: pretreatment; saccharification; fermentation; product extraction. Typically, algae biomass is pretreated with acids and/or alkalis for bioethanol production. Depending on the purpose of pretreatment, chemical, biological, thermochemical or thermophysical methods are used, sometimes in combination [12]. Algae do not contain lignin, so lignin removal is not required. The removal of lignin is effectively a rate-limiting step for other feedstocks, so its absence reduces the cost, time, and complexity of the conversion process. The efficiency of fermentation for the production of bioethanol is highly dependent on the pretreatment and saccharification conditions of algae biomass [13].

Algae carbohydrates are a mixture of neutral sugars, amino sugars, and uronic acids, and their composition varies depending on the species and growth conditions. Therefore, efficient pretreatment should be aimed at increasing the production of bioethanol by improving the availability of carbohydrates and, therefore, accelerating the rate of fermentation [14].

Typically, pretreatment involves lysing the algae cells to release stored carbohydrates within the cells. Whereas saccharification, leading to the destruction of α-(1,4), α-(1,6), β-(1,3), and β-(1,6) glycosidic bonds between monomers, is intended to separate complex carbohydrates into their monosaccharide components [15] (fermenting microorganisms can only convert fermentable forms of sugars such as disaccharides and monosaccharides, usually hexoses and pentoses, into ethanol).

The aim of this study was to investigate the features of producing biofuel from algae of the Baltic Sea. The novelty of this study is that it investigates for the first time the methods of pretreatment of Baltic Sea algae, yeast fermentation, and the content of reducing sugars that undergo bioconversion into biofuel (bioethanol, etc.).

Methods and materials

Characteristics of macroalgae

For this study, we used samples of macroalgae collected in the Kaliningrad region in the Baltic Bay (59°43′ N 28°24′ E), on the Baltic Sea coast (54° 42.4' 0" N, 20° 30.4' 0" E).

Capillary sequencing of variable loci of the mitochondrial cytochrome oxidase (COI) subunit 1 gene [16], plastid elongation factor gene Tu (tufA), and the transcribed spacer ITS1+5.8S+ITS2 was used to identify macroalgae samples.

The DNA was isolated from the biomass of macroalgae samples using phenol-chloroform extraction [17]. Prior to isolation, samples of green macroalgae were treated with a 1 M KOH solution in a ratio of 3:1 to a dry sample and incubated for 10 minutes at 94°C [17]. Gene amplification was performed using specific primers (Table 1) and Tersus polymerase (Evrogen, Moscow, Russia) in a volume of 100 μL according to the manufacturer's protocol. Cycling parameters are presented in Table 2.

The results of amplification were visualized by horizontal electrophoresis on a 1% agarose gel. Amplicons were purified using a commercial mini-purification kit (Eurogen, Moscow, Russia). Concentrations of purified amplicons were measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Libraries prepared for virus sequencing included molecular cloning of a 10 μM amplicon in the PAL2-T vector (insertion: vector) from the commercial Rapid TA kit (Eurogen, Moscow, Russia) in a 3:1 ratio. Chemically competent E. coli DH5 α cells were used for cloning [17].

All experiments were performed on ice using live cells. Tubes with cell suspension stored at -80°C were thawed on ice for 30 min. Then 5 μL of supernatant was added to 50 μL of cells and incubated on ice for 30 min followed by incubation at 42°C to 90°C. The cells were then transferred to ice and incubated for 2 min. 200 μL of liquid LB nutrient medium was added to the tube and incubated for 120 min with stirring. The temperature of the plasmids was raised to 37°C. The obtained cell suspension was inoculated into 200 mL of dense LB nutrient medium (1.5% agar, 1.0% tryptone, 1.0% NaCl, 0.5% yeast extract). Ampicillin at a concentration of 50 mg/L and X-Gal + IPTG were used as selective markers. Petri dishes were incubated at 37°C for 8 h. Multivariate analysis was performed using blue-white analysis [17].

White colonies containing the insertion vector were subjected to PCR using standard primers for the PAL2-T vector M13 forward (5-GTTGTAAAACGACGGGGCCAGTG-3) and reverse M13 (5-AGCGGATAAACAATTTTTTCACACAGGA-3). The qPCR mix probe using 15 μL of HS reaction mixture (Eurogen, Moscow, Russia). PCR results were analyzed using horizontal electrophoresis in agarose gel [17].

Overnight cultures were obtained using active plasma biomass. A suspension of single colonies was transferred into 5 ml of LB medium containing ampicillin, and the optical density of OD600 at 37°C was 0.6-0.8. Plasmids were isolated using a commercial Plasmid Miniprep kit (Eurogen, Moscow, Russia). Plasma concentrations were measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) [17].

Sequencing reactions were performed using BigDye Terminator v3.1 with M13Forward and M13Reverse (Applied Biosystems, Foster City, CA, USA). The sequence of reactions was repeated in forward and reverse replications for each sample. The volume of the reaction mixture was 10 μl. 3 μL of buffer, 1 μL of ends, 100-200 ng of plasma DNA, and water were added to the final mQ volume. The library was amplified in a C1000 Touch thermal cycler. The cycle parameters are presented in Table 3.

The reaction mixture was purified using a commercial X-Terminator kit (Applied Biosystems, Foster City, CA, USA). Capillary sequencing was performed on a 3500 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing results were processed using 4 Peaks and CLC Genomics Workbench software. Four peaks were used to align the two repeats relative to each other to unambiguously identify nucleotides that were not read in the sample [17].

Further processing in the CLC Genomics Workbench included trimming the 5'- and 3'-end sequences and assembly, which led to the analysis of discordant groups. Sequence analysis was performed using the proprietary BLAST algorithm [18] using nr/nt, 18S rRNA, and ITS databases. A phylogenetic tree was plotted using the MEGA software package [19] and the Neighbor-Joining method [20].

Characteristics of the yeast

The following yeast species were used in this study: *Saccharomyces cerevisiae* (Y-4246), *Lachancea thermotolerans* (Y-4532), and *Pachysolen tannophilus* (Y-3270). They were purchased from the collection of GosNIIgenetika (Moscow, Russia). The optimum temperature for their cultivation is 26°C.

Czapek medium is the typical cultivation medium for *S. cerevisiae*. The nutrient medium was sterilized by autoclaving at 0.5 atm for 30 minutes at 26°C. We used a nutrient medium containing 20 g of glucose, 10 g of peptone, 5 g of yeast extract, and 20 g of agar per 1 liter of distilled water to cultivate *L. thermotolerans* and *P. tannophilus*. The nutrient medium was sterilized by autoclaving at 121°C for 20 min.

Cultivation was performed using by the quadrant or continuous streak method on Petri dishes, in test tubes with a cotton-gauze stopper. The passage was performed using a bacteriological loop.

Characteristics of the enzymes

In this study, the enzymes cellulase and xylanase (GMBKh, Lyubertsy, Russia), the characteristics of which are presented in Table 4, were used for enzymatic treatment.

Cellulase is an enzyme that hydrolyzes cellulose, a linear polysaccharide molecule consisting of repeating β-(1,4)-glycosidic bonds. Xylanase is an enzyme complex capable of breaking down xylose and xylan to xylooligosaccharide.

Determination of reducing sugar content

The content of reducing sugars in samples of macroalgae was determined using 3,5-dinitrosalicylic acid (DNS) according to the following method. A 1% solution of DNS was prepared at room temperature, followed by the gradual addition of 16.05 g of sodium hydroxide and 300 g of potassium sodium tartrate (in a water bath at 47±1°C). The resulting solution had a bright yellow color.

Prior to analysis, the optical density of the DNS solution was measured on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) to refine the absorption maximum. To assess the content of reducing sugars, 2 mL of the DNS solution was mixed with 1 mL of the sample and heated on a water bath for 5 minutes. After cooling, the sample volume was adjusted to 25 mL with pH 8.2 buffer and analyzed on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 530 nm in cuvettes with a light-absorbing layer thickness of 10 mm.

Photometric reactions were performed with glucose solutions prepared in volumetric flasks with a capacity of 100 mL from weighed portions of varying weights (from 50 to 150 mg) in distilled water to plot a calibration graph. After 3 minutes of photometric reaction, the absorbance at 530 nm was measured using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The calibration curve was used to determine the reducing sugar content.

