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Phytochemical and antioxidant composition of crude water extracts of *Chlorella vulgaris* and its effects on *Saccharomyces cerevisiae* growth in an ethanolic medium

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Abstract.
Chlorella vulgaris is rich in secondary metabolites that defend against environmental stress and aid in detoxification. In particular, bioactive compounds extracted from *C. vulgaris* may enhance the growth of microorganisms and detoxify them in an ethanolic medium. We aimed to effectively extract and characterize bioactive compounds found in *C. vulgaris* and further test them for their beneficial effects on the growth of *Saccharomyces cerevisiae* cultured in an ethanolic medium.
Bioactive compounds in *C. vulgaris* were extracted using ultrasound and water as solvents. The extracts were analyzed for total phenol and flavonoid contents as part of their phytochemical composition. Their DPPH radical activity and Hydrogen peroxide scavenging activity were examined to determine their antioxidant properties and protective potential for *S. cerevisiae* in an ethanolic medium. Further, the extracts were added at 0.1, 0.5, 1, 2, 3, and 4% w/v concentrations into *S. cerevisiae* culture induced with 1% v/v ethanol for 23 days. The yeast cells' density and viability were measured after 2, 5, 9, 13, 17, and 23 days.
The extracts of *C. vulgaris* were rich in phenols and flavonoids, which are important bioactive compounds. Higher concentrations of the extracts increased total phenols up to 47.67 GAE mg/L and total flavonoids up to 218.67 QE mg/L. The extracts' antioxidant composition showed high DPPH activity (70.12%) and H_2O_2 scavenging activity (4.97%). After 23 days, the samples treated with *C. vulgaris* extracts maintained a high viability of the yeast cells. In particular, the samples with 2, 4, 0.1, and 1% of the extract had a cell viability of 95.75, 94.04, 89.15, and 74%, respectively. The positive control (1% ethanol alone) and negative control (yeast alone) had 47.71 and 21.01% viability, respectively. This drastic reduction in viability was due to lysis of the yeast cells caused by ethanol.
Ultrasound extraction with water as a solvent produced abundant beneficial secondary metabolites from *C. vulgaris*. The addition of *C. vulgaris* extract increased the viability and cell density of *S. cerevisiae* after 27 days, thereby protecting the yeast cells from the toxic effects of ethanol.

Keywords. *Chlorella vulgaris*, phytochemicals, antioxidants, microalgae, yeast, ultrasound-assisted extraction, *Saccharomyces cerevisiae*, viability, water extracts

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Влияние фитохимического и антиоксидантного составов необработанных водных экстрактов *Chlorella vulgaris* на рост *Saccharomyces cerevisiae* в спиртовой среде

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Аннотация.
Хлорелла обыкновенная имеет высокое содержание вторичных метаболитов, которые защищают от воздействия внешней среды и способствуют детоксикации. Биоактивные соединения, экстрагированные из *Chlorella vulgaris*, могут усиливать рост микроорганизмов и детоксицировать их в спиртовой среде. В данном исследовании описали биологически активные соединения, обнаруженные в *C. vulgaris*, и их влияние на рост *Saccharomyces cerevisiae*, культивируемых в этанольной среде.
Биоактивные соединения извлекались из *C. vulgaris* при помощи ультразвука; в качестве растворителя применялась вода. В экстрактах анализировали общее содержание фенолов и флавоноидов. Антиоксидантные свойства и защитный потенциал для *S. cerevisiae* в спиртовой среде изучали через радикальную активность ДФПГ и активность по удалению перекиси водорода. В течение 23 дней экстракты в концентрациях 0,1, 0,5, 1, 2, 3 и 4 % мас./об. добавляли в культуру *S. cerevisiae*, индуцированную 1 % об./об. этанола. Плотность и жизнеспособность дрожжевых клеток измеряли через 2, 5, 9, 13, 17 и 23 дня.
Экстракты хлореллы обыкновенной богаты фенолами и флавоноидами, которые являются важными биологически активными соединениями. Высокие концентрации экстрактов увеличивали общее количество фенолов до 47,67 GAE мг/л, а общее количество флавоноидов до 218,67 QE мг/л. Антиоксидантный состав экстрактов показал высокую активность ДФПГ (70,12 %) и активность по связыванию H_2O_2 (4,97 %). Через 23 дня образцы, обработанные экстрактами *C. vulgaris*, сохраняли высокую жизнеспособность дрожжевых клеток. Образцы, содержащие 2, 4, 0,1 и 1 % экстракта, продемонстрировали жизнеспособность клеток в объеме 95,75, 94,04, 89,15 и 74 % соответственно. Положительный контроль (1 % этанол) и отрицательный контроль (дрожжи) имели жизнеспособность 47,71 и 21,01 % соответственно. Такое снижение жизнеспособности произошло из-за лизиса дрожжевых клеток, вызванного этанолом.
Ультразвуковая экстракция с водой в качестве растворителя привела к образованию обильных полезных вторичных метаболитов *C. vulgaris*. Добавление экстракта *C. vulgaris* на протяжении 27 дней повысило жизнеспособность и плотность клеток *S. cerevisiae*, что защищало дрожжевые клетки от токсического воздействия этанола.

Ключевые слова. *Chlorella vulgaris*, фитохимические вещества, антиоксиданты, микроводоросли, дрожжи, ультразвуковая экстракция, *Saccharomyces cerevisiae*, жизнеспособность, водные экстракты

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Introduction

Microalgae, or microscopic algae, are unicellular photosynthetic organisms that can grow in diverse environmental conditions, including deserts, polar regions, as well as marine and freshwater reserves [1]. They contain considerable amounts of carbohydrates, lipids, proteins, and secondary metabolites, including phytochemicals, pigments, antioxidants, and detoxicants [2]. These components serve as defense mechanisms during growth [3]. For example, antioxidant molecules regulate free radical formation in organisms and prevent oxidative damage, while detoxicants remove harmful substances from heavy metals and prevent oxidative stress [4].

Chlorella vulgaris, a unicellular green microalga of the genus *C. vulgaris*, is one of the most popular photosynthetic protists distributed in diverse terrestrial, freshwater, and marine habitats [5]. This microscopic (2–15 µm in diameter) non-mobile green alga is a solitary and high-performing producer when compared to both aquatic and terrestrial systems. It has gained widespread

use as a food supplement in various countries of the world [6, 7]. *C. vulgaris* contains various nutrients, including carotenes, protein, fiber, vitamins, minerals, nucleic acids, polysaccharides, chlorophyll, dietary antioxidants, chlorella growth factor, and bioactive peptide [8]. Since it can tolerate several heavy metals and metalloids, this microalga has been extensively used in Japan for detoxification purposes. Considered a most effective detoxifying agent, *C. vulgaris* is capable of binding and removing alcohol from the liver. Also, it can remove heavy metals (cadmium and mercury), certain pesticides, herbicides, and polychlorobiphenyls from the tissues of the human body [9, 10]. Its detoxification capability is attributed to its unique cell composition and the presence of metal-chelating compounds such as phytochelatin and metallothionein [9, 10]. In addition, the compounds extracted from *C. vulgaris* have functional benefits in food biotechnology (such as improving beer functional properties) and in microbial biotechnology (such as enhancing microbial growth and fermentation).

