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Potential of *Chlorella vulgaris* powder to enhance ethanol-cultured *Saccharomyces cerevisiae*

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ABSTRACT

Chlorella vulgaris is a highly nutritious single cell microalgae reported to alleviate oxidative-stress induced damage. Although *Saccharomyces cerevisiae* is highly renounced for its fermentation capacity is susceptible to ethanol toxicity. In this context, the potential of *C. vulgaris* powder to help improve the life span of *S cerevisiae* cultured in the presence of ethanol was studied. The growth characteristic including cell viability and vitality of *S. cerevisiae* cultured in a media supplemented with *C. vulgaris* powder (0.1 and 1% w/v) and induced with 5% ethanol was monitored over 5 days. Results showed that from days 1 to 5, the yeast media supplemented with 1% *C. vulgaris* powder showed enhanced growth compared to that with 0.1% chlorella, 5% ethanol alone, and control media. By day 5, ATP production enhanced significantly ($p < 0.05$) in the 1% chlorella-supplemented (180.45 nmol), compared to control (86.84 nmol) and 0.1% chlorella-supplemented sample (105.185 nmol).

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Saccharomyces cerevisiae; Chlorella vulgaris; fermentation; cell metabolic activity; media supplementation

1. Introduction

Alcoholic fermentation involves the conversion of wort or broth sugars into ethanol, volatile organic compounds, other metabolites, and the release of carbon dioxide by active yeast. Besides influencing yeast growth and its performance/efficiency by decreasing cell division, the accumulation of ethanol during fermentation could result in cell lysis and death [1]. Brewer yeast (*Saccharomyces cerevisiae*) is reported not to tolerate more than 10–11% ethanol produced during fermentation [2]. Considered among valuable bio-research model candidates, *S. cerevisiae* remains very vital in the fermentation of sugars into alcohol and other metabolites of food and pharmaceutical relevance. Notably, the application of *S. cerevisiae* in the beverage and food industry is massive, especially in the production of alcoholic, and distilled beverages [3]. Microalgae, on the other hand, are unicellular, photosynthetic organisms that thrives in diverse environmental conditions, from deserts, and polar regions, to marine and freshwater reserves [4,5]. Marine microalgae can offset the biochemical disparities induced by various compounds associated with free radicals [6]. Single-cell green microalga like *Chlorella vulgaris* can tolerate several heavy metals and metalloids which appears of particular interest to researchers [7,8]. *Chlorella* is highly exploited due to its high nutritional content such as protein (51%–58% dry weight;) essential amino acids, among others [9]. It also contains dietary antioxidants, including β-1,3-glucan, vitamins (B complex and ascorbic acid), minerals (potassium, sodium, magnesium, iron, and calcium), β-carotene, lutein, chlorella growth factor (CGF), and bioactive peptides [6,10].

The metabolic process of ethanol entails its oxidation along with (microsomal) nicotinamide adenine dinucleotide phosphate (NADPH), and xanthine oxidase-catalyzed reactions [11]. Along with inhibiting yeast growth, ethanol would alters the membrane lipid bilayer via the endoplasmic reticulum thus deregulating metabolism with concomitant oxidative stress and damage to mitochondrial DNA [1,12,13]. Ethanol-induced oxidative stress is underpinned by the homolytic cleavage yielding free radicals (CCl_3,

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$CCl_3OO\cdot$, etc.) that interact with neighbouring molecules, such as proteins, nucleic acid, thiols, and membrane unsaturated fatty acids [11]. Several stress conditions can influence yeast during fermentation. Ethanol-induced stress decreases yeast growth hence, causes considerable damage to the cell morphology, function of the cell membrane, which changes the membrane structure and permeability that allow leakage of essential cofactors and coenzymes [13,14]. In addition, ethanol-induced stress influences the macromolecular biosynthesis, and transport mechanism in yeast. As the intracellular proteins are affected, the glycolytic enzymes would lower the rate of RNA and protein synthesis [1,2,12]. Besides, ethanol-induced stress stimulates heat shock response in cells leading to accumulation of trehalose which inhibits cell division, metabolic activity, and decreased viability [15]. The oxidative stress in ethanol toxicity is likely caused by its conversion to a free radical intermediate during metabolism, or its reaction with some nucleophile in an antioxidant molecule, able to decrease the antioxidant potential [11]. Bioactive peptide glutathione and its oligomer phytochelatin in *C. vulgaris* may alleviate the damage caused by reactive oxygen species and other free radicals. Glutathione is a key factor that enhances tolerance against biotic and abiotic stress in plants and other microorganisms (i.e. *C. vulgaris*) [16] whereas phytochelatin is important for heavy metal detoxification [7,8,17].