Determination of alcohol content

To determine the alcohol content, three flasks were prepared, each with 5 g of macroalgae samples and 250 mL of the solution, depending on the processing method. One flask contained 250 mL of 2% sulfuric acid solution (H_2SO_4), the other contained 250 mL of 2% sodium hydroxide solution (NaOH). 250 mL of distilled water was added to the third flask. The flasks were sterilized by autoclaving at 121°C for 20 min. Next, 5 mL of cellulase enzyme was added. This solution was thermostated at 30°C for 72 h.

All three flasks were fermented for 144 hours with the yeast *S. cerevisiae*, which was added in a volume of 5 mL. The optical density of hydrolysates was measured on a spectrophotometer at a wavelength of 540 nm and compared with unfermented algae suspensions.

The alcohol content was determined using a packed column with a 5% Carbowax® sorbent (Aquakhim, St. Petersburg, Russia) on CarboBlackTM B (Akvakhim, St. Petersburg, Russia) with a 2 m long SilcoSmoothTM tube (ITEK, Moscow, Russia). Analysis conditions: temperature from 65 to 150 °C, exposure for 5 minutes, nitrogen was the carrier gas, a photoionization detector was used. The content of ethanol, methanol, butanol, ethyl acetate, isoamyl alcohol was determined using calibration curves.

Pretreatment of algae before fermentation

Three methods of pretreating macroalgae biomass were considered in this study: acidic, alkaline, and enzymatic.

1 g of dry macroalgae biomass and 30 mL of 2% sulfuric acid (H_2SO_4) solution were used for acid treatment. The resulting suspension was heated on a water bath for 30 minutes at 120°C. The prepared solution was sterilized by autoclaving at 121°C for 120 minutes.

In parallel, 1 g of macroalgae samples were weighed, and a 2% solution of sulfuric acid and 2.5% manganese sulfate was prepared. For this treatment method, 15 mL of a manganese sulfate solution and 10 mL of a sulfuric acid solution were additionally used. The solution was heated on a water bath at 120°C for 40 minutes. The prepared solution was sterilized by autoclaving at 121°C for 120 minutes.

1 g of macroalgae samples were used for alkaline treatment, and two portions of 2% sodium hydroxide solution (NaOH) were prepared. 35 mL of sodium hydroxide solution was added to one portion, and 30 mL to the other. The solutions were heated on a water bath at 120°C for 30 minutes. The prepared solutions were sterilized by autoclaving at 121°C for 120 minutes.

Cellulase and xylanase enzymes were used for enzymatic treatment. A water ratio of 1:100 was used (1 g of macroalgae samples and 100 mL of distilled water). Suspensions were sterilized by autoclaving for 120 minutes at 121°C. After addition of enzymes (2 ml of cellulase and 2 ml of xylanase) to the suspension, the resulting mixture was thermostated at 30°C for 72 h.

After using all the processing methods, samples of the macroalgae suspension were fermented with the yeast *S. cerevisiae* (Y-4246) in a volume of 5 mL, the fermentation duration was 144 hours. Next, the optical density of the hydrolyzates was measured on a spectrophotometer at a wavelength of 540 nm and compared with the optical density values of unfermented macroalgae.

Statistical analysis

Statistical processing was performed using Excel (Microsoft Co, 2019, Redmond, Washington, USA) and IBM SPSS Statistics 22 (IBM Company, 2013, Chicago, Illinois, USA). All experiments were performed in three replications. Data were presented as median ± standard deviation. The Kruskal–Wallis test was used to compare the medians of the samples (significance of differences at $p<0.05$). For intergroup comparison, the Mann–Whitney U-test was used with the Bonferroni correction (significance of differences at $p<0.01$). The Spearman correlation coefficient was used to test for a correlation between UV exposure time and quantitative indicators of cellulolytic activity (significance of differences at $p<0.05$).

Results

Results of macroalgae identification

The macroalgae samples were identified as *Ulva clathrata, Ulva prolifera, Cladophohora fracta,* and *Ulva intenstinalis*. According to the BLAST results, the resulting sequences had 98–99% identity (Figure 1-3).

The results of determining the rational fermentation parameters

At the beginning of the studies, enzymatic hydrolysis of macroalgae samples was performed. The rational parameters of fermentation were similar for all studied samples of macroalgae; the results for *U. clathrata* at different hydromodules in the presence of yeast S. cerevisiae (Y-4246) cultivated for 72 hours were selected as indicative and presented. Figure 4 depicts changes in the optical density of a macroalgae suspension.

Next, the content of reducing sugars was calculated (Figure 5).

A similar series of experiments was carried out at the next stage of research, with the duration of fermentation increased up to 120 h. Figures 6-7 shows the results of the optical density and reducing sugars measurements.

Because the yeast Saccharomyces cerevisiae produced the highest yield of reducing sugars, it was used for further fermentation of macroalgae biomass in the production of bioethanol.

Influence of the pretreatment method on the bioconversion degree of macroalgae from the Baltic Sea

The influence of the pretreatment method on the degree of bioconversion was studied for all types of macroalgae, however, due to the identity of the results, the research data are presented for the macroalgae *U. clathrata*.

Figure 8 demonstrates the results of studying the effect of pretreatment methods on the optical density of macroalgae *U. clathrata* using the yeast *S. cerevisiae*.

Figure 9 depicts the content of reducing sugars in macroalgae *U. clathrata* suspensions using the yeast *S. cerevisiae* after various treatment methods.

At the next stage, the biomass of macroalgae *U. clathrata* fermented by the yeast S. cerevisiae was distilled to obtain alcohols. Distillation was performed at a temperature of 78°C for 30–40 minutes.

Only 0.6 mL of a clear liquid with a similar odor to ethyl alcohol was obtained as a result of distillation. This result was obtained from the contents of a flask with a suspension of algae after alkaline treatment.

The most effective and illustrative results were obtained using the treatment of *U. clathrata* and *U. intenstinalis* macroalgae.

Figure 10 demonstrates the results of studying the effect of the processing method on the optical density of the *U. intenstinalis* macroalgae suspension using the yeast *S. cerevisiae*.

The results of studying the influence of the treatment method on the content of reducing sugars in suspensions of macroalgae *U. clathrata* using the yeast *S. cerevisiae* are presented in Figure 11.

Influence of the macroalgae pretreatment method on the yield of alcohols (biofuels)

Six samples were prepared for analysis on a gas chromatograph: alkaline treatment (sample No. 1), acid treatment (sample No. 2), enzymatic treatment (sample No. 3) of macroalgae *U. clathrata*; treatment of macroalgae *U. clathrata* with distilled water (sample No. 4), treatment of macroalgae *U. intenstinalis* with distilled water (sample No. 5), alkaline treatment (sample No. 6) and enzymatic treatment (sample No. 7) of macroalgae *U. intenstinalis*.

Chromatograms for samples No. 1-6 are presented in Figures 12-18.

Table 7 shows the total alcohol content in samples No. 1-7 of macroalgae.

Based on this research, a method for producing bioethanol from marine macroalgae with the highest ethanol yield was developed. Figure 19 depicts a bioethanol production scheme using the macroalgae *U. clathrata* as an example.

Discussion

The research results indicate the high efficiency of using the biomass of marine macroalgae U. *clathrata, U. prolifera, C. fracta,* and *U. intenstinalis* collected on the coast of the Baltic Sea for biofuel production. Based on the results obtained with the macroalgae *U. clathrata*, it is possible to conclude that bioconversion by yeast S. cerevisiae is most effective at a hydromodulus

of 1:30, or 1 g of crushed biomass per 30 mL of distilled water. It is also worth noting that this solution is quite convenient for further research after autoclaving.

It has been established that with a longer fermentation (within 144 hours), the content of reducing sugars decreases, implying that the bioconversion of marine macroalgae is more efficient and complete, implying a higher bioethanol yield. Studies have shown that Saccharomycetes are well suited for enzymatic hydrolysis. A large amount of reducing sugars are formed during fermentation under the influence of *S. cerevisiae* on all species of macroalgae studied, which are converted into biofuel as a result of bioconversion. The concentration of reducing substances after fermentation with *S. cerevisiae* was 0.136 mg/mL, and the bioethanol yield was 32%, indicating that macroalgae biomass is almost completely transformed. The rational duration of fermentation was 144 hours.

According to a number of studies, the main disadvantages of marine multicellular algae in terms of obtaining fuel are their low lipid content, large size, and rigid cell wall, which prevents them from being used directly as a raw material in a one-stage mode and necessitates a pretreatment step [21].

