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Research Article

Process optimization for third-generation bioethanol production from *Chlorella vulgaris* as a feedstock by *Candida boidinii*

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Abstract

Background: Microalgae offer significant advantages for third-generation bioethanol production due to their rapid growth rates, high photosynthetic efficiency, and ability to accumulate substantial amounts of carbohydrates. Unlike agricultural food crops, microalgae can be cultivated on non-arable land using saline or wastewater resources, thereby avoiding competition with food crops. Moreover, their low lignin contained cell wall structure enables milder pretreatment requirements and more efficient enzymatic hydrolysis, which ultimately leads to improved sugar production and higher ethanol yields. In addition, microalgae-based bioethanol production contributes to carbon dioxide mitigation through CO_2 fixation, enhancing the overall environmental sustainability of the process. For the mentioned reasons *Chlorella vulgaris* biomass was used as a feedstock for third-generation bioethanol production in the present study.

Aims: The aim of this study is to develop a sustainable and integrated process for third generation bioethanol production by utilizing domestic food waste. Specifically, the research focuses on: investigating the effects of ZnO nanoparticles on the fermentation process; evaluating the performance of *C. boidinii* yeast in the presence of nanoparticle catalysts; optimizing cultivation conditions to achieve efficient microalgal growth and enhanced bioethanol production by *C. boidinii*; and examining the influence of key parameters, such as pretreatment methods (1% H_2SO_4 and 1% NaOH), biomass loading (50, 100, 200 g/L), and media composition, on the ethanol yield.

Methods: In this study, *C. vulgaris* was used as a feedstock for bioethanol several key parameters were optimized, including microalgal cultivation conditions (photoautotrophic, photoheterotrophic with glucose, and photoheterotrophic with carrot pomace), pretreatment

Özet

Dayanak: Mikroalgler; hızlı büyüme oranları, yüksek fotosentetik verimlilikleri ve önemli miktarda karbonhidrat biriktirme yetenekleri nedeniyle üçüncü nesil biyoetanol üretimi için önemli avantajlar sunmaktadır. Tarımsal gıda ürünlerinin aksine, mikroalgler tarıma elverişli olmayan arazilerde, tuzlu su veya atık su kaynakları kullanılarak yetiştirilebilir; bu sayede gıda ürünleriyle rekabetten kaçınılır. Ayrıca, düşük lignin içeriğine sahip hücre duvarı yapıları, daha ılımlı ön işlem koşullarına ve daha verimli bir enzimatik hidrolize olanak tanır, bu da sonuç olarak şeker üretiminin artmasına ve daha yüksek etanol verimine yol açar. Ek olarak, mikroalg tabanlı biyoetanol üretimi, CO_2 fiksasyonu yoluyla karbondioksit azaltımına katkıda bulunarak sürecin genel çevresel sürdürülebilirliğini artırır. Bahsedilen bu nedenlerden dolayı, mevcut çalışmada üçüncü nesil biyoetanol üretimi için hammadde olarak *Chlorella vulgaris* biyokütlesi kullanılmıştır.

Amaçlar: Bu çalışmanın amacı, evsel gıda atıklarından yararlanarak üçüncü nesil biyoetanol üretimi için sürdürülebilir ve entegre bir süreç geliştirmektir. Araştırma spesifik olarak şu konulara odaklanmaktadır: ZnO (Çinko Oksit) nanopartiküllerinin fermantasyon süreci üzerindeki etkilerinin araştırılması; nanopartikül katalizörlerin varlığında *C. boidinii* mayasının performansının değerlendirilmesi; *C. boidinii* ile verimli mikroalgal büyüme ve artırılmış biyoetanol üretimi sağlamak için kültivasyon koşullarının optimize edilmesi, ön işlem yöntemleri (%1 H_2SO_4 ve %1 NaOH), biyokütle yüklemesi (50, 100, 200 g/L) ve besiyeri bileşimi gibi temel parametrelerin etanol verimi üzerindeki etkisinin incelenmesi.