The ethanol tolerance mechanism in yeasts may come from complex inhibitory mechanisms, with the plasma membrane phospholipids playing a crucial role as membrane unsaturated fatty acids increase with yeast ethanol tolerance. Besides physiological factors like intracellular ethanol accumulation, mode of substrate uptake, osmotic pressure and temperature enhance the ethanol tolerance of yeast. The complex nature of ethanol toxicity suggest that different genes are likely involved in the ethanol tolerance mechanism [18]. Both biochemical and physiological determinants of ethanol tolerance specific to yeast, would primarily require the identification of genes responsible for the tolerance [12]. Previous report showed that *Chlorella* extracts improved the viability of yeast cultured in a liquid ethanol-free media [19]. Others have reported that media supplemented with enzymes (i.e. *Neutrase*, *Flavorzyme,* and *Protamex*) improved the ethanol tolerance in *S. cerevisiae* which enhanced fermentation performance [20]. Co-cultured *S. cerevisiae* with other yeast strains can improve ethanol tolerance [21]. Thus *C. vulgaris* could potentially comprise antioxidant-like bioactive compounds and peptides could potentially protect the yeast's cell wall and membrane against ethanol-induced oxidative stress [19]. For emphasis, this work was directed to establish the potential of *C. vulgaris* powder to help mitigate the deleterious effects of ethanol on cultured *S. cerevisiae* serving as a gateway for further studies on its potential tomitigate ethanol-induced oxidative stress on cultured *S. cerevisiae*. Therefore, this work aimed to understand the potential effects of supplementing *C. vulgaris* powder on the life span of *S. cerevisiae* cultured in the presence of ethanol.

2. Materials and methods

2.1. Schematic overview of the experimental programme

Figure 1 shows a schematic overview of the experiment, depicting the steps, from yeast preculture/culture, cell harvesting, different treatments, and analytical measurements.

2.2. Procurement of experimental materials

1272 American Ale II yeast (Wyeast Laboratories, OR, USA) was gifted by Beersfan microbrewery (Yekaterinburg, Russia). Food grade *C. vulgaris* powder was sourced from Zhengzhou Sigma Chemical Co., Ltd (Zhengzhou, China). Sabouraud growth media was purchased from the Federal Budgetary Institution of Science "State Research Center for Applied Microbiology" (Oblonesk, Russia) and absolute ethanol (95% purity) was from RusBio (St. Petersburg, Russia). Cell Counting Kit-8 was purchased from Oz Biosciences (San Diego, CA, USA), whereas the BacTiter-Glo^{TM} Microbial Cell Viability Assay kit was from Promega (Madison, WI, USA).

2.3. Introducing C. vulgaris into the cultured S. cerevisiae

For precultures, a single yeast colony was transferred from an agar slant to solidified Sabouraud agar media and incubated (Memmert GmbH + Co. KG, Schwabach, Germany) for 48 h at 28°C. The cultured yeast were uniformly suspended in Sabouraud broth media (250 mL) in a 500-mL Erlenmeyer flask and incubated at 28°C for 48 h with constant shaking at 160 rpm in an orbital shaker-incubator (Biosan, Riga, Latvia). The spent media was discarded by centrifugation and the exponential phase cells resuspended in Sabouraud media (100 mL). Cells were inoculated (1×10^6 cells/mL) in Sabouraud media supplemented (100 mL) with 0.1 and 1% w/v *chlorella* powder in 250 mL Erlenmeyer flasks and ethanol was added to the cultures to a final volume of 5%v/v. Similarly, ethanol (5%v/v) supplemented Sabouraud media served as a positive control, whereas cultures in Sabouraud media alone served as the negative control. All treated samples were incubated on a rotary shaker at 160 rpm (26°C) for 5 days.