As a result of the research, it was discovered that alkaline treatment of macroalgae biomass with sodium hydroxide (NaOH) in a volume of 35 mL is the most effective method of pretreatment of macroalgae, as demonstrated by the example of *U. clathrata*. The highest bioethanol yield was obtained with alkaline treatment of macroalgae (32%). The enzymatic method of treating microalgae biomass with cellulase and xylanase enzymes yielded sufficiently good results. Acid treatment showed less effectiveness compared to alkaline treatment. This can be explained by the formation of byproducts such as formic, acetic, and other acids. In addition to ethyl alcohol, the substances released during acid treatment inhibit the growth, reproduction, and activity of the yeast *S. cerevisiae*.

Neveux et al. [22] studied several marine macroalgae species for biofuel production and determined that the algae *Derbesia* sp., *Ulva* sp., and *Oedogonium* sp. are promising candidates in terms of high biofuel yields and its quality.

The use of zeolites as a catalyst (dose from 10-20% by mass) allowed the group of authors [23] to increase the biofuel yield during processing of macroalgae *Ulva prolifera* from 16.6 wt.% to 29.3 wt.%, to increase the calorific value of biofuel from 21.2 MJ/kg to 32.2-34.8 MJ/kg under identical other conditions.

Recently, one of the main trends in biomass processing has been the concept of cascade conversion, which involves sequentially obtaining the maximum number of valuable products. Macroalgae can potentially be used in various biotechnology sectors, such as biofuels, food, cosmetics, and medical products [24,25].

This strategy for macroalgae consists in the initial production of bioactive compounds (lipids, vitamins) and subsequent conversion of biomass (hydrolysis, pyrolysis, fermentation) to produce biofuel, followed by disposal of fermented or recycled waste in the form of compost, sorbents, etc. [25].

There are a number of different methods to reduce the content of nitrogenous compounds in macroalgae biomass, including enzymatic, acid or alkaline hydrolysis, but these methods involve costly subsequent separation of proteins and their derivatives [26].

Several authors [27,28] suggested the use of low-temperature pretreatment of macroalgae in aqueous medium, leading to the transfer of part of the nitrogen-containing compounds into the aqueous phase without significant degradation of lipids.

Fine-tuning the reaction conditions of enzymatic hydrolysis can enhance favorable reaction pathways for selective extraction of compounds such as polysaccharides, proteins, amino acids, pigments, and inorganic nutrients. Moreover, after preliminary removal, the mass exchange between water and lipid molecules is improved, which ultimately increases the efficiency of biofuel extraction.

The approach of cascade extraction of valuable components fully implements the Zero Waste approach, which is a fundamental requirement of the EU countries for the implementation of the bioeconomy [29]. The primary requirement for macroalgae biomass processing is the extraction of the most valuable bioactive components and nutrients, followed by the production of biofuel from residues [30].

Studies by Wi [31] show that macroalgae biomass contains a large amount of sugars (not less than 50%), which can be used for the production of ethanol fuel.

Khoo, et al. [32] reported that macroalgae are promising raw materials for bioethanol production due to high biomass yields and higher yields compared to various land crops.

The potential of macroalgae for ethanol production can be estimated based on the following postulates: carbohydrate content of 60% of dry weight and 90% conversion level to ethanol through fermentation. Thus, fermentation of 1 g of sugar can produce 0.4 g of ethanol. Ideally, it is possible to obtain 0,22 kg or 0,27 L of ethanol from 1 kg of dry macroalgae biomass, which is equivalent to about 0,05 L of ethanol per kg of crude mass [33].

According to one study [34], macroalgae fermentation produces approximately 25,000 liters of bioethanol per hectare, whereas rapeseeds produce 1,500 liters, sunflower produces 950 liters, and soybeans produce only 446 liters.

Borines et al [35] produced bioethanol from *Sargassum* spp. with a conversion rate of 89%. *Gracilariaverrucosa*, red macroalgae, were fermented to produce bioethanol with a yield of 0.43 g/g sugars [36]. Yoza and Masutani [37] experimented with the production of bioethanol from the

biomass of *Ulva reticulate* macroalgae in which the concentration of bioethanol was 0.37% v/v. per 1 g of biomass. The authors also reported that the above results correspond to about 90 L of ethanol produced from a ton of dried macroalgae. According to a study by Osman et al. [38], Ulva intestinalis can produce 0.081 g of bioethanol per gram of dry weight. A bioethanol yield of 90.9% was obtained by treating marine macroalgae waste with saccharification and fermentation methods [39]. Anaerobic fermentation using *B. custersii* resulted in about 27.6 g/L ethanol from 72.2 g/L sugar in a continuous reactor [40]. In addition, the results of Offei et al. [41] led to the conclusion that *E. cottonii* can be a potential raw material for bioethanol production. Stefan Kraan et al. [42] reported that washing macroalgae in acidic water (0.09 M HCl in H_2O) at 65°C enhanced laminarin hydrolysis.

Conclusion

Various Baltic Sea macroalgae species were chosen and subjected to enzyme and yeast hydrolysis; various methods of treating selected macroalgae were investigated; and the content of reducing sugars was determined using 3,5-dinitrosalicylic acid solution. It was found that ethyl alcohol could be extracted from marine macroalgae samples using any treatment method, but alkaline pretreatment yielded the highest content – 32%.

Samples of macroalgae *U. clathrata*, *U. prolifera*, *C. fracta*, and *U. intenstinalis* fermented with yeasts *L. thermotolerans* (Y-4532) and *S. cerevisiae* (Y-4246) actively secreted alcohols, including ethanol. The fermentation of the macroalgae *U. clathrata* with the yeast *S. cerevisiae* (Y-4246) yielded the highest ethyl alcohol content (32%). Chemical and enzymatic treatment of marine macroalgae can also produce ethyl alcohol, which proves the prospect of using this raw material for bioethanol production.

Thus, macroalgae are one of the best raw materials for bioethanol production; however, the efficiency of using macroalgae is dependent on a variety of factors, ranging from the structure of the cell wall to the method of cultivation. Based on the results, it can be concluded that the direct fermentation method is well suited for producing bioethanol, which can then be used as a biofuel, which is currently important for improving the global environmental situation. The diversity of macroalgae allows for different combinations with different microorganisms to maximize reducing sugar yield, which influences biofuel yield.

Another benefit of using macroalgae in the production of biofuel is that it is highly biodegradable and does not exhibit toxicity because it does not contain sulfur compounds.

Macroalgae are a potential source of commercial biogas products such as biohydrogen and biomethane, which can be used as gas fuel or for power generation. Biohydrogen produced by macroalgae is a promising product in the renewable energy industry.

Disclosure statement

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Author contributions

SS and OB conceived and designed the research; SI, OB, RS and PM analyzed and interpreted the data; SS, AD and OK contributed reagents, materials, analysis tools or data; SI, OB and O. Kriger wrote the paper

Data availability statement

The data supporting the findings of this study are available from the corresponding author IS upon reasonable request.

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Tables

Table 1. Primer sequences for macroalgae identification

Gene	F primer	R primer	Division
ITS1+5.8S+ITS2	5′-GTCGCTCCTACCGATTGGGTGTG -3′	5′-TCCCTTTTCGCTCGCCGTTACTA-3′	Green algae
Tu (tufA)	5′- GGNGCNGCNCAAATGGAYGG-3′	5′-CCTTCNCGAATMGCRAAWCGC-3′	Green algae

Table 2. Cycling parameters during amplification of variable genes

No.	Stage	Incubation temperature, °C	Time, seconds
1	Pre-denaturation	95	60
2	Denaturation	95	30
3	Annealing	55–60	30
4	Elongation	72	60
Repetition of stages 2-4 for 29 times			
5	Final elongation	72	300

Table3. Sequence reaction cycling parameters

No.	Stage	Incubation temperature, °C	Time, seconds
1	Pre-denaturation	96	60
2	Denaturation	96	10
3	Annealing	50	4
4	Elongation	72	240
Repetition of stages 2-4 for 29 times			
5	Final elongation	72	300

Table 4. Characteristics of cellulase and xylase

Enzyme	Activity, U/g	Optimal pH	Operating pH range	Optimal temperature, °C	Operating temperature range, °C
Cellulase	10000	3.5-4.5	2.0-6.5	50-65	30-75
Xylanase	10000	5.0-7.0	4.5-8.0	50-60	40-65