Fermentation is an important process in food production. In alcoholic fermentation, yeast transforms fermentable sugars in wort into ethanol, volatile organic compounds, and other metabolites. *Saccharomyces cerevisiae* is a yeast that is traditionally used to produce beer, wine and other higher alcohols, as well as bioethanol. However, ethanol buildup caused by the conversion of fermentable sugars harms the performance/efficiency of the yeast cells, as well as their viability and vitality [11]. Ethanol is known to inhibit the growth of microorganisms by dissolving their membrane lipid bilayer and denaturing their proteins [12]. There has been extensive research to enhance the fermentative properties of the yeast and protect its cells from the toxic effects of ethanol buildup. Studies have shown that ethanol damages the mitochondrial DNA in the yeast cells and inactivates some of the enzymes such as hexokinase and dehydrogenase [13]. *S. cerevisiae* generally cannot tolerate an environment with more than 10–11% ethanol. Higher concentrations of ethanol during fermentation reduce the yeast's effectiveness in fermenting sugars and limit fermentation productivity and ethanol yield [14]. However, certain types of yeasts can withstand higher concentrations of ethanol, which is evident in some specialist brews. Cost-effective ethanol fermentation depends on, among other factors, rapid and high-yielding conversion of carbohydrates to ethanol. This, in its turn, entirely depends on the survival and performance of yeast cells under industrial conditions [15]. Monitoring yeast survival and performance, as well as yeast cell viability and vitality (the physiological state of viable cells), is a critical factor in alcoholic fermentation. Effective brewery fermentation requires appropriate conditions for maintaining yeast vitality and producing high-quality beer [11]. Understanding the impact of ethanol toxicity on *S. cerevisiae* and its cells' response to ethanol stress is a key factor in optimizing fermentation productivity and maximizing ethanol production without affecting the yeast fermentation activity [11]. This can be achieved through developing approaches to improve the ethanol tolerance of the yeast cells during fermentation, including the use of secondary metabolites such as antioxidants and detoxicants from microalgae.

Many studies have investigated the beneficial effects of lactic acid bacteria and other prebiotics on the growth of microorganisms and preventing oxidative damage. However, fewer studies have looked into the detoxication capabilities of *C. vulgaris*. In our previous study, *C. vulgaris* biomass enhanced the growth and survival of *S. cerevisiae* cultured in an ethanolic medium for 5 days [13]. Further, chlorella extracts improved the viability of the yeast cultured in a liquid ethanol-free medium [6]. We are yet to understand what effects the extracts from chlorella would have on a long-term culture of yeasts in an ethanolic medium. Also, these compounds are produced as secondary metabolites in response to environmental factors and may not be easily accessible for the rigid cell wall. For this reason, it is important to understand not only how selective an extraction process is but also how it may affect the composition of bioactive compounds. Therefore, we aimed to identify an effective and optimal method for extracting phytochemicals from *C. vulgaris*, characterize them for their antioxidant properties, and test their protective effects on yeasts cultured in ethanolic media.

Study objects and methods

Materials and chemicals. Food-grade *Chlorella vulgaris* biomass was obtained from Zhengzhou Sigma Chemical Co., Ltd. (Zhengzhou, China). Wyeast 1272 American Ale II yeast (Wyeast Laboratories, OR, USA) was provided by Beersfan microbrewery (Yekaterinburg, Russia). Sabouraud growth medium was purchased from the State Research Center for Applied Microbiology (Obolensk, Russia). Ethanol and methanol were purchased from Rosbio (St. Petersburg, Russia). Anhydrous gallic acid (anhydrous), Folin-Ciocalteu solution, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (Darmstadt, Germany). Sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), aluminum nitrite ($Al(NO_3)_3$), and sodium nitrite ($NaNO_2$) were sourced from Bashkir Soda Company (Ufa, Bashkortostan, Russia). Quercetin was purchased from Conscientia Industrial Co., Ltd. (Zhejiang, China). All the chemicals used were of analytical grade.

Water extraction of *C. vulgaris*. Bioactive compounds of *C. vulgaris* were extracted using an ultrasonic extractor with distilled water as a solvent, as previously described [6]. Briefly, the *C. vulgaris* biomass (1 g) was measured in a beaker containing water (10 mL). The beaker was placed on an ultrasonic-assisted extractor (Elma, Schmidbauer GmbH, Germany) and sonicated at 30°C, 37 kHz, 60% power for 30 min. After sonication, all the samples were mixed and centrifuged with an

IEC-CL Multispeed centrifuge (Rotoflox 32A, Hettich, Tuttlingen, Germany) at 6000 rpm for 10 min, and the supernatant was transferred into a clean sterilized beaker. The solvent (water) was evaporated using an IKA Rv8 rotary evaporator (IKA Werke GmbH and Co. KG, Staufen, Germany) until a viscous *C. vulgaris* water extract was obtained and stored at −18°C until further use. The extraction yield, %, was calculated as follows:

$$\text{Extraction yield} = \left[\frac{\text{Weight of extract after evaporation}}{\text{Weight of the dry sample}}\right] \times 100 \text{ (l)}$$

Phytochemical analysis. ***Determination of total phenolic content.*** The total phenolic content of the *C. vulgaris* water extracts (CWE) was determined according to a previously described method with slight modifications [16]. Briefly, diluted CWE samples (0.25 mL) without pretreatment, CWE, and standard gallic acid (0, 50, 100, 150, 250, and 500 mg/L) were pipetted into assay tubes. Folin-Ciocalteu solution (0.5 mL) and distilled water (5.5 mL) were mixed and homogenized. The mixture was allowed to incubate for 5 min, and 1 mL of Na_2CO_3 (20%) solution was added. The assay tubes were further incubated at 20°C for 2 h. Absorbance was measured at 765 nm against a blank (distilled water) using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The total phenolic content was calculated from the standard curve of gallic acid ($y = 0.0038x + 0.0487$, $R^2 = 0.9982$), and the results were expressed as milligrams of gallic acid equivalents per liter of CWE (GAE mg/L).

Determination of total flavonoid content. The total flavonoid content of the *C. vulgaris* water extracts was determined based on a modified nano2–Al $(NO_3)_3$–NaOH colorimetric procedure, as previously described [17]. Briefly, 1 mL of the extract was mixed with 4 mL of 30% ethanol and 0.3 mL of nano2 (5%, w/v). After 5 min, the mixture was reacted with 0.3 mL of $Al(NO_3)_3$ (10%, w/v) for 6 min. Then, 4 mL of 1 M NaOH was added, and the mixture was adjusted to 10 mL with 0.4 mL of 30% ethanol. After incubation at room temperature for 10 min, absorbance was measured at 510 nm. The $Al(NO_3)_3$ and NaOH solutions were substituted with the same amount of 30% ethanol in the blank. The total flavonoid content of the samples was expressed as quercetin equivalents, and the calibration curve ranged from 0 to 500 µg/mL.

Antioxidant activity. ***In vitro DPPH antioxidant activity.*** *In vitro* antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, as previously described [18, 19]. Two milliliters of the *C. vulgaris* water extract (CWE) (1:10 dilution) was mixed with 0.1 mm DPPH methanol solution (2 mL). Similarly, control samples were prepared by mixing ethanol (2 mL) and 0.1 mm DPPH methanol solution (2 mL). The mixture was incubated in the dark for 30 min at room temperature. Absorbance was measured at 515 nm using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). DPPH antioxidant activity was calculated as follows:

$$\%\ \text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad (2)$$

where $A_{control}$ is the absorbance of control; A_{sample} is the absorbance of sample.