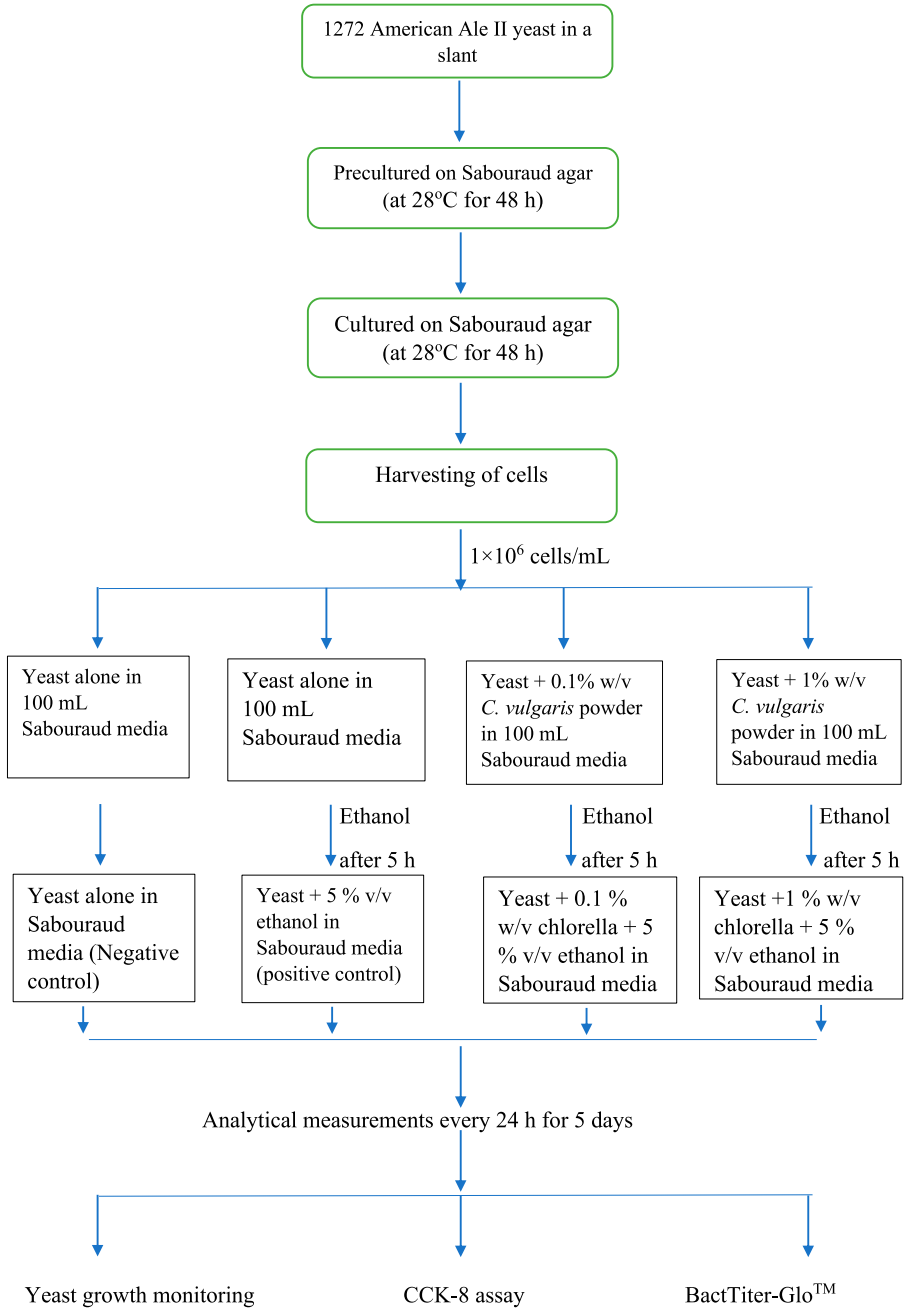


Figure 1. A schematic overview of the experimental programme, depicting the major stages, from preparation of yeast preculture/culture stages, cell harvesting, the allocation of four different treatments, and thereafter, analytical measurements.

2.4. Analytical measurements

2.4.1. Determination of yeast growth, viability, and vitality

Yeast growth was monitored using a Shimadzu ultraviolet (UV)-1800 spectrophotometer (Kyoto, Japan) at 600 nm and light microscopy (400× magnification) respectively. Yeast viability and vitality were assessed using the cellular adenosine triphosphate (ATP) content based on the luciferin reaction, and yeast enzyme activity on monosodium salt (WST-8) [22] with some modifications. Briefly, yeast cells from the cultured media were pelleted (6000 × g, 10 min) and washed twice with sterile water. Pelleted cells were resuspended in 100 mM phosphate buffer (pH 7.0) with 0.1% glucose, 1 mM ethylenediaminetetraacetic acid (EDTA) to dilute cells for WST-8 (1 × 107 cells/mL) and ATP (1 × 106 cells/mL) assays.

2.4.2. Cell counting kit-8 assay

Cell proliferation was determined based on the monosodium salt WST-8 using the Cell Counting Kit-8 as described by the manufacturer. Briefly, the buffered suspended yeast (1×10^7 cells/mL) was pipetted (100 µL) into a 96-well plate, and an assay reagent (10 µL) added. Absorbance was measured after 3 h using a Perkin Elmer 2300 microplate reader (Perkin Elmer, MA, USA) at $\lambda = 450$ nm. WST-8 was reduced by yeast cellular dehydrogenase to an orange formazan product. The amount

of formazan formed was directly proportional to the number of live cells (Equation 1).

$$\text{Survival rate (\%)} = \frac{A_{\text{sample}} - A_b}{A_c - A_b} * 100 \qquad (1)$$

where A_{sample} = absorbance of treated yeast cells; Ab = absorbance of blank (assay only); A_c = absorbance of the control (yeast cells alone).

2.4.3. BactTiter-Glo™ microbial cell viability assay

The ATP content of the cells was assessed using BactTiter-Glo™ was used to measure the ATP content based on the manufacturer instructions. Briefly, the buffered suspended yeast (1×10^6 cells/mL) was pipetted (100 µL) into a 96-well plate and an assay reagent (100 µL) added. Luminescence in Relative Light Units (RLU) was measured after 5 min with a Perkin Elmer 2300 microplate reader. The luminescent signal was proportional to the amount of ATP present, which was also directly proportional to the number of viable cells. The RLU resulting from the BacTiter-Glo™ Assay of each sample was converted to ATP concentration using an ATP calibration curve (y = 114.7x (R^2 = 0.9991)) prepared from a BactTiter-Glo™ ATP solution.