Table 6. Influence of the pretreatment method on the bioconversion degree of macroalgae *U. clathrata* from the Baltic Sea

Pretreatment method	Optical absorbance		Concentration, mg/mL	
	before fermentation	after fermentation	before fermentation	after fermentation
Without treatment (treatment with distilled water)	0.06±0.009	0.17±0.01	0.885±0.05	1.042±0.08
H_2SO_4 (2%, 30 mL)	0.247±0.008	0.658±0.02	1.195±0.036	3.230±0.097
H_2SO_4 (2%, 10 mL) + $MnSO_4$ (10%, 15 mL)	0.395±0.012	0.528±0.017	1.928±0.090	2.586±0.078
NaOH (2%, 30 mL)	0.467±0.014	0.834±0.025	2.284±0.069	4.101±0.123
NaOH (2%, 35 mL)	0.613±0.019	0.853±0.026	3.007±0.009	4.195±0.126
EC	0.344±0.011	0.724±0.02	1.675±0.051	3.556±0.107

Data are presented as median±standard deviation (n = 3). EC - enzymatic complex cellulase : xylase (1:1)

Table 7. The content of alcohols in the studied samples

Name	Content, %						
	Sample No.1	Sample No.2	Sample No.3	Sample No.4	Sample No.5	Sample No.6	Sample No.7
Ethanol	32.80±0.98	11.19±0.34	6.39±0.19	31.25±0.9	12.10±0.34	6.51±0.19	5.37±0.16
Methanol	-	0.76±0.02	0.76±0.02	0.76±0.02	-	-	-
Butanol	0.40±0.01	21.37±0.64	4.82±0.14	4.82±0.14	-	-	-
Ethyl acetate	1.80±0.05	37.20±1.10	1.95±0.05	0.43±0.01	-	-	-
Isoamyl alcohol	4.51±0.13	-	-	-	-	-	-

Data are presented as median±standard deviation (n = 3)

Figures

a

ATGGCTCGCGAAAAATTTGAAAGATCAAAACCACACGTTAATATTGGAACTATCG
GTCATGTTGATCACGGTAAAACAACATTAACTGCCGCTATTACTATGGCATTACAA
AAATTTAGTGGAAACACCGGTAAAAAATATGATGAAATTGACTCTGCGCCTGAAG
AAAAAGCACGAGGTATTACTATTAATACAGCACATGTAGAATACGAAACAGAAA
ATCGCCATTATGCTCATGTTGATTGTCCCGGTCATGCTGATTATGTTAAAAATATG
ATTACAGGTGCAGCTCAAATGGATGGTGCTATTCTAGTTGTATCTGGTGCTGATGG
CCCTATGCCACAAACAAAAGAACATTTATTATTAGCTAAACAAGTAGGTGTTCCT
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ATAG

b

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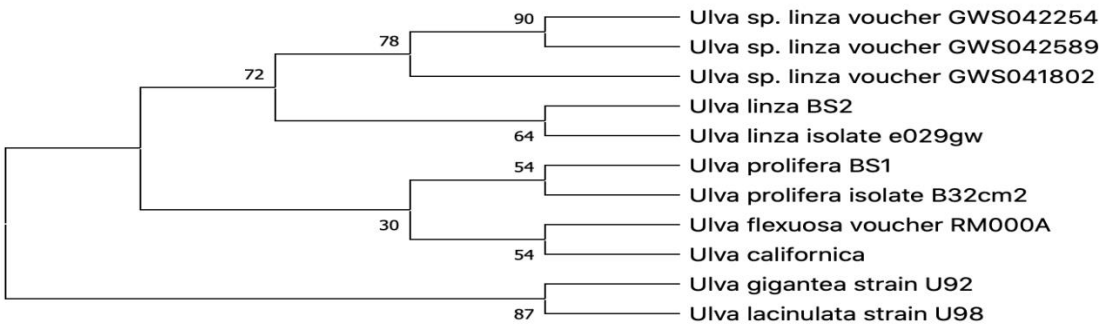

Figure 1.

a

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b

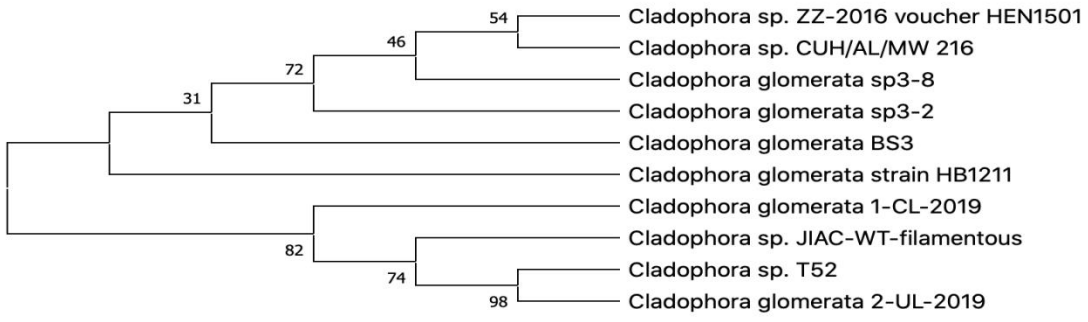

Figure 2.

a

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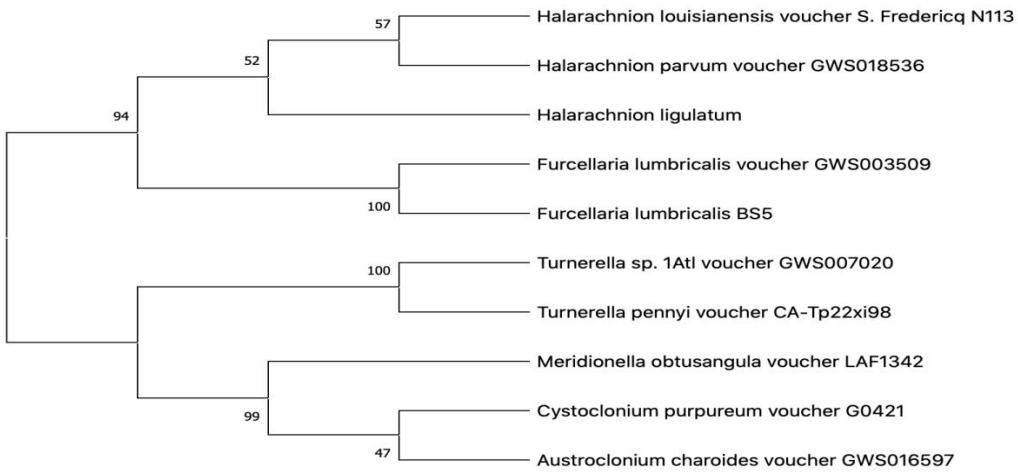
b