Hydrogen peroxide (H_2O_2) scavenging activity. The hydrogen peroxide scavenging capacity of the *C. vulgaris* water extracts (CWE) was assayed according to the method described by, with slight modifications [20]. Briefly, an H_2O_2 solution (43 mm) was prepared in a 1 M phosphate buffer (pH 7.4). An aliquot (3 mL) of the diluted CWE samples (50 times) was transferred into separate test tubes, and H_2O_2 solution (1 mL) was added. The reaction mixture was incubated for 10 min at room temperature. After incubation, absorbance was measured using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) at 230 nm against a blank solution (phosphate buffer only). The experiment was performed in triplicate. The percentage of hydrogen peroxide scavenging of the CWE samples was estimated as follows:

$$\%\ H_2O_2\ \text{scavenging activity} = \left[\frac{A_{\text{control}}\ (\text{without CWE}) - A_{\text{sample}}}{A_{\text{control}}}\right] \times 100 \qquad (3)$$

where $A_{control}$ is the absorbance of control; A_{sample} is the absorbance of sample; $A_{control}$ (without CWE) is the absorbance of control (without CWE).

Identification of individual phenols. The samples were analyzed using high-performance liquid chromatography on an Agilent 1290 Infinity II liquid chromatograph coupled to an Agilent 6545 Q-TOF LC/MS quadrupole time-of-flight mass spectrometer. Chromatographic separation was performed on a Zorbax Eclipse Plus C18 RRHD column with dimensions of 2.1 mm × 50 mm × 1.8 µm (959757-902, Agilent Technologies), additionally protected by a pre-column with dimensions of 2.1 mm × 5 mm × 1.8 µm. The mobile phase consisted of a mixture of a 0.1% (v/v) solution of formic acid in water (solvent A) and a 0.1% solution of formic acid in acetonitrile (solvent B). Chromatographic separation was performed in a gradient mode with a linear change in the content of eluent B in the mobile phase from 5 to 100 vol.% for 15 min. Then, the final content of eluent B was maintained for 2 min. The flow rate was 0.4 mL/min. The temperature of the column thermostat was set at 35°C. The prepared solution was injected in a volume of 1 µL. A quadrupole time-of-flight detector was used with an electrospray ionization source in the negativeion detection mode. The scan range (m/z) was 100–1700 in the MS mode and 30–500 in the MS/MS mode. Spectra from the MS/MS experiments were obtained due to thecollision-induced dissociation (DIS) with nitrogen molecules at a collision energy of 20 eV. The device was automatically adjusted using standard

calibration solutions recommended by the device manufacturer. Preliminary identification of the compounds was carried out by determining their elemental compositions (gross formulas) and comparing the DIS spectra obtained during the MS/MS experiments with literature data and spectral libraries.

Protective effect of *C. vulgaris* extracts on the growth of *Saccharomyces cerevisiae. S. cerevisiae* culture and growth. Precultured *S. cerevisiae* cells were inoculated (1×10^6 cells/mL) in Sabouraud medium (100 mL) supplemented with 0.1, 0.5, 1, 2, 3, and 4% w/v *C. vulgaris* extracts (coded YEC 1-6 respectively) in 250 mL Erlenmeyer flasks, and ethanol was added to the cultures at a final volume of 5% v/v. Similarly, the ethanol (5% v/v)-supplemented cultures served as a positive control, the cultures in the media alone, without treatment, served as a negative control, and the cultures treated with only 0.1% w/v *C. vulgaris* extract served as a normal control. All the samples were transferred into a rotary shaker at 160 rpm and incubated at 26°C for 23 days. Aliquots from the samples were collected from the media on days 2, 5, 9, 13, 17, and 23 to check for yeast viability.

***S. cerevisiae* growth and viability quantification.** *S. cerevisiae* cell numbers were determined using a Nikon labophot-2 light microscope (×400 magnification) with methylene blue staining. The numbers of viable yeast cells were calculated by pipetting 1 mL of the yeast suspension into 9 mL of water. The diluted suspension (1 mL) was mixed with methylene blue stain (1:1 ratio) and allowed to rest for 1 min in a 2 mL microcentrifuge tube. The sample (10 μL) was then transferred to a hemocytometer slide (Goryaev chamber) using a pipette and examined under a microscope. The yeast count (million cells/mL viable cells) was determined by counting five small squares (each consisting of 16 smaller cells) from a total of 25 squares. The cell density, cells/mL, and yeast viability, %, were calculated using the following Eqs. (4)–(5):

$$\text{Cell density } M = \frac{a \times 10^3 \times n}{h \times S} \quad (4)$$

where M is the number of cells in 1 mL of suspension; a is the average number of cells in a square grid; 10^3 is the conversion factor mm^3 to mL; n is the dilution factor of the suspension used; h is the depth of the chamber, mm; and S is the area of the grid square, mm^2.

$$\text{Yeast viability} = \frac{\left(C_{\text{cells}} - C_{\text{dead cells}}\right)}{C_{\text{cells}}} \times 100 \quad (5)$$

where C_{cells} is the total counted cells; $C_{dead\ cells}$ is the s total counted dead cells.

Statistical analysis. The data generated from triplicate measurements were subjected to analysis of variance (ANOVA) and significant ($p \leq 0.05$) means were separated with Tukey's test using Minitab 21 (Minitab Ltd., Coventry, UK). Microsoft Excel was used in plotting the line graph to show the progression of the cells over time. The results were presented as mean ± standard deviation (SD).

Results and discussion

Extraction yield. To obtain functional compounds from *Chlorella vulgaris*, ultrasound-assisted extraction was employed with water used as a solvent to maximize the extract content. Ultrasound-assisted extraction uses ultrasonic waves to extract compounds from the compound matrix through the cavitation phenomenon, which allows the solute to diffuse into the solvent. In our study, 0.67 g of nutraceutical compounds was extracted from *C. vulgaris*, with a yield of 67%. Kitada *et al.* performed hydrothermal extraction in a semi-batch and batch-type extractor (120–200°C, 2–10 mPa, 30–300 min) and conventional hot-water extraction (95–100°C, 25 min) [21]. They observed that the extraction yield increased significantly with increasing temperature and extraction time. Furthermore, hot-water extraction had the lowest yield at both temperatures and a lower extraction time of 30 min [21]. However, Plaza *et al.*, who performed pressurized liquid extraction, reported much higher extraction yields than those obtained by ultrasound-assisted extraction, when water and higher temperatures were employed [5]. According to both results, microalgae require medium-high polar solvents to extract bioactive compounds, as *C. vulgaris* is mostly composed of medium-to-high-polarity compounds [5]. Higher temperature resulted in an increase in the ion product of water, which enhanced the ability of water to penetrate *C. vulgaris* and extract its extractable components. However, high temperature can also cause extractable components to decompose into carbon, which reduces the yield [21].

Bioactive compounds from *C. vulgaris* biomass can be potentially used as functional food supplements or to improve microbial growth [7, 22, 23]. *C. vulgaris* has rigid cell walls which are difficult to break down. As a result, extracting intracellular components becomes a challenge and a costly operation [5]. Therefore, for efficient extraction, cell lysis must be achieved before other fractionation procedures. Some of the best methods to lyse the cell walls include mechanical agitation using ultrasonic sounds, high-pressure homogenizers, bead mills, etc. [24]. Other methods include thermal, enzymatic, chemical, and osmotic shock treatments. Nonetheless, the quality of the target molecules is likely to be different compared to the cell disruption method [25].

Phytochemical composition and antioxidant properties. The phytochemical composition and antioxidant properties of *C. vulgaris* extract are shown in Tables 1 and 2, respectively. Based on our previous studies, we established concentrations of *C. vulgaris* biomass which had beneficial effects on yeast growth and beer [6, 13, 26]. This study showed that higher concentrations caused an increase in both phytochemical composition and antioxidant activity.