2.5. Statistical analysis

All data from triplicate measurements were subjected to analysis of variance (ANOVA) using Minitab® 21.0 (Minitab Ltd., Coventry, UK). Results are presented as the mean ± standard deviation (SD). Turkey's test was used to identify the differences between means ($p < 0.05$).

3. Results

3.1. Yeast cell growth

Figure 2a shows yeast growth based on optical density (OD_{600nm}) and cell count (Figure 2b) across the three treatment conditions compared to control. The yeast media supplemented with 1% *C. vulgaris* powder significantly increased ($p < 0.05$) the yeast growth more compared to 0.1% chlorella-supplemented media, as well as both controls from day 1–5. The least growth was found in the positive control (growth medium containing 5% ethanol without chlorella supplementation) over the 5 days. The OD of both positive (broth with 5% ethanol) and negative (only broth) controls were similar throughout the study. More so, the cell population peaked at day 2 for 1% *C. vulgaris* powder (8.20×10^9 cells/mL), which resembled cultures supplemented with ($p > 0.05$) 0.1% *C. vulgaris* powder (3.48×10^9). Besides, the cell population of these two groups decreased by day 4 (15.55×10^8 and 16.8×10^8 cells/mL for 0.1% and 1% chlorella-supplemented groups respectively) and day 5 (16.05×10^8 and 17.55×10^8 for 0.1% and 1% chlorella-supplemented groups respectively), to levels comparable to the negative control, especially on day 4 ($p > 0.05$). Like that of *C. vulgaris* powder-treated samples, the cell population of the positive and negative controls peaked on day 2(1.83×10^8 and 1.63×10^8 cells/mL respectively) and day 3(2.24×10^8 and 1.87×10^8 cells/mL respectively).

3.2. Viability of yeast culture

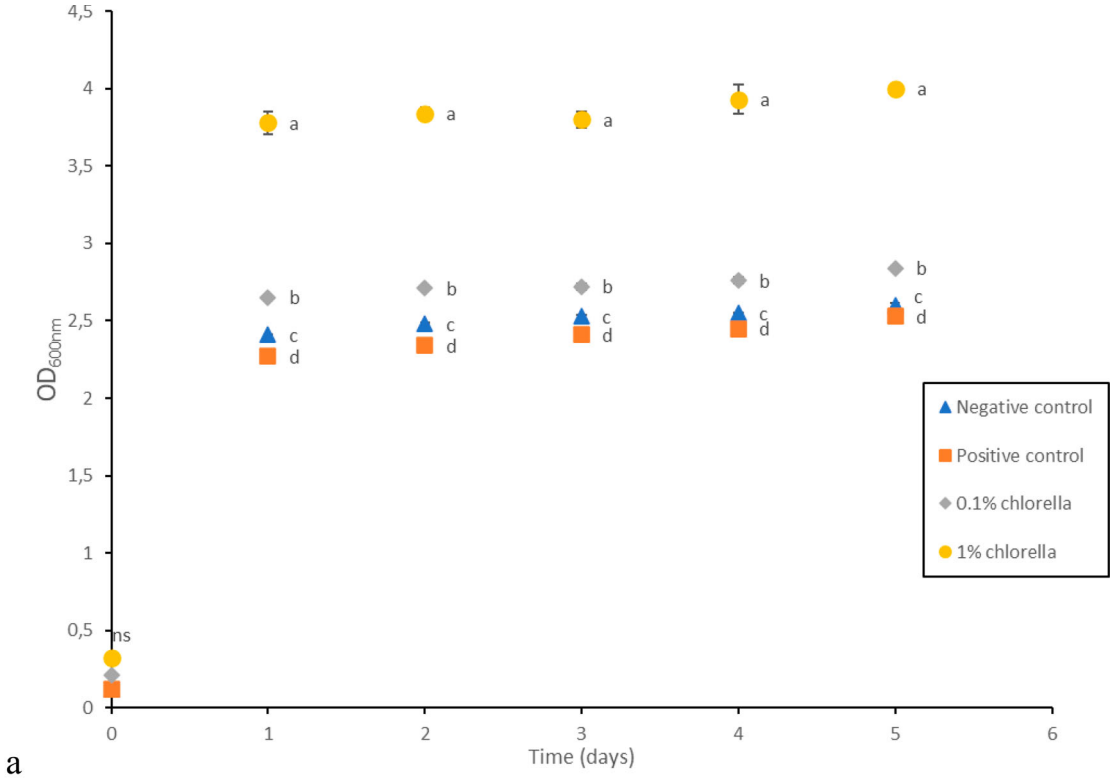
Figure 3 shows the viability of yeast cultures under the three treatment conditions compared to that of the control. By day 3, the peak viability for yeast culture supplemented with 0.1% *C. vulgaris* powder (504.84%), together with yeast culture supplemented with 1% powder (923.44%) significantly differed ($p < 0.05$) compared to control. However, the viability of those of the positive and negative controls were similar ($p > 0.05$). By day 5, the viability of those positive control (~29%) significantly differed ($p < 0.05$) from the negative control. Also, samples treated with 0.1 and 1% *C. vulgaris* powder (102.86 and 145.65% respectively), significantly differed ($p < 0.05$) on day 5.