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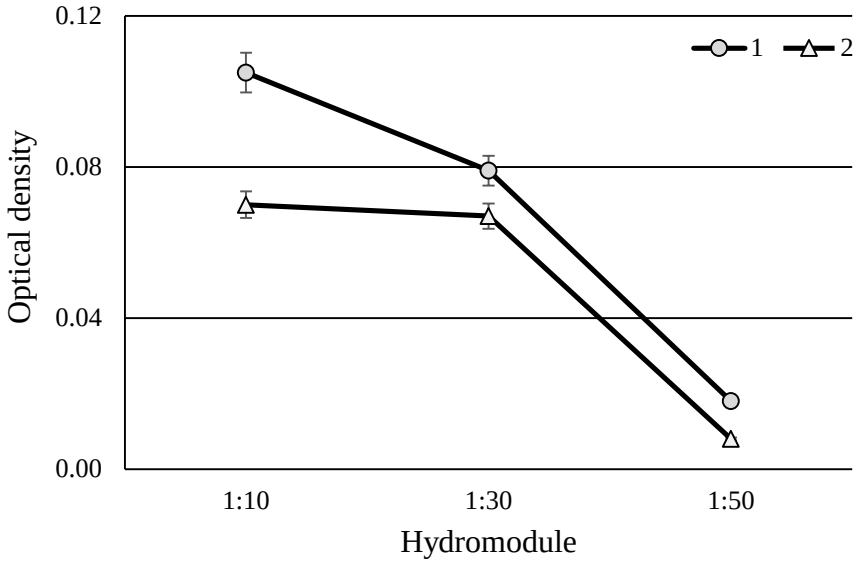
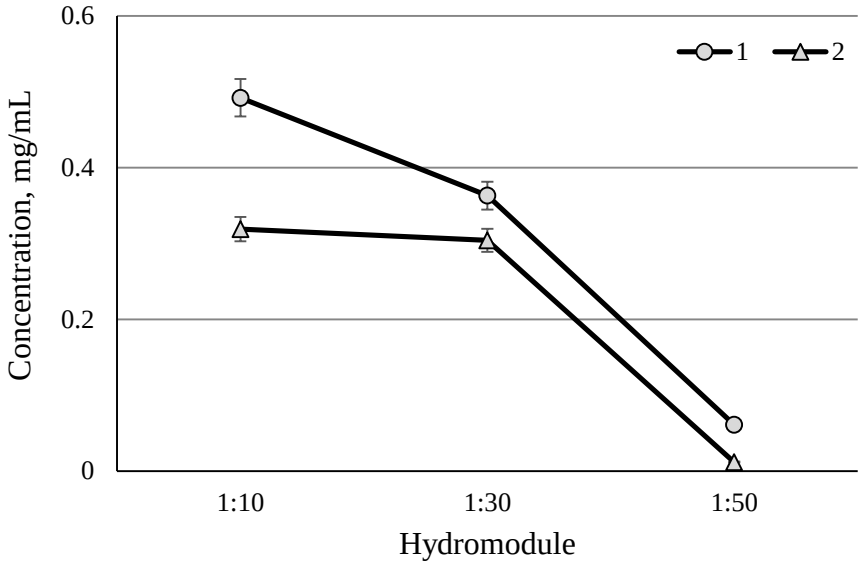


Figure 4.



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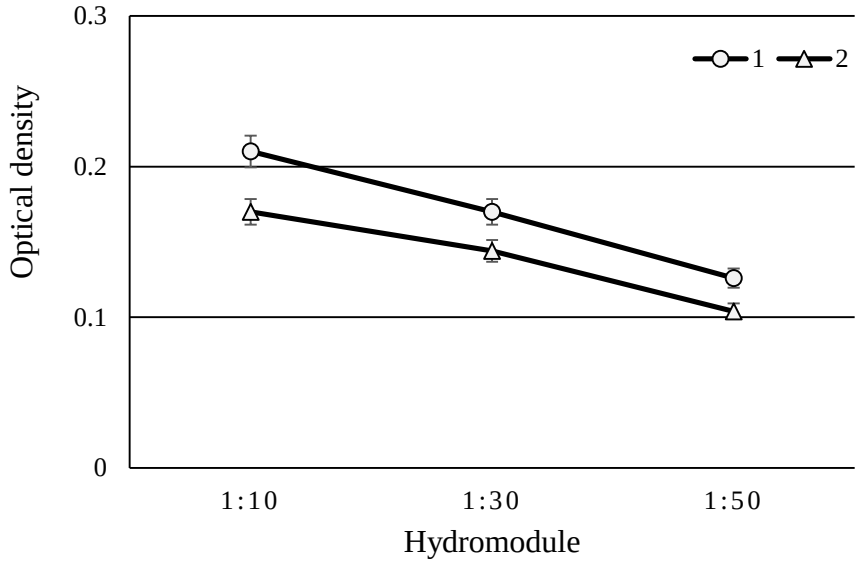

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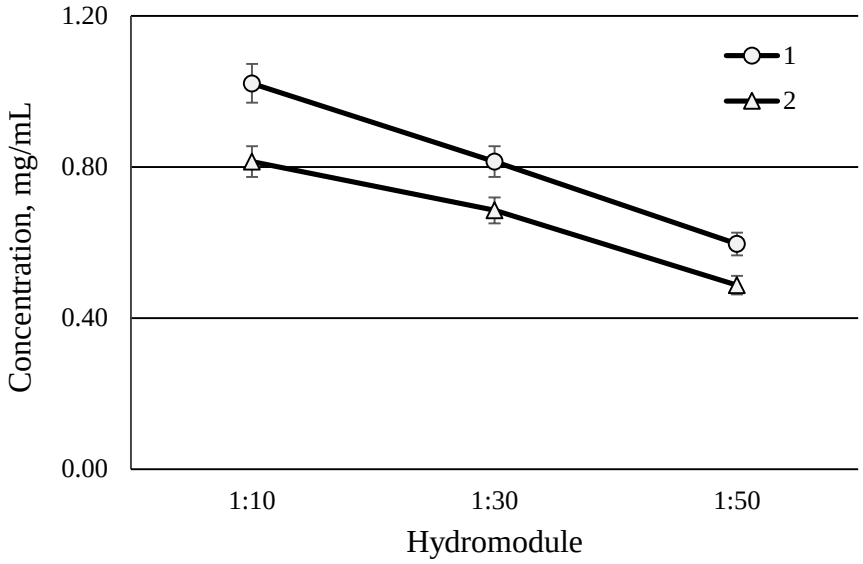
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0.80
0.40
0.00
Concentration, mg/mL
1
2
1:10
1:30
1:50
Hydromodule

Figure 7.

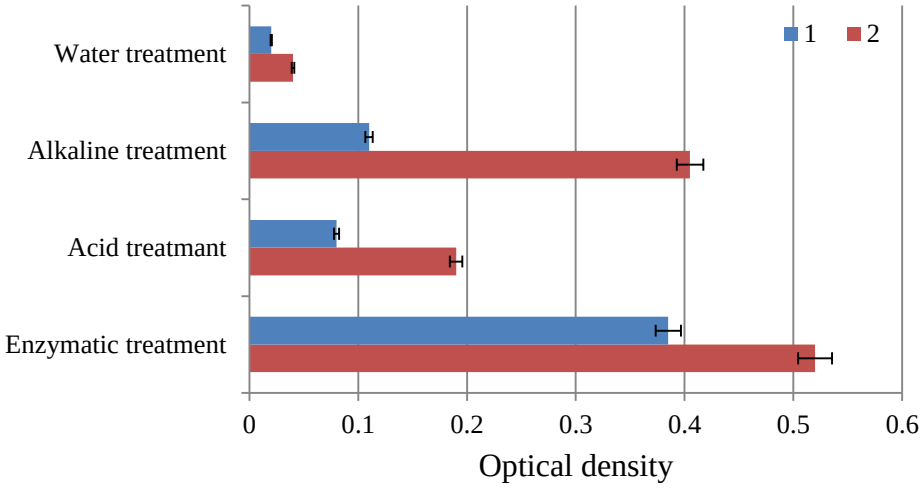

Water treatment
Alkaline treatment
Acid treatmant
Enzymatic treatment
1
2
0
0.1
0.2
0.3
0.4
0.5
0.6
Optical density

Figure 8.

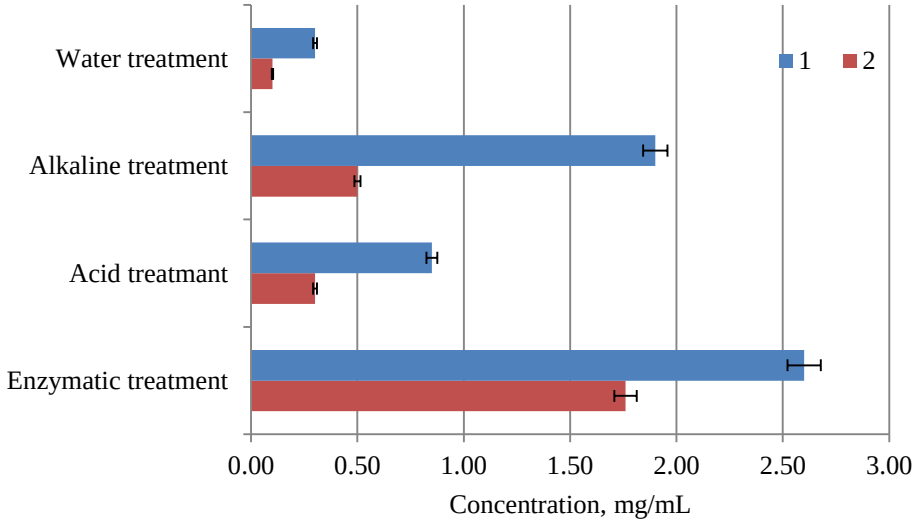

Water treatment
Alkaline treatment
Acid treatmant
Enzymatic treatment
1
2
0.00
0.50
1.00
1.50
2.00
2.50
3.00
Concentration, mg/mL

Figure 9.