As can be seen in Table 1, the CWE3 sample (5 g/L *C. vulgaris* extract) had the highest total phenol content and total flavonoid content (47.67 mg GAE/L and 218.67 mg QE/L, respectively). These contents were

Table 1. Phytochemical properties of *Chlorella vulgaris* extracts

Таблица 1. Фитохимические свойства экстрактов хлореллы обыкновенной (*Chlorella vulgaris*)

Samples	Total phenol content, mg GAE/L	Total flavonoid content, mg QE/L
CWE1	4.60 ± 0.06^{c}	30.81 ± 17.85^{c}
CWE2	11.66 ± 0.74^{b}	272.10 ± 27.95^{b}
CWE3	47.67 ± 1.05^{a}	218.67 ± 17.51^{a}

The results represent the mean ± SD of triplicate measurements. The means with different letters in each column denote significant differences ($p < 0.05$) using Tukey's test, where CWE is the *C. vulgaris* extract wort (CWE1 = 0.5 g/L *C. vulgaris* extract, CWE2 = 1 g/L *C. vulgaris* extract, CWE3 = 5g/L *C. vulgaris* extract).

Результаты представляют собой среднее значение ± SD трехкратных измерений. Средние значения в одном столбце с разными буквенными обозначениями маркируют достоверные различия ($p < 0,05$) с использованием критерия Тьюки, где CWE – экстракт сусла *C. vulgaris* (CWE1 = 0,5 г/л экстракта *C. vulgaris*, CWE2 = 1 г/л экстракта *C. vulgaris*, CWE3 = 5 г/л экстракта *C. vulgaris*).

Table 2. Antioxidant activities of *Chlorella vulgaris* extracts

Таблица 2. Антиоксидантная активность экстрактов хлореллы обыкновенной (*Chlorella vulgaris*)

Samples	DPPH Antioxidant activity, %	H_2O_2 Scavenging activity, %
CWE1	47.30 ± 0.64^{c}	2.24 ± 0.05^{c}
CWE2	50.45 ± 0.64^{c}	4.25 ± 0.11^{b}
CWE3	70.12 ± 1.49^{b}	4.97 ± 0.05^{a}
ASA1	96.40 ± 0.00^{a}	3.72 ± 0.21^{b}
ASA2	95.35 ± 1.06^{a}	5.01 ± 0.00^{a}
ASA3	96.40 ± 0.00^{a}	5.09 ± 0.11^{a}

The results represent the mean ± SD of triplicate measurements. The means with different letters in each column denote significant differences ($p < 0.05$) using the Tukey's test, where CWE is the *C. vulgaris* extract wort (CWE1 = 0.5 g/L *C. vulgaris* extract, CWE2 = 1 g/L *C. vulgaris* extract, CWE3 = 5 g/L *C. vulgaris* extract, ASA1 = 0.5 g/L ascorbic acid, ASA2 = 1 g/L ascorbic acid, ASA3 = 5 g/L ascorbic acid).

Результаты представляют собой среднее значение ± SD трехкратных измерений. Средние значения в одном столбце с разными буквенными обозначениями маркируют достоверные различия ($p < 0,05$) с использованием критерия Тьюки, где CWE – экстракт сусла *C. vulgaris* (CWE1 = 0,5 г/л экстракта *C. vulgaris*, CWE2 = 1 г/л экстракта *C. vulgaris*, CWE3 = 5 г/л экстракта *C. vulgaris*, ASA1 = 0,5 г/л аскорбиновой кислоты, ASA2 = 1 г/л аскорбиновой кислоты, ASA3 = 5 г/л аскорбиновой кислоты).

both significantly different ($p \leq 0.05$) from those in the samples CWE1 and CWE 2 (0.5 and 1 g/L *C. vulgaris* extracts, respectively).

According to Table 2, the antioxidant properties of the extracts increased with higher concentrations. Ascorbic acid, which is a natural antioxidant, was used as a reference standard. The ascorbic acid standard showed significantly higher DPPH scavenging activity in the samples ASA1, ASA2, and ASA3 (0.5, 1, and 5 g/L ascorbic acid, respectively) at 96.40, 95.35, and 96.40%, respectively. All of these percentages were statistically significant ($p \leq 0.05$) compared to those for the *C. vulgaris* extracts (47.30, 50.45, and 70.12% for CWE1, CWE2, and CWE3, respectively). Likewise, higher concentrations of the ascorbic acid standard showed higher H_2O_2 scavenging activity (5.01 and 5.09% for ASA2 and ASA3, respectively), but the difference was not statistically significant ($p \geq 0.05$) from CWE3 (4.97%). CWE1 had the lowest H_2O_2 scavenging activity (2.24%) and was significantly different ($p \leq 0.05$) from the other samples.

Our study showed that *C. vulgaris* extracts contain considerable amounts of phytochemicals and antioxidant compounds due to the presence of hydrophilic molecules in the water extract. Free radicals can be neutralized or stabilized by antioxidants before they damage biological cells. Antioxidants can either create stable compounds by combining with other radicals or be absorbed by other antioxidants if they are unable to initiate a chain reaction [27]. Our findings are in line with the work of Dantas *et al.*, who recorded higher total phenol content and total flavonoid content values (3.34 and 1.48 mg/mL, respectively) of the water extracts from *C. vulgaris* when compared to other solvents [27]. Vieira *et al.* recorded a total phenol content of 114.32 mg/100 g dried biomass of *C. vulgaris* extracted with a solvent mixture of methanol, water, acetic acid, and ascorbic acid [28]. Likewise, Dantas *et al.* found that *C. vulgaris* water extracts had 68.5% DPPH inhibition when compared to other solvents, which is consistent with our results [27].

Several techniques have been recently created to assess the overall antioxidant activity of bioactive compounds. Measuring each antioxidant independently is comparatively challenging since crude extracts contain a variety of antioxidant components [29, 30]. Due to their ability to prevent product oxidation, free radical scavenging with DPPH is frequently employed as a measure of antioxidant activity [30, 31]. Hydrogen peroxide (H_2O_2) in excess amounts can be harmful to cells [32]. This damage increases as free radicals are combined with Fe^{+2}, generating hydroxyl radicals through the Fenton reaction, which involves lipid peroxidase [33]. The scavenging of hydrogen peroxide may be associated mostly with the phenolic compounds which can contribute electrons to hydrogen peroxide, thus neutralizing it into water.

Phenolic composition. High-performance liquid chromatography-mass spectrometry (HPLC/MS) was used to detect and identify bioactive compounds in the *C. vulgaris* extracts (Fig. 1). Exact mass measurements of pseudomolecular ions in the analytes performed with a time-of-flight mass spectrometer enabled us to determine molecular formulas. Of 36 compounds identified in the extracts with HPLC/MS, only 6 could be characterized using the mass spectra database (Table 3). They include hydroxycinnamic acids, flavan-3-ol, flavanol, and benzoic acids.