3.3. ATP production and yeast cell viability

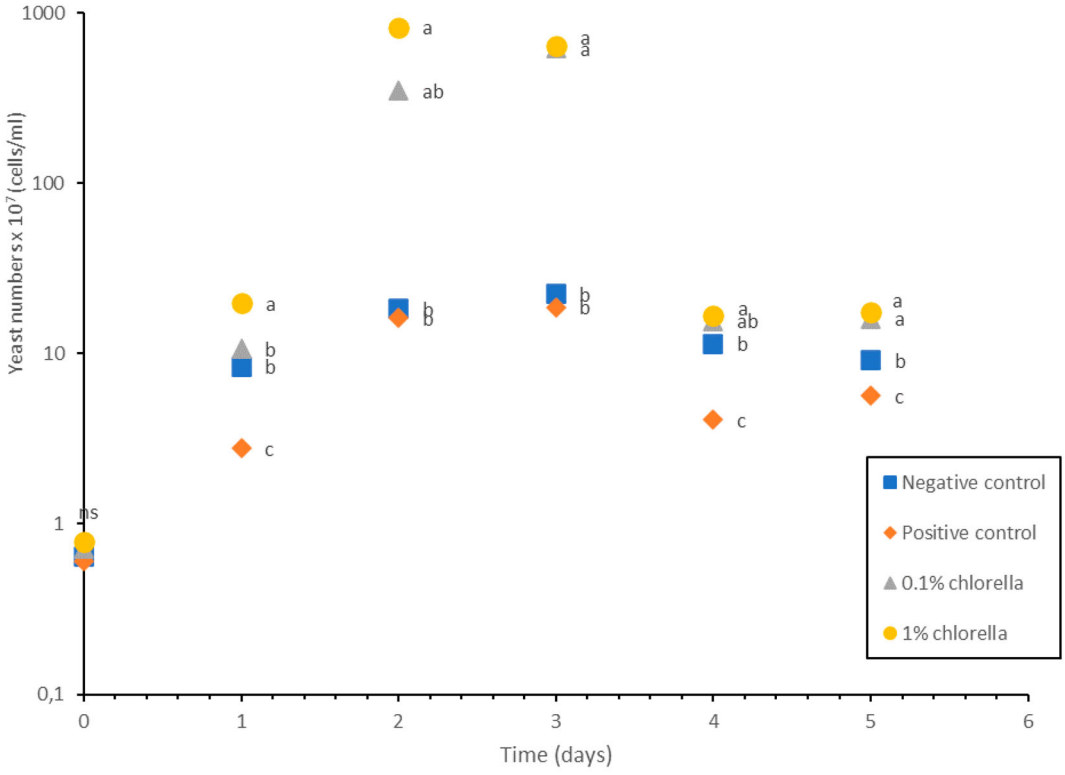
Figure 4 shows the yeast cell capacity to produce ATP (a) and remain viable during the five days of culturing (b) under treatment conditions and control. A relative increase in ATP production for *C. vulgaris* powder-treated samples can be seen. By day 5, ATP production appeared enhanced in the samples treated with 1% *C. vulgaris* powder (ATP content = 180.45 nmol) which significantly differed ($p < 0.05$) from controls (ATP content = 86.84 nmol) and 0.1% *C. vulgaris* powder (ATP content = 105.185 nmol). Although the ATP production in cultures with 0.1% chlorella powder was higher than than the controls, the difference was not statistically significant ($p < 0.05$). Figure 4(b) shows *C. vulgaris* powder 0.1% and 1% could respectively improve yeast cells' viability up to 150.86% and 368.19% by day 2. Between days 4 and 5, the positive controls significantly differed ($p < 0.05$) when compared to the negative controls. For both the Cell-Counting Kit-8 and ATP assay, the initial survival baseline reflected the daily cell viability numbers from the control samples, which helped to normalize all cell measurements from other cultures.