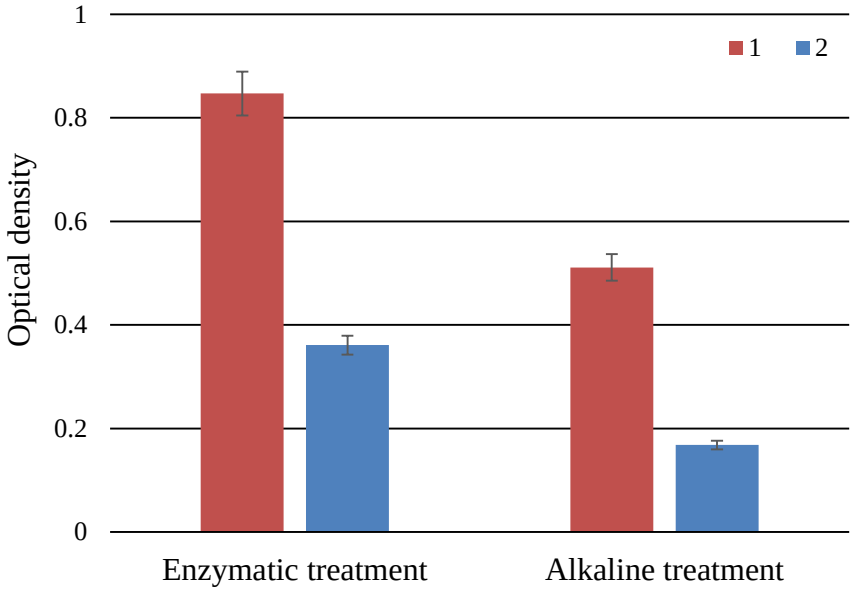

1
0.8
0.6
0.4
0.2
0
Optical density
1
2
Enzymatic treatment
Alkaline treatment

Figure 10.

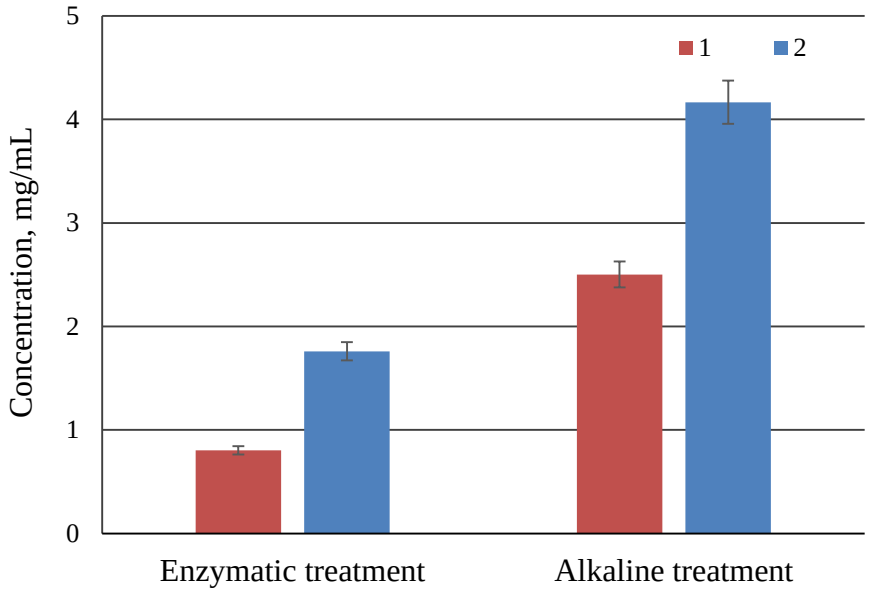
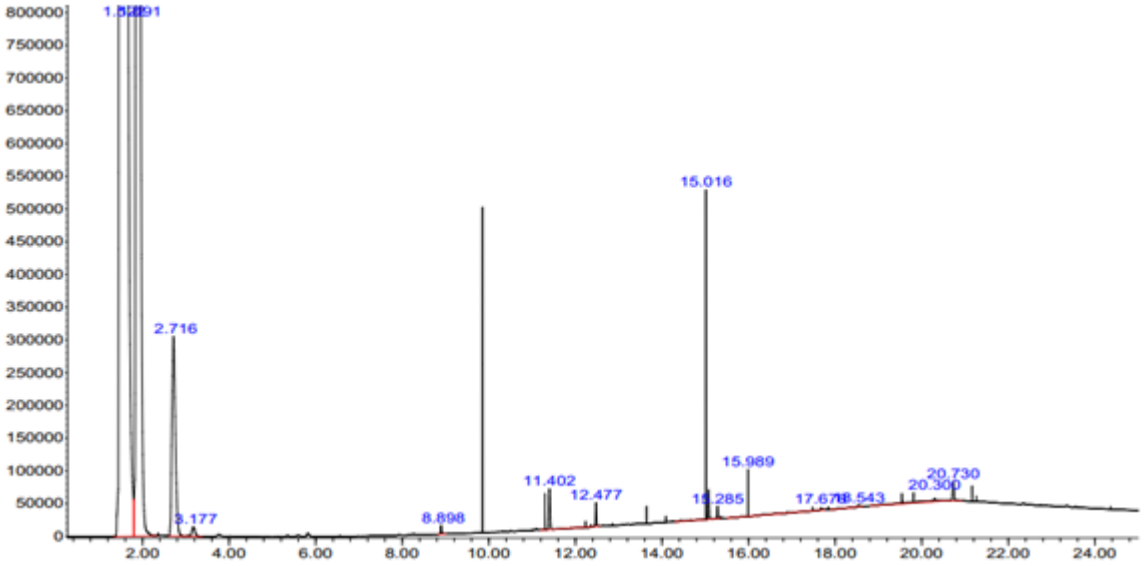

5
4
3
2
1
0
Concentration, mg/mL
1
2
Enzymatic treatment
Alkaline treatment

Figure 11.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650000
600000
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500000
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400000
350000
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250000
200000
150000
100000
50000
0
2.716
3.177
8.898
11.402
12.477
15.016
15.285
15.989
17.678
18.543
20.300
20.730
2.00
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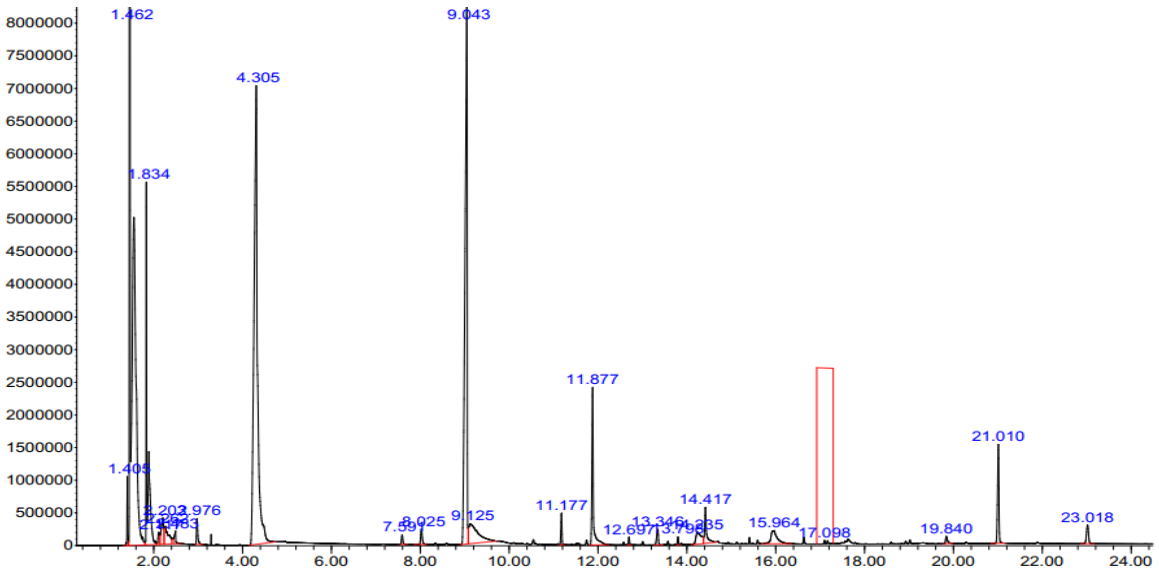
16.00
18.00
20.00
22.00
24.00