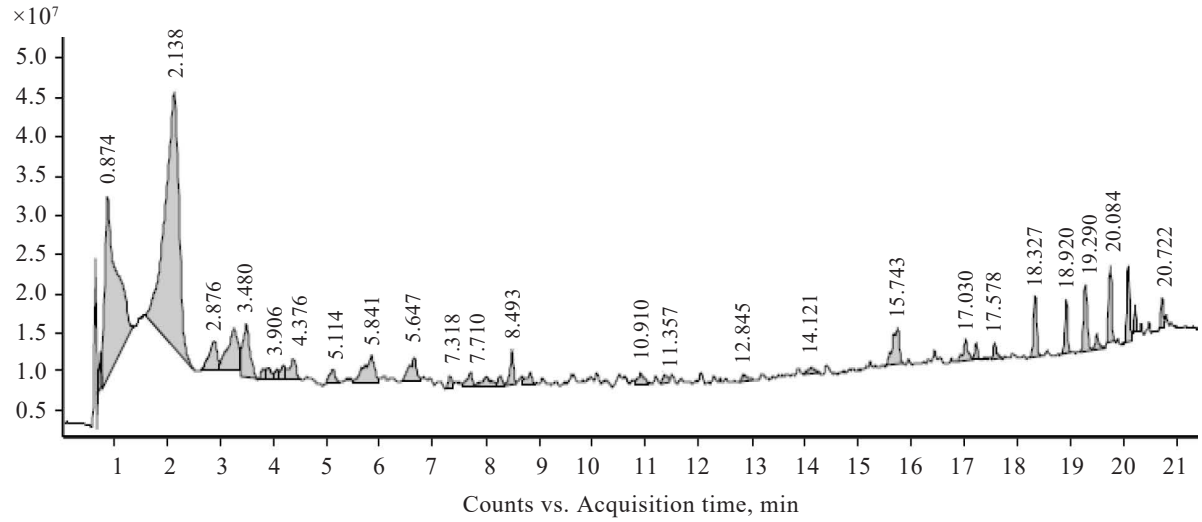

Figure 1. HPLC/MS chromatogram for *Chlorella vulgaris* extracts

Рисунок 1. Хроматограмма ВЭЖХ/МС экстрактов *Chlorella vulgaris*

Table 3. Phenolic compounds identified in *Chlorella vulgaris* extracts

Таблица 3. Фенольные соединения в экстрактах *Chlorella vulgaris*

Mass, m/z	Name	Formula
182.0576	4-Hydroxyphenyllactic acid	$C_9H_{10}O_4$
290.0785	Epicatechin	$C_{15}H_{14}O_6$
164.0472	Coumaric acid	$C_9H_8O_3$
154.0264	2,6-Dihydroxybenzoic acid	$C_7H_6O_4$
194.0577	(E)-Ferulic acid	$C_{10}H_{10}O_4$
302.0424	Quercetin	$C_{15}H_{10}O_7$

Some of our results are consistent with those in several other studies that characterized phenols in microalgae. For example, Bhuvana *et al.* identified different phenolic acids and flavonoids using HPLC in methanolic extracts of *C. vulgaris*, including chlorogenic and caffeic acids, hydroxycinamic acid derivative, quercetin pentosidehexoside, quercetin-7-o-hexoside3-o-hexoside, and luteolin 7-Orutinoside [34]. Goiris reported the presence of hydroxycinnamic (ferulic and p-coumaric) acids, while another study identified caffeic, ferulic, and p-coumaric acids in *C. vulgaris* [35, 36]. Similarly, Vieira *et al.* identified mostly flavan-3-ols in *C. vulgaris* using ultra-performance liquid chromatography [28]. *C. vulgaris* contains a lot of phytochemicals with antioxidant and antimicrobial properties. Their types and amounts may depend on the species and the solvent/extraction technique employed, as well as on the growth parameters and environmental factors [28].

Phenols are a big family of phytochemicals with a wide range of chemical variations. Studies indicate that their benefits to human health and the food industry are mainly due to their antioxidant activity, as well as some biological features. Liu and Chen found that astaxanthin, a carotenoid isolated from *Chlorella zofingiensis*, has potential to protect organisms against a wide range of diseases, with promising applications in healthcare [37]. For example, ProTec Ingredia created a commercial product based on *C. vulgaris* extract which stimulates the synthesis of collagen in the skin, regenerating tissues and slowing down aging [38].

Effects of *C. vulgaris* extracts on the viability of *S. cerevisiae* in an ethanolic medium. *Morphology of cultured S. cerevisiae.* To determine the effects of *C. vulgaris* extracts on *S. cerevisiae*, we analyzed the physical appearance (shape, size, and dispersion) of their cells under the microscope after 2 and 23 days of storage (Figs. 1 and 2, respectively). The *S. cerevisiae* cells were big and either clustered together or dispersed, except for the sample with 0.1% of the extract which was small and dispersed. However, we cannot explain the reason behind their size and dispersion. Investigating the cells' viability, we noticed that they had a rapid division rate in the samples with the *C. vulgaris* extract, as compared to the normal and negative control samples.

The growth and viability of S. cerevisiae cells. To quantify the effect of *C. vulgaris* extracts on the cell growth of *S. cerevisiae* in an ethanolic medium, we determined the viability and density of the *S. cerevisiae* cells (Fig. 3a and b, respectively). After 2 days of culturing, the viability of the negative (YA) and positive (YE) controls reduced to 93.36 and 61.11%, respectively. All the *C. vulgaris*-treated samples showed 100% cell viability, except for the YEC1 sample with 96.69% viability on the 2nd day of storage. The viability of the *S. cerevisiae* cells was still high in the treated samples after 23 days of storage. The YEC4 sample had the highest viability of 95.75%, followed by YEC6 and YEC1 (94.04 and 89.15%, respectively), and YEC3 with the lowest viability

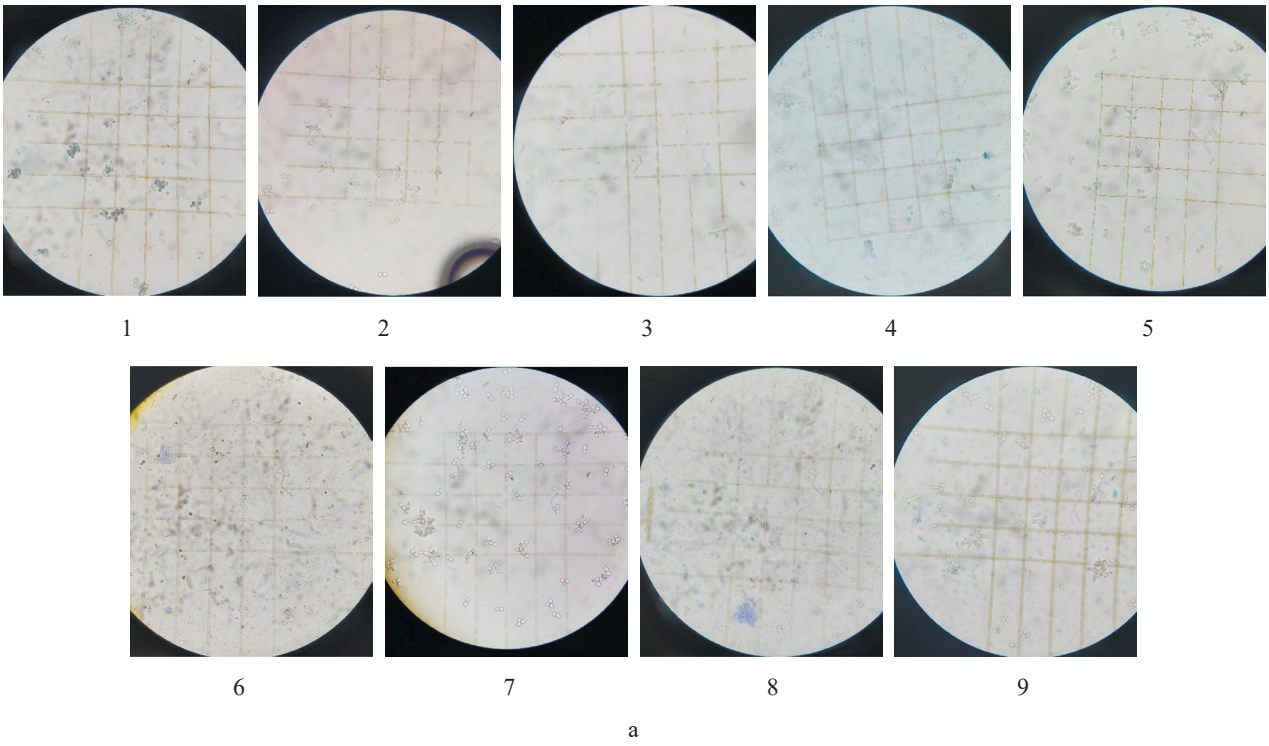

Figure 2. Morphology of yeasts under different extract conditions after (a) 2 days, (b) 23 days of storage, where 1: yeast alone; 2: yeast + 1% ethanol; 3: yeast + 0.1% *Chlorella vulgaris*, 4: yeast + 0.1% *Chlorella vulgaris* + 1% ethanol; 5: yeast + 0.5% *Chlorella vulgaris* + 1% ethanol; 6: yeast + 1% *Chlorella vulgaris* + 1% ethanol; 7: yeast + 2% *Chlorella vulgaris* + 1% ethanol; 8: yeast + 3% *Chlorella vulgaris* + 1% ethanol; 9: yeast + 4% *Chlorella vulgaris* + 1% ethanol