4. Discussion

The potential of *C. vulgaris* powder to mitigate ethanol-induced stress in yeast cells cultures was investigated in the current work. This required exposure of yeast cells to ethanol in both the presence and absence of *C. vulgaris* powder and subsequently, monitoring the cell growth, viability and vitality. Yeast cells improved noticeably



a



b

Figure 2. Yeast growth based on (a)optical density (OD_{600nm}) and (b) microscopic cell count across three treatment conditions compared to control. Results shown are mean ± SD of six measurements (3 analytical x 2 biological replicates). Means that do not share the same letter (a, b, or c) on each time point (days) are significantly different ($p < 0.05$) using the Tukey's test. (ns = no significance).

when supplemented with *C. vulgaris* powder. Moreover, the *C. vulgaris* powder induced significant changes in yeast growth and physiological activity under this current study' conditions. Different concentrations of *C. vulgaris* powder could extend the mean chronological life span (CLS) of the yeast cultured under ethanolic conditions (Refer to Figures 2a and b). The mean lifespan directly reflects the survival rates of microbial

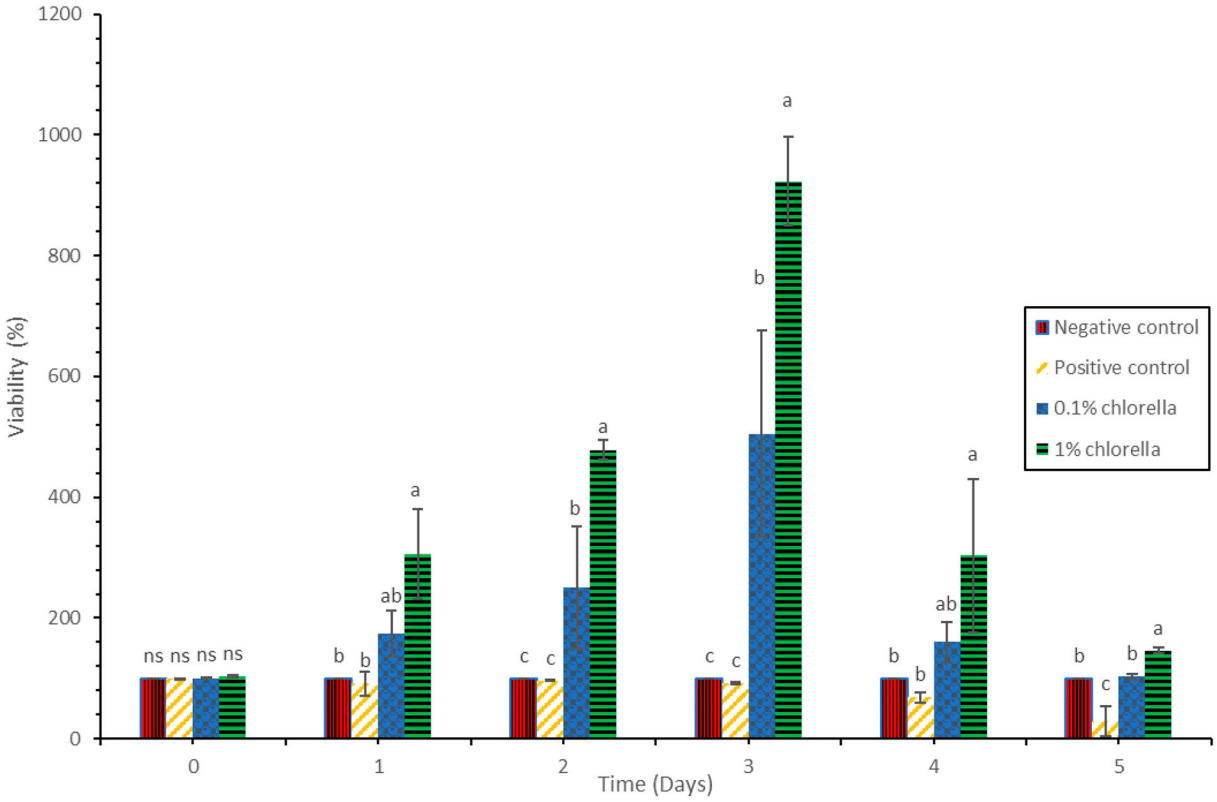


Figure 3. Viability of yeast culture across three treatment conditions compared to control. Results shown are mean ± SD of six measurements (3 analytical x 2 biological replicates). Means that do not share the same letter (a, b, or c) on each time point (days) are significantly different ($p < 0.05$) using the Tukey's test. (ns = no significance).

populations specific to both development and maturity stages. Whereas the maximum lifespan reflects the duration of the "healthy" life period (i.e. health duration) during the quiescence/senescence stage of organismal aging, that of maximum lifespan would likely be controlled by certain intrinsic (cellular and organismal) longevity modifiers [23,24]. Before cell entry into quiescence or senescence, the addition of *C. vulgaris* powder could decrease the chronological/intrinsic aging rate of yeast triggered by ethanol-induced stress.