Figure 12.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6000000
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5000000
4500000
4000000
3500000
3000000
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1500000
1000000
500000
0
2.00
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8.00
10.00
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14.00
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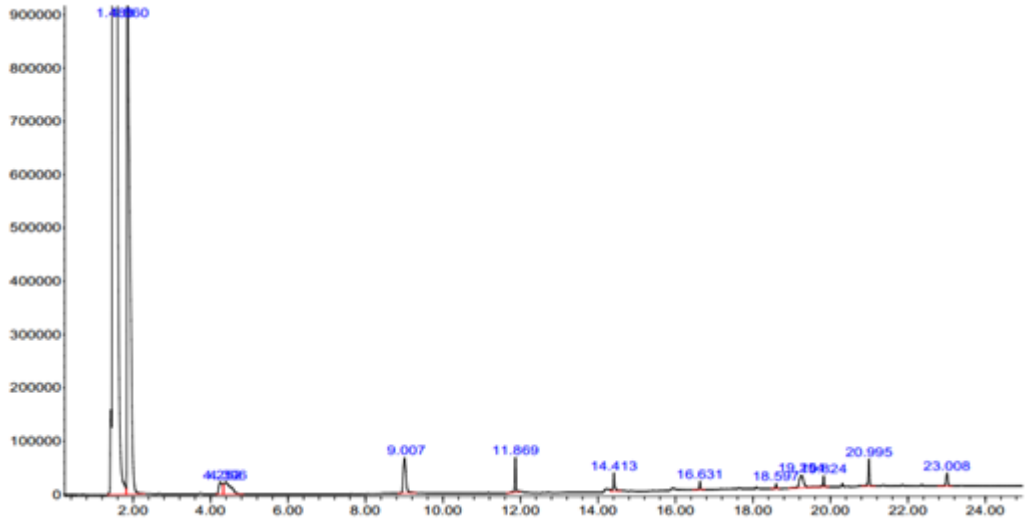
18.00
20.00
22.00
24.00

Figure 13.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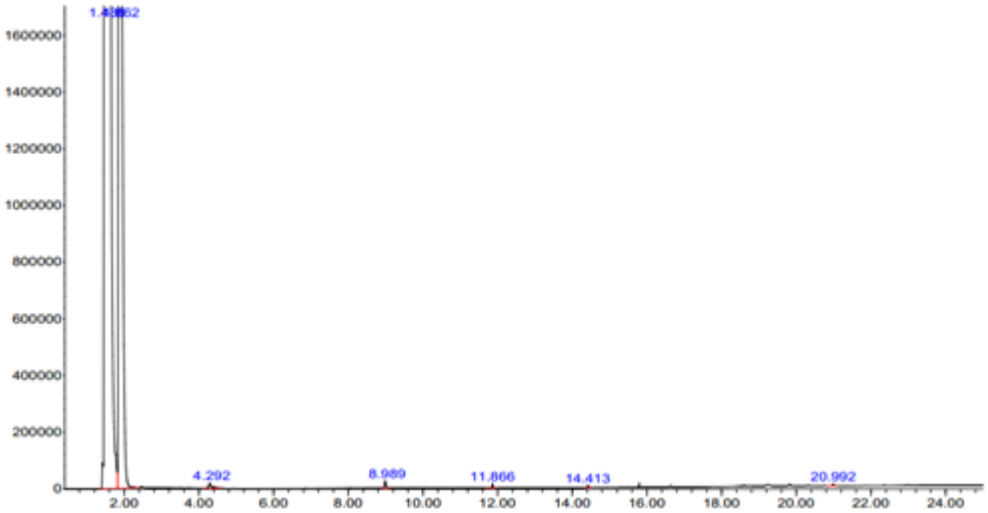
20.00
22.00
24.00

Figure 14.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20.992
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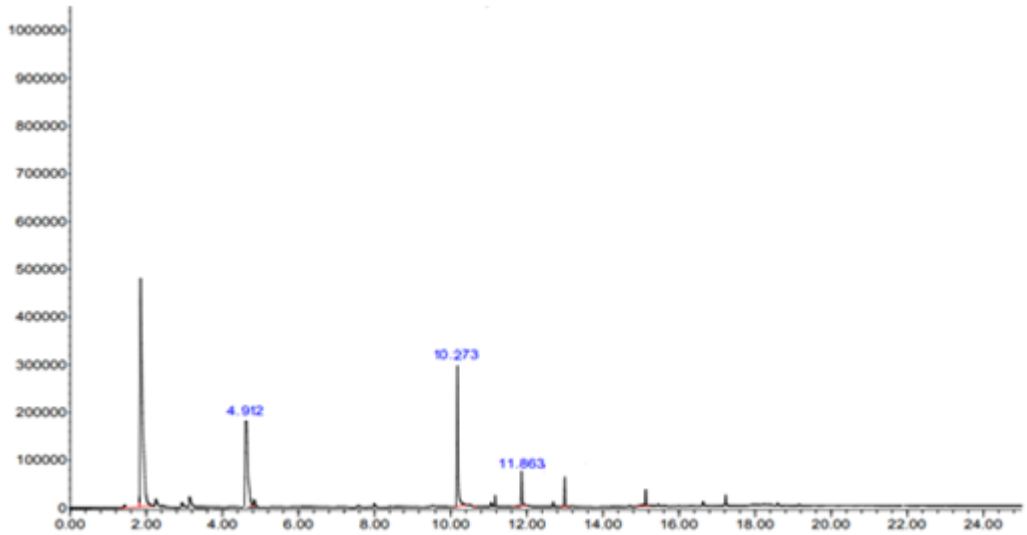
16.00
18.00
20.00
22.00
24.00

Figure 15.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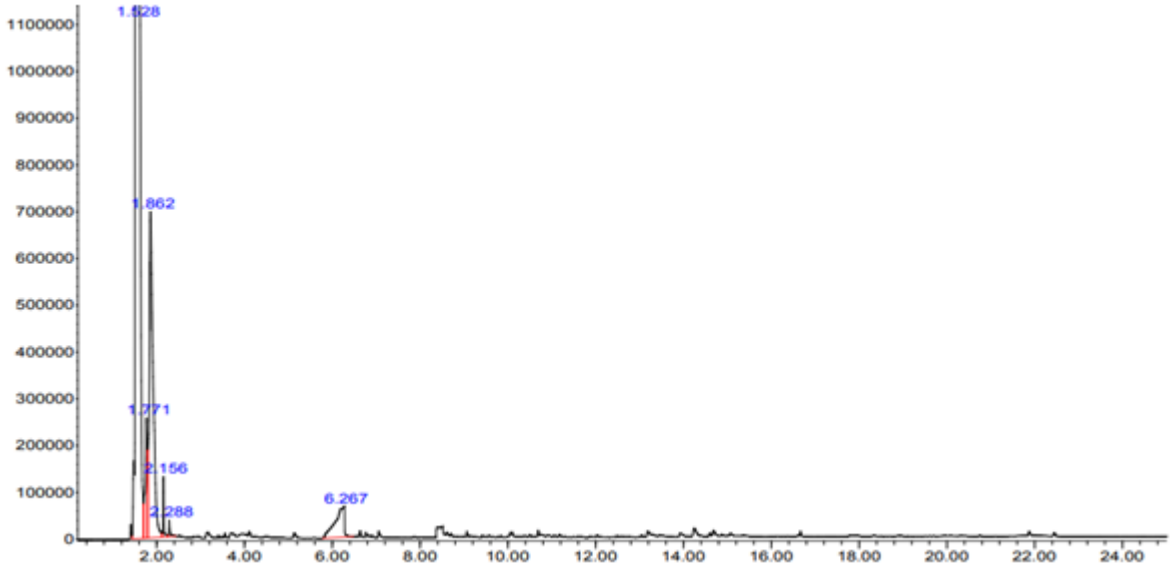
22.00
24.00

Figure 16.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22.00
24.00

Figure 17.