Yöntemler: Bu çalışmada, *C. vulgaris* biyoetanol için hammadde olarak kullanılmış ve mikroalg yetiştirme koşulları (fotootrofik, glikozlu fotoheterotrofik ve havuç posası ile fotoheterotrofik), ön

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type (1% H_2SO_4 and 1% NaOH), biomass loading (50, 100, and 200 g/L), and nutrient supplementation (Medium 1 and Medium 2).

Results: *Candida boidinii* exhibited the highest bioethanol production and productivity at 3.29 ± 0.14 g/L and 0.26 ± 0.01g/L.h, respectively. When Medium 1 was applied, bioethanol concentration and productivity further increased to 4.54 ± 0.18 g/L and 0.38 ± 0.01 g/L.h, respectively.

Conclusion: These findings demonstrate that fermentable sugars derived from *C. vulgaris* can be effectively converted into third-generation bioethanol by *C. boidinii*.

işlem türü (1% H_2SO_4 ve 1% NaOH), biyokütle yüklemesi (50, 100 ve 200 g/L) ve besin takviyesi (Ortam 1 ve Ortam 2) gibi önemli parametreler optimize edilmiştir.

Bulgular: *Candida boidinii*, sırasıyla 3,29 ± 0,14 g/L ve 0,26 ± 0,01 g/L.h ile en yüksek biyoetanol üretimi ve verimliliğini sergilemiştir. Orta 1 uygulandığında, biyoetanol konsantrasyonu ve verimliliği sırasıyla 4,54 ± 0,18 g/L ve 0,38 ± 0,01 g/L.h'ye yükseldi.

Sonuç: Bu bulgular, *C. vulgaris*'ten elde edilen fermente edilebilir şekerlerin *C. boidinii* tarafından üçüncü nesil biyoetanola etkili bir şekilde dönüştürülebileceğini göstermektedir.

Keywords: Microalgae, fermentation, supplement, bioethanol, carrot pomace

Introduction

In recent decades, the energy crisis and global warming have emerged as some of the most critical global concerns. These challenges are closely associated with population growth and the excessive consumption of fossil fuels. Consequently, the exploration of renewable energy sources has become a key factor in achieving sustainability (Medipally et al., 2015). Solar, wind, biomass, and geothermal energy are commonly referred to as alternative renewable sources, and they possess significant potential to reduce both environmental pollutants and greenhouse gas emissions (Panwar et al., 2011).

Biomass-based energy sources offer several advantages, including renewability, wide availability, and cost-effectiveness. Among these, bioethanol is the most extensively studied biofuel and can be produced from various raw materials such as corn, rice, lignocellulosic biomass, photosynthetic organisms, and genetically modified microorganisms (Dutta et al., 2014; Srilatha et al., 2019).

Edible raw materials used in the food industry, including sugar beet, rice, corn, and cassava, are classified as first-generation bioethanol sources. In contrast, non-edible lignocellulosic feedstocks are utilized for second-generation bioethanol production (Kiran et al.,2014; Lavanya et al., 2020). Photosynthetic organisms, particularly microalgae, serve as feedstocks for third-generation bioethanol production. Microalgae utilize sunlight and CO_2 as carbon sources for growth, which offers a distinct advantage by potentially lowering production costs (Sarkar & Shimizu, 2015). They are easy to cultivate and possess high lipid, protein, and carbon contents. Moreover, the low lignin content of microalgae allows for the release of fermentable sugars without requiring harsh pretreatment conditions (Jambo et al., 2016). In addition to their lipid content, microalgae contain significant amounts of carbohydrates, such as glucose and xylose. Several genera, including *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, and *Spirulina*, have been reported to accumulate carbohydrates comprising up to approximately 50% of their dry biomass, making them strong candidates for bioethanol production (John et al., 2011).

Numerous studies on bioethanol production have demonstrated that *Chlorella vulgaris* contains carbohydrates accounting for 37%–55% of its dry biomass. These carbohydrates include glucose, xylose, galactose, arabinose, mannose, fucose, and rhamnose (Agwa et al., 2017; Caetano et al., 2022).