In the presence of ethanol, there appeared increases in the viability and vitality of *C. vulgaris* powder-treated cells (Refer to Figure 3). Potentially, the presence of ethanol may not necessarily result in yeast cell death but could hasting the loss of physiological function. This situation might be attributed to the concentration of ethanol, which aligns with the alcohol content found in most beers. The fact that yeast still stays alive most of the time, and undergoes several morphological, intracellular, or metabolic alterations that prevents cell division [22], further reiterates the relevance of this current study, which has been directed to know more about the potentials of *C. vulgaris* powder on the physiological capabilities of yeast cells. To estimate the vitality (which is the physiological state of the yeast cells), this current work determined the cellular ATP content using BactTiter-Glo™ Microbial Cell Viability Assay. This is based on the reaction of luciferin with ATP in the presence of luciferase, Mg^{2+} ions, and oxygen, resulting in the emission of light [22]. For emphasis, ATP primarily in the mitochondria, helps the cell store the chemical energy required to power its biochemical reactions [22]. From the improved ATP production found in the samples supplemented with *C. vulgaris* powder (Refer to Figure 4), the measured luminescent signal appeared proportional to both ATP present and the number of yeast cells. The *C. vulgaris* powder probably maintained growth and cellular integrity of yeast thus, they remain viable in producing more energy required for biochemical reactions. To estimate the metabolic activity of yeast cells, the cell counting Kit-8 assay was employed, which is based on the reduction of sodium WST-8 salt by cellular dehydrogenase (oxidoreductases) to water-soluble orange formazan [22]. The increased amount of formazan produced compared well with the number of living cells. More so, the use of cell counting Kit-8 enables the determination of the number of viable yeast cells in suspension. The addition of *C. vulgaris* powder to the cell culture seemed to improve their metabolism, which perhaps lead to the ability of supplemented cultures to rapidly convert WST-8 to formazan. The proportion of cell survival and viability, obtained from both assays, reveals the promising antiapoptotic property of *C. vulgaris* powder, which is capable of protecting the cells