Рисунок 2. Морфология дрожжей в различных условиях экстрагирования после (a) 2 суток, (b) 23 суток хранения, где 1 – только дрожжи; 2 – дрожжи + 1 % этанола; 3 – дрожжи + 0,1 % хлореллы обыкновенной; 4 – дрожжи + 0,1 % хлореллы обыкновенной + 1 % этанола; 5 – дрожжи + 0,5 % хлореллы обыкновенной + 1 % этанола; 6 – дрожжи + 1 % хлореллы обыкновенной + 1 % этанола; 7 – дрожжи + 2 % хлореллы обыкновенной + 1 % этанола; 8 – дрожжи + 3 % хлореллы обыкновенной + 1 % этанола; 9 – дрожжи + 4 % хлореллы обыкновенной + 1 % этанола

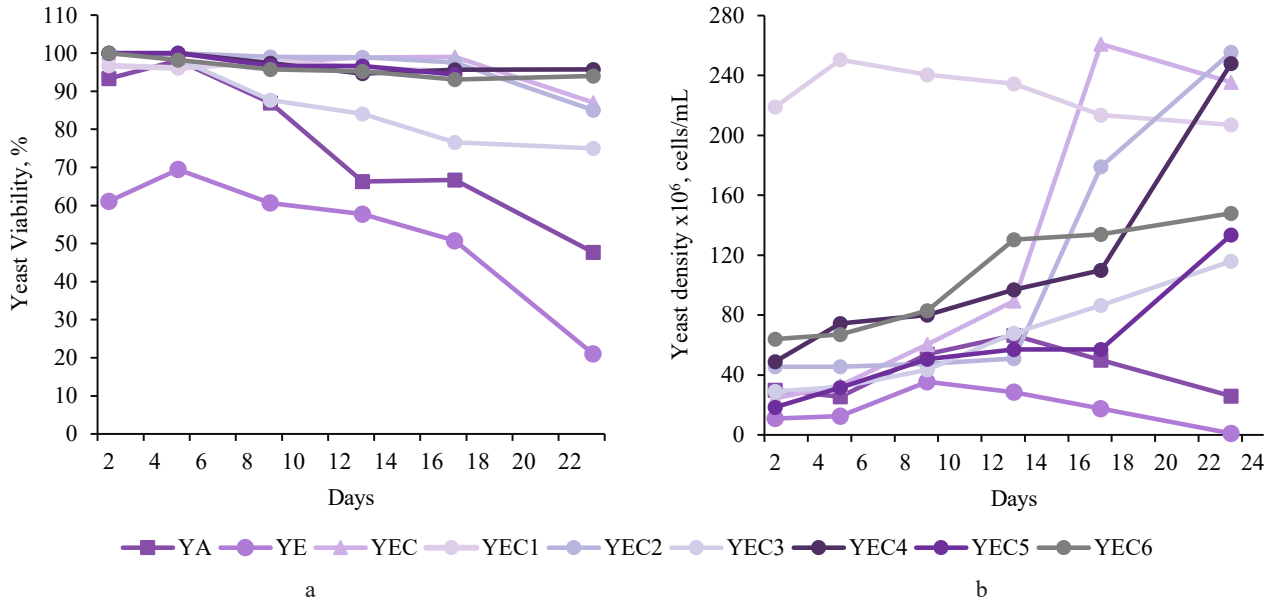

Figure 3. Yeast growth based on (a) cell viability and (b) density across the treated samples compared to the control. The results are mean ± SD of six measurements, where YA: yeast alone; YE: yeast + 1% ethanol; YEC: yeast + 0.1% *Chlorella vulgaris*; YEC1: yeast + 0.1% *Chlorella vulgaris* + 1% ethanol; YEC2: yeast + 0.5% *Chlorella vulgaris* + 1% ethanol; YEC3: yeast + 1% *Chlorella vulgaris* + 1% ethanol; YEC4: yeast + 2% *Chlorella vulgaris* + 1% ethanol; YEC5: yeast + 3% *Chlorella vulgaris* + 1% ethanol; YEC6: yeast + 4% *Chlorella vulgaris* + 1% ethanol

Рисунок 3. Рост дрожжей на основе (a) жизнеспособности клеток и (b) плотности в обработанных образцах по сравнению с контролем. Результаты представляют собой среднее значение ± стандартное отклонение шести измерений, где YA – только дрожжи; YE – дрожжи + 1 % этанола; YEC – дрожжи + 0,1 % хлореллы обыкновенной; YEC1 – дрожжи + 0,1 % хлореллы обыкновенной + 1 % этанола; YEC2 – дрожжи + 0,5 % хлореллы обыкновенной + 1 % этанола; YEC3 – дрожжи + 1 % хлореллы обыкновенной + 1 % этанола; YEC4 – дрожжи + 2 % хлореллы обыкновенной + 1 % этанола; YEC5 – дрожжи + 3 % хлореллы обыкновенной + 1 % этанола; YEC6 – дрожжи + 4 % хлореллы обыкновенной + 1 % этанола

of 74%. The positive (YA) and negative (YA) controls had 47.71 and 21.01% viability, respectively. This drastic reduction in viability was due to lysis of the yeast cells caused by ethanol.

The cell density followed a similar upward trend with the addition of *C. vulgaris* extracts (Fig. 3b). The negative (YA) and positive (YE) controls had cell densities of 30 and 11×10^6 cells/mL, respectively, on the 2nd day of storage. Their cell densities gradually increased until day 13 to mark a lag phase (66.5 and 28.5×10^6 cells/mL for YA and YE, respectively). On day 17, both controls showed a marked decrease in the cell density (from 50 and 17.5×10^6 cells/mL for YA and YE, respectively). By day 23, they entered a death phase (26 and 1×10^6 cells/mL for YA and YE, respectively). Among the extract-treated samples, YEC1 maintained a higher cell density than the others.

Although ethanol is a final product of anaerobic fermentation of sugars by *S. cerevisiae*, it is toxic to yeast cells. Moreover, ethanol induces stress responses such as the expression of heat shock proteins and the accumulation of trehalose [39]. Cell death is the most obvious irreversible effect of ethanol on yeast. The cell density is an aggregate of total yeast cells (both living and dead), whereas the cell viability is a percentage of viable yeast cells in the medium. We observed the viability of the yeast culture in a Sabura medium for 23 days by comparing the percentages of living and dead cells. We also calculated the cell density (cells/mL) at daily intervals to determine how the cells multiply.