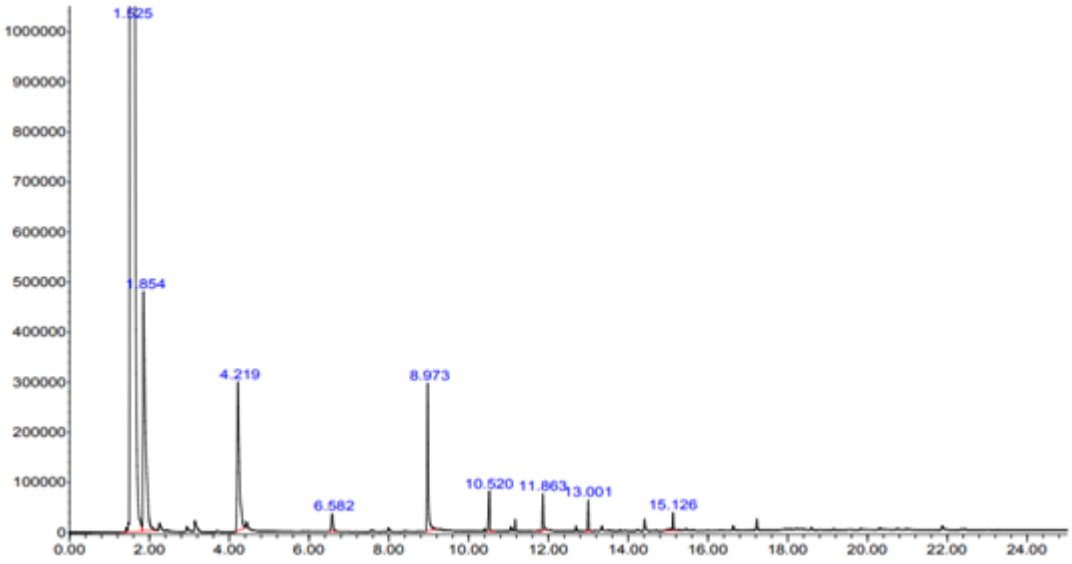
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Figure 18.

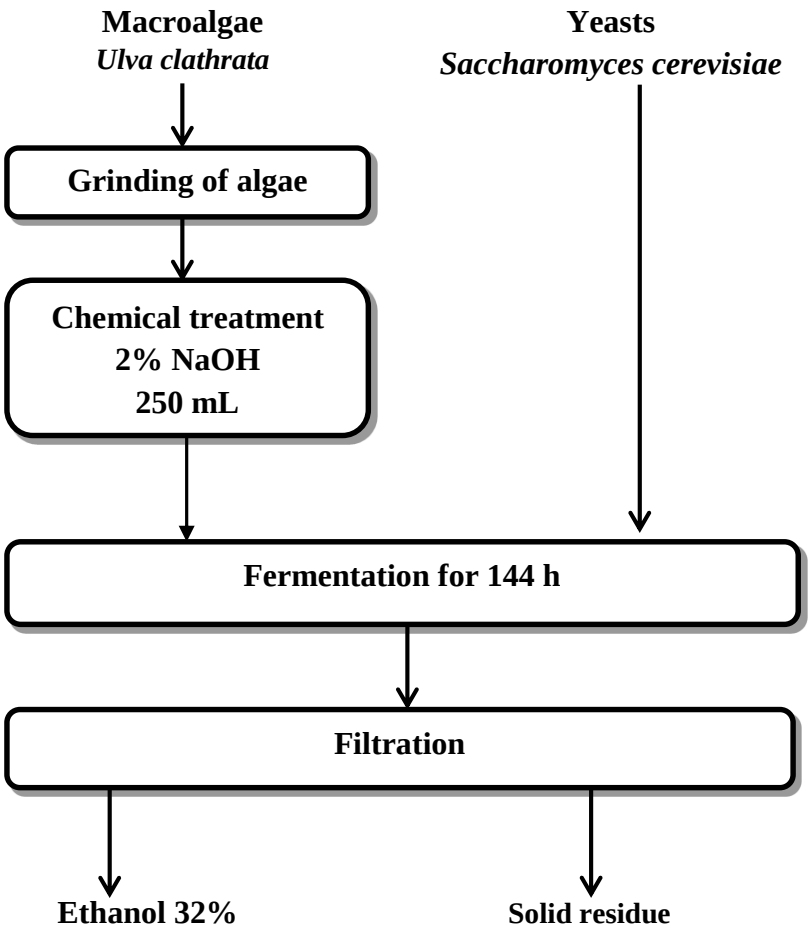
Macroalgae
Ulva clathrata
Yeasts
Saccharomyces cerevisiae
Grinding of algae
Chemical treatment
2% NaOH
250 mL
Fermentation for 144 h
Filtration
Ethanol 32%
Solid residue

Figure 19.

Figure captions

Figure 1. Results of identification of the green macroalgae *U. intestinalis* (linza) BS2 and *U. prolifera* BS1 by the Tu gene (tufA). A. Tu (tufA) gene sequence of the alga *U. intestinalis (linza)* BS2; B. Tu (tufA) gene sequence of the macroalga *U. prolifera* BS1; C. phylogenetic position of the green macroalga *U. intestinalis (linza*) BS2 and *U. prolifera* BS1 by the Tu gene (tufA).

Figure 2. Results of identification of the green macroalga *C. fracta* BS3 by the TS1+5.8S+ITS2 spacer. A. spacer sequence; B. phylogenetic position of the green macroalga BS3 by spacer.

Figure 3. Results of identification of the green macroalga U. clathrata BS5 by the COI gene. A COI gene sequence; B Phylogenetic position of the COI gene.

Figure 4. Changes in the optical density of the suspension of macroalgae *U. clathrata* at different hydromodules in the presence of yeast *S. cerevisiae* (Y-4246) at a fermentation time of 72 h: 1 – optical absorption before fermentation; 2 – optical absorption after fermentation. Data are presented as median±standard deviation (n = 3).

Figure 5. Changes in the reducing sugar concentration in the suspension of macroalgae *U. clathrata* at different hydromodules in the presence of yeast *S. cerevisiae* (Y-4246) at a fermentation time of 72 h: 1 – reducing sugar concentration before fermentation; 2 – reducing sugar concentration after fermentation. Data are presented as median±standard deviation (n = 3).

Figure 6. Changes in the optical density of the suspension of macroalgae *U. clathrata* at different hydromodules in the presence of yeast *S. cerevisiae* (Y-4246) at a fermentation time of 120 h: 1 – optical absorption before fermentation; 2 – optical absorption after fermentation. Data are presented as median±standard deviation (n = 3).

Figure 7. Changes in the reducing sugar concentration in the suspension of macroalgae *U. clathrata* at different hydromodules in the presence of yeast *S. cerevisiae* (Y-4246) at a fermentation time of 120 h: 1 – reducing sugar concentration before fermentation; 2 – reducing sugar concentration after fermentation. Data are presented as median±standard deviation (n = 3).

Figure 8. Changes in the optical density of suspensions of macroalgae *U. clathrata*, using the yeast *S. cerevisiae* after different treatment methods: 1 – before treatment; 2 – after treatment. Data are presented as median±standard deviation (n = 3).

Figure 9. Changes in the concentration of reducing sugars in suspensions of macroalgae *U. clathrata* using the yeast *S. cerevisiae* after treatment: 1 – before treatment; 2 – after treatment. Data are presented as median±standard deviation (n = 3).

Figure 10. Changes in the optical density of suspensions of macroalgae *U. intenstinalis* using the yeast *S. cerevisiae*: 1 – before treatment; 2 – after treatment. Data are presented as median±standard deviation (n = 3).

Figure 11. Changes in the concentration of reducing sugars in suspensions of macroalgae *U. clathrata* during treatment: 1 – before treatment; 2 – after treatment. Data are presented as median±standard deviation (n = 3).

Figure 12. Chromatogram of sample No. 1 (alkaline treatment of macroalgae *U. clathrata*).

Figure 13. Chromatogram of sample No. 2 (acid treatment of macroalgae *U. clathrata*).

Figure 14. Chromatogram of sample No. 3 (enzymatic treatment of macroalgae *U. clathrata*).

Figure 15. Chromatogram of sample No. 4 (treatment of macroalgae *U. clathrata* with distilled water).

Figure 16. Chromatogram of sample No. 4 (treatment of *U. intenstinalis* macroalgae with distilled water).

Figure 17. Chromatogram of sample No. 6 (alkaline treatment of *U. intenstinalis*).

Figure 18. Chromatogram of sample No.7 (enzymatic treatment of *U. intenstinalis*).

Figure 19. Scheme of bioethanol production from marine microalgae.