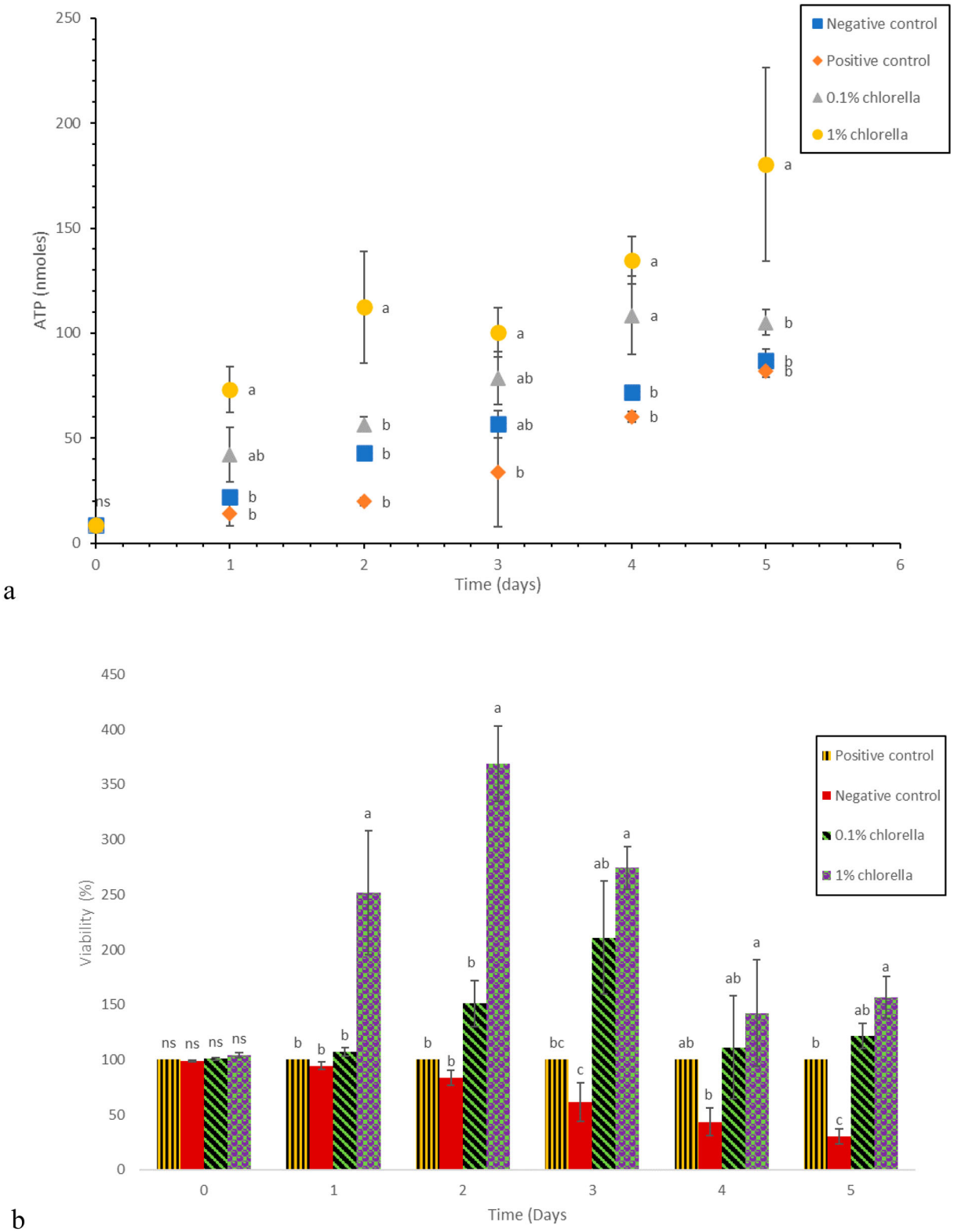


Figure 4. Yeast cell capacity to (a) produce ATP and (b) to remain viable during the five days culturing under three treatment condition and a control. Results are mean ± SD of six measurements (3 analytical × 2 biological replicates). Means that do not share the same letter (a, b, or c) on each time point (days) are significantly different ($p < 0.05$) using the Tukey's test. (ns = no significance).

against death, and decreasing physiological activities caused by ethanol.

The ethanol tolerance of yeast is overly complicated, and the detailed mechanisms are not yet fully understood. Some researches underscore that ethanol tolerance to associate with multiple membrane components [25]. It has been shown that ethanol can stimulate the production of reactive oxygen and nitrogen species, as well as decrease several antioxidant mechanisms in the liver [26]. Intrinsic to cellular functioning, ROS is present at basal levels in normal cells. However, when ROS concentration is in excess it can lead to the oxidization and

modification of cellular components hindering their original activity, thus leading to irreversible DNA damage [27]. Ethanol accumulation at toxic concentrations during fermentation increases ROS generation at the cellular and mitochondrial levels, with the release of hydrogen peroxide and superoxide [28]. As mitochondrial morphology and activity are closely related to the cellular ATP content, preventing ethanol-induced mitochondrial dysfunction could maintain cellular ATP production and overall the physiology of yeast cells.

C. vulgaris is rich in nutrients and metabolites that help to defend itself against biotic and abiotic stress. These nutrients and metabolites might be beneficial for the growth and survival of microorganisms. *Chlorella* could stimulate the growth of *Lactobacillus acidophilus*, an important bacterium that promotes the good health of the intestines [5]. Considerable amounts of chlorophyll and fibrous cell walls found in *Chlorella vulgaris* can serve as prebiotics for these beneficial lactic acid bacteria, causing the microflora in the gut to reasonably multiply compared to the usual rate [10]. *Chlorella* – a first-class detoxifying agent, can help bind and remove alcohol from the liver, as well as certain heavy metals (cadmium and mercury), pesticides, herbicides, and polychlorobiphenyls (PCB) from human body tissues [7,8,29]. The detoxification capability of *Chlorella* has been associated with its unique cell wall, with the main functional groups such as -NH_2 and -COOH involved, adding the polypeptides glutathione (GSH) and its derivative phytochelatins (PCs), which are metal chelating cystine-rich thiol group [29]. Glutathione, a tripeptide, and its oligomer phytochelatin are peptides found in higher plants and are understood to possess detoxifying and antioxidant properties [30]. These polypeptides are produced as intracellular cell defense mechanisms against heavy metal ions in the cytoplasm [7,31]. Moreover, the reduced form of glutathione can help remove the xenobiotics in the liver and heavy metals in plants [7,8,16]. *Chlorella* grown with arsenate was able to remove approximately 70% of Arsenate (AS^{5+}), with glutathione (GSH) playing the key role in AS^{5+} chelation [7]. Elsewhere, *C. vulgaris* cultivated under five different conditions was able to bio-absorb hexavalent chromium Cr (VI) into less hazardous trivalent Cr (III). Specifically, Cr (VI) bio absorption increased with the protein content of microalgae biomass [8,29]. In line with the above using Chlorella to improve yeast tolerance to ethanol particularly in the beverage industry could additionally offer other functional attributes.

5. Conclusions

Based on the cell viability/vitality assays in this study, introducing ethanol result in a considerable degree of yeast cell death. However, the addition of *C. vulgaris* powder shows high promise to protect the yeast cells from damage caused by ethanol. Notably, *C. vulgaris* being enriched with carbohydrates, proteins, and lipids as well as antioxidants, would make it not only an effective nutrient source for the yeast cells but also, a bioactive metabolite resource for protection of yeast cells from ethanol during fermentation. *C. vulgaris* powder would be effective in maintaining cell growth and proliferation, and potentially improve the longevity of *S. cerevisiae*.

However, this study did not show the exact mechanism(s) through which *Chlorella* can help mitigate ethanol-induced stress in yeast cells as it only shows it can improve yeast viability and vitality in the presence of ethanol. Future work should examine the bioactive compounds obtained from *Chlorella* especially those involved in ethanol detoxification, which will help to reveal the underpinning knowledge about how the ethanol-induced stress could be mitigated in a model microorganism.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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