Kubota *et al.* showed how different concentrations of ethanol reduced cell viability within 6 h during the log phase of cell growth [40]. The bioactive compounds (phytochemicals) from the dry *C. vulgaris* have important characteristics that enhance yeast viability. In our study, different concentrations of *C. vulgaris* extract greatly extended the mean chronological life span of the yeast cultured in ethanolic media (Fig. 3a and b). The mean lifespan is directly proportional to the survival rates of organisms in a population during the development and maturity stages of organismal aging. It is also likely to be affected by certain extrinsic (environmental) factors [41, 42]. From this, we can assume that the extracts decrease the extrinsic rate of yeast chronological aging before the cells enter quiescence or senescence. The maximum lifespan is referred to a "healthy" life period during the quiescence/senescence stage of organismal aging. It is likely to be controlled by certain intrinsic (cellular and organismal) longevity modifiers [43]. We can, therefore, conclude that the extracts also decrease

the intrinsic rate of yeast chronological aging after the cells enter quiescence or senescence.

Our previous works showed that *C. vulgaris* extracts enhanced yeast viability for 17 days and *C. vulgaris* powder improved yeast characteristics in the presence of 5% ethanol after 5 days [6, 13]. In a study where the brewer's yeast was treated with lethal doses of four highly toxic substances (mercury, copper, cadmium, and polychlorobiphenyl), the yeast remained alive when these poisonous substances were supplemented with *C. vulgaris* extract [9]. Lutchman *et al.* found that plant extracts increased the chronological lifespan of yeast more significantly than any of the longevity-extending chemical compounds known to date [44]. They noted that the extracts decreased the rate of yeast aging by eliciting a hormetic stress response. A recent study found that *C. vulgaris* stimulates the growth of *Lactobacillus acidophilus*, an important bacterium that keeps the intestines healthy [23]. This can be explained by large amounts of chlorophyll and fibrous cell walls in *C. vulgaris* that cause the beneficial lactic acid bacteria in the gut to multiply four times the usual rate and remove the foul smell of the stools. As a result, digestion and assimilation of nutrients are vastly improved. In our study, these compounds were responsible for the improved growth of yeast cells in the ethanolic medium. *C. vulgaris* has also been shown to stimulate the production of beneficial bacteria in the bowel, which in turn has a probiotic effect of strengthening the gut flora and preventing disease.

Conclusion

Chlorella vulgaris is an alga of significant interest to scientists due to its nutritive components such as bioactive compounds. However, care needs to be taken as to which extraction method to use for each group of these compounds. Our results showed greater efficiency in using water as a solvent to extract aqueous compounds. We concluded that the abundance of phenols and flavonoids in the extracts was due to the hydrophilic character of the solvent. Also, the phenolic compounds in *C. vulgaris* proved beneficial for the growth of *Saccharomyces cerevisiae* cells in an ethanolic medium, protecting them from the toxic effects of ethanol. However, there is a need for further research to identify individual compounds responsible for decreasing the chronological aging of *S. cerevisiae* and explore the mechanism behind this protective effect. Also, viability alone is not an accurate measure of the cells' effectiveness in fermentation – it is critical to understand their vitality. Finally, *C. vulgaris* extracts need to be added during the brewing process to understand if they will have any adverse physiological effects on the beer quality.

Conflict of interest

The author declares no conflict of interest.

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Конфликт интересов

Автор заявляет об отсутствии конфликта интересов, связанного с публикацией данной статьи.

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References

1. Rani K, Sandal N, Sahoo PK. A comprehensive review on chlorella – its composition, health benefits, market and regulatory scenario. The Pharma Innovation Journal. 2018;7(7):584–589.

2. Okechukwu QN, Adepoju FO, Hassani MI, Kovaleva EG, Rao AR, Ravishankar GA. Suitability of microalgae and fungi in meat analogs: an overview. In: Ravishankar GA, Rao AR, Tahergorabi R, Mohan A, editors. Handbook of plant-based meat analogs. Innovation, technology and quality. Academic Press; 2024. pp. 121–146. https://doi.org/10.1016/B978-0-443-21846-0.00017-4

3. Dolganyuk V, Belova D, Babich O, Prosekov A, Ivanova S, Katserov D, *et al.* Microalgae: A promising source of valuable bioproducts. Biomolecules. 2020;10(8):1153. https://doi.org/10.3390/biom10081153

4. Kumar N, Goel N. Phenolic acids: Natural versatile molecules with promising therapeutic applications. Biotechnology Reports. 2019;24:e00370. https://doi.org/10.1016/j.btre.2019.e00370

5. Plaza M, Santoyo S, Jaime L, Avalo B, Cifuentes A, Reglero G, *et al.* Comprehensive characterization of the functional activities of pressurized liquid and ultrasound-assisted extracts from *Chlorella vulgaris*. LWT – Food Science and Technology. 2012;46(1):245–253. https://doi.org/10.1016/j.lwt.2011.09.024

6. Okechukwu QN, Yama I, Kovaleva EG. Enzymatic extraction of growth factor in *Chlorella* and possible protective effects of *Chlorella* extracts on yeast growth. AIP Conference Proceedings. 2020;2280(1):030013. https://doi.org/10.1063/5.0018029

7. Ścieszka S, Gorzkiewicz M, Klewicka E. Innovative fermented soya drink with the microalgae *Chlorella vulgaris* and the probiotic strain *Levilactobacillus brevis* ŁOCK 0944. LWT. 2021;151:112131. https://doi.org/10.1016/j.lwt.2021.112131

8. Dantas DMM, Cahú TB, Oliveira CYB, Abadie-Guedes R, Roberto NA, Santana WM, *et al. Chlorella vulgaris* functional alcoholic beverage: Effect on propagation of cortical spreading depression and functional properties. PLoS ONE. 2021;16(8):e0255996. https://doi.org/10.1371/journal.pone.0255996

9. Chlorella – the most exciting nutritional discovery on planet earth. Abeille d'Or Corporation; 2014. 56 p.

10. Pantoja Munoz L, Purchase D, Jones H, Raab A, Urgast D, Feldmann J, *et al.* The mechanisms of detoxification of As(III), dimethylarsinic acid (DMA) and As(V) in the microalga *Chlorella vulgaris*. Aquatic Toxicology. 2016;175:56–72. https://doi.org/10.1016/j.aquatox.2016.02.020

11. Laopaiboon L, Suporn S, Klanrit P, Phukoetphim N, Daengbussadee C, Laopaiboon P. Novel effective yeast strains and their performance in high gravity and very high gravity ethanol fermentations from sweet sorghum juice. Energies. 2021; 14(3):557. https://doi.org/10.3390/en14030557

12. Coffman RE, Kraichely KN, Kreutzberger AJB, Kiessling V, Tamm LK, Woodbury DJ. Drunken lipid membranes, not drunken SNARE proteins, promote fusion in a model of neurotransmitter release. Frontiers in Molecular Neuroscience. 2022;15:1022756. https://doi.org/10.3389/fnmol.2022.1022756

13. Okechukwu QN, Kanwugu ON, Adadi P, Okpala COR, Kovaleva EG. Potential of *Chlorella vulgaris* powder to enhance ethanol-cultured *Saccharomyces cerevisiae*. Journal of Taibah University for Science. 2023;17(1):2187602. https://doi.org/10.1080/16583655.2023.2187602

14. Karabín M, Jelínek L, Kotrba P, Cejnar R, Dostálek P. Enhancing the performance of brewing yeasts. Biotechnology Advances. 2018;36(3):691–706. https://doi.org/10.1016/j.biotechadv.2017.12.014

15. Samakkarn W. Ratanakhanokchai K, Soontorngun N. Reprogramming of the ethanol stress response in *Saccharomyces cerevisiae* by the transcription factor Znf1 and its effect on the biosynthesis of glycerol and ethanol. Applied and Environmental Microbiology. 2021;87(16):e00588-21. https://doi.org/10.1128/AEM.00588-21

16. Kupina S, Fields C, Roman MC, Brunelle SL. Determination of total phenolic content using the Folin-C assay: Single-laboratory validation, first action 2017.13 Journal of AOAC International. 2018;101(5):1466–1472. https://doi.org/10.5740/jaoacint.18-0031

17. Huang R, Wu W, Shen S, Fan J, Chang Y, Chen S, *et al.* Evaluation of colorimetric methods for quantification of citrus flavonoids to avoid misuse. Analytical Methods. 2018;10(22):2575–2587. https://doi.org/10.1039/C8AY00661J

18. Adadi P, Kovaleva EG, Glukhareva TV, Barakova NV. Production and investigations of antioxidant rich beverage: Utilizing *Monascus purpureus* IHEM LY2014-0696 and various malts. Agronomy Research. 2018;16(S2):1312–1321. https://doi.org/10.15159/AR.18.028

19. Essiedu JA, Adadi P, Kovaleva EG. Production and characterization of beer supplemented with *Hibiscus sabdariffa* (Malvaceae). Food Frontiers. 2021;3(2):328–338. https://doi.org/10.1002/fft2.127

20. Haida Z, Hakiman M. A comprehensive review on the determination of enzymatic assay and nonenzymatic antioxidant activities. Food Science and Nutrition. 2019;7(5):1555–1563. https://doi.org/10.1002/fsn3.1012

21. Kitada K, Machmudah S, Sasaki M, Goto M, Nakashima Y, Kumamoto S, *et al.* Antioxidant and antibacterial activity of nutraceutical compounds from *Chlorella vulgaris* extracted in hydrothermal condition. Separation Science and Technology. 2009;44(5):1228–1239. https://doi.org/10.1080/01496390902729056

22. Scieszka S, Klewicka E. Influence of the microalga *Chlorella vulgaris* on the growth and metabolic activity of *Lactobacillus* spp. bacteria. Foods. 2020;9(7):959. https://doi.org/10.3390/foods9070959

23. Csatlos N-I, Simon E, Teleky B-E, Szabo K, Diaconeasa ZM, Vodnar D-C, *et al.* Development of a fermented beverage with *Chlorella vulgaris* powder on soybean-based fermented beverage. Biomolecules. 2023;13(2):245. https://doi.org/10.3390/biom13020245

24. Krishnamoorthy A, Rodriguez C, Durrant A. Sustainable approaches to microalgal pre-treatment techniques for biodiesel production: A review. Sustainability. 2022;14(16):9953. https://doi.org/10.3390/su14169953

25. Kulkarni S, Nikolov Z. Process for selective extraction of pigments and functional proteins from *Chlorella vulgaris*. Algal Research. 2018;35:185–193. https://doi.org/10.1016/j.algal.2018.08.024

26. Okechukwu QN, Adadi P, Kovaleva EG. Production and analysis of beer supplemented with *Chlorella vulgaris* powder. Fermentation. 2022;8(11):581. https://doi.org/10.3390/fermentation8110581

27. Dantas DMM, Costa RMPB, Carneiro-da-Cunha MG, Galvez AO, Drummond AR, Bezerra RS. Bioproduction, antimicrobial and antioxidant activities of compounds from *Chlorella vulgaris*. Research and Reviews: Journal of Botanical Sciences. 2015;4(2):12–18.

28. Vieira MV, Turkiewicz IP, Tkacz K, Fuentes-Grünewald C, Pastrana LM, Fuciños P, *et al.* Microalgae as a potential functional ingredient: Evaluation of the phytochemical profile, antioxidant activity and in-vitro enzymatic inhibitory effect of different species. Molecules. 2021;26(24):7593. https://doi.org/10.3390/molecules26247593

29. Munteanu IG, Apetrei C. Analytical methods used in determining antioxidant activity: A review. International Journal of Molecular Sciences. 2021;22(7):3380. https://doi.org/10.3390/ijms22073380

30. Baliyan S, Mukherjee R, Priyadarshini A, Vibhuti A, Gupta A, Pandey RP, *et al.* Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 2022;27(4):1326. https://doi.org/10.3390/molecules27041326

31. Christodoulou MC, Palacios JCO, Hesami G, Jafarzadeh S, Lorenzo JM, Domínguez R, *et al.* Spectrophotometric methods for measurement of antioxidant activity in food and pharmaceuticals. Antioxidants. 2022;11(11):2213. https://doi.org/10.3390/antiox11112213

32. Pak VV, Ezeriņa D, Lyublinskaya OG, Pedre B, Tyurin-Kuzmin PA, Mishina NM, *et al.* Ultrasensitive genetically encoded indicator for hydrogen peroxide identifies roles for the oxidant in cell migration and mitochondrial function. Cell Metabolism. 2020;31(3):642–653.e6. https://doi.org/10.1016/j.cmet.2020.02.003

33. Collin F. Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases. International Journal of Molecular Sciences. 2019;20(10):2407. https://doi.org/10.3390/ijms20102407

34. Bhuvana P, Sangeetha P, Anuradha V, Syed Ali M. Spectral characterization of bioactive compounds from microalgae: *N. oculata* and *C. vulgaris*. Biocatalysis and Agricultural Biotechnology. 2019;19:101094. https://doi.org/10.1016/j.bcab.2019.101094

35. Goiris K, Muylaert K, Voorspoels S, Noten B, de Paepe D, Baart GJE, *et al.* Detection of flavonoids in microalgae from different evolutionary lineages. Journal of Phycology. 2014;50(3):483–492. https://doi.org/10.1111/jpy.12180

36. Zakaria SM, Kamal SMM, Harun MR, Omar R, Siajam SI. Subcritical water technology for extraction of phenolic compounds from *Chlorella* sp. microalgae and assessment on its antioxidant activity. Molecules. 2017;22(7):1105. https://doi.org/10.3390/molecules22071105

37. Liu J, Chen F. Biology and industrial applications of *Chlorella*: Advances and prospects. In: Posten C, Chen SF, editors. Microalgae biotechnology. Cham: Springer; 2016. pp. 1–35. https://doi.org/10.1007/10_2014_286

38. Michalak M, Pierzak M, Kręcisz B, Suliga E. Bioactive compounds for skin health: A review. Nutrients. 2021;13(1):203. https://doi.org/10.3390/nu13010203

39. Postaru M, Tucaliuc A, Cascaval D, Galaction A-I. Cellular stress impact on yeast activity in biotechnological processes – A short overview. Microorganisms. 2023;11(10):2522. https://doi.org/10.3390/microorganisms11102522

40. Kubota S, Takeo I, Kume K, Kanai M, Shitamukai A, Mizunuma M, *et al.* Effect of ethanol on cell growth of budding yeast: Genes that are important for cell growth in the presence of ethanol. Bioscience, Biotechnology, and Biochemistry. 2004;68(4):968–972. https://doi.org/10.1271/bbb.68.968

41. Flatt T, Partridge L. Horizons in the evolution of aging. BMC Biology. 2018;16:93. https://doi.org/10.1186/s12915-018-0562-z

42. Li S, Vazquez JM, Sudmant PH. The evolution of aging and lifespan. Trends in Genetics. 2023;39(11):830–843. https://doi.org/10.1016/j.tig.2023.08.005

43. Kumari R, Jat P. Mechanisms of cellular senescence: Cell cycle arrest and senescence associated secretory phenotype. Frontiers in Cell and Developmental Biology. 2021;9:645593. https://doi.org/10.3389/fcell.2021.645593

44. Lutchman V, Medkour Y, Samson E, Arlia-Ciommo A, Dakik P, Cortes B, *et al.* Discovery of plant extracts that greatly delay yeast chronological aging and have different effects on longevity-defining cellular processes. Oncotarget. 2016;7:16542–16566. https://doi.org/10.18632/oncotarget.7665