Phototrophic, heterotrophic, photoheterotrophic, and mixotrophic conditions represent the primary cultivation strategies for microalgae, each supporting growth under different carbon and energy sources (Tandon & Jin, 2017). In phototrophic systems, microalgae rely exclusively on light and CO_2 for metabolic activity. In contrast, heterotrophic cultivation enables growth through the utilization of organic carbon sources, in the absence of light. Photoheterotrophic cultivation combines illumination with organic substrates to support cellular growth (Abreu et al., 2012). Although photoheterotrophic cultivation has received comparatively limited attention in bioethanol-focused studies, existing reports suggest its potential to enhance biomass accumulation and increase lipid content (Selvakumar & Umadevi, 2014).

Agricultural and industrial food wastes are rich in fermentable sugars and growth-promoting factors such as proteins and minerals (Roy et al., 2023). Carrot pomace represents an important raw material, as it contains fermentable sugars including xylose, glucose, and galactose along with mineral salts (Mg, Ca, K, P, Na), carotenoids, and vitamins (Barzee et al., 2019). For these reasons, carrot pomace was employed as an organic carbon source to support microbial growth and fermentable sugar accumulation during the photoheterotrophic cultivation of *C. vulgaris*.

C. boidinii is a methylotrophic yeast characterized by considerable intraspecies variability and significant biotechnological relevance. It can be isolated from diverse natural habitats as well as environments influenced by human activities. The organism is capable of growth across a broad temperature range (15 °C–37 °C) and is widely distributed across various geographic regions (Camiolo et al., 2017; da Silva Almeida et al., 2024). Importantly, its metabolic capacity extends beyond hexose sugars, as it can also efficiently utilize pentose sugars. This metabolic versatility positions *C. boidinii* as a promising alternative ethanologenic yeast to *Saccharomyces cerevisiae* (Fehér et al., 2021).

Additives such as nitrogen sources and mineral salts play a crucial role in supporting microbial growth and ethanol tolerance.

For example, mineral salts act as cofactors in various metabolic reactions during fermentation (Rees & Stewart, 1997). Conversely, nitrogen sources, including amino acids, improve cell viability and increase ethanol tolerance (Yamaoka et al., 2014).

In the first phase of this study, the effects of different cultivation conditions were investigated to achieve more efficient microalgal growth and enhanced bioethanol production by *C. boidinii*. Subsequently, the influence of key parameters, including pretreatment methods (1% H_2SO_4 and 1% NaOH), biomass loading (50, 100, 200 g/L), and media composition, on bioethanol production by *C. boidinii* was examined. This study represents the first report on microalgal-based bioethanol production using *C. boidinii*.

Materials and Methods

Microalgae and Cultivation Conditions

C. vulgaris was obtained from the culture collection of Ankara University, Department of Biology, Biotechnology Research Laboratory Culture Collection. To initiate cultivation, 10 mL of pre-cultured microalgae was inoculated into 250 mL flasks containing 100 mL of BG-11 medium. The composition of BG-11 medium was as follows (per liter): 1.5 g $NaNO_3$, 75 mg $MgSO_4.7H_2O$, 40 mg K_2HPO_4, 36 mg $CaCl_2.2H_2O$, 6 mg ferric ammonium citrate, 6 mg citric acid H_2O, 1 mg $Na_2EDTA.2H_2O$, 2.86 mg H_3BO_3, 20 mg Na_2CO_3, 1.81 mg $MnCl_2.4H_2O$, 0.39 mg $Na_2MoO_4.2H_2O$, 0.22 mg $ZnSO_4.7H_2O$, 0.0494 mg $Co(NO_3)_2.6H_2O$, and 0.079 mg $CuSO_4.5H_2O$ (Park et al., 2014; Rippka, 1988). The working volume was maintained at 100 mL in 250 mL Erlenmeyer flasks. The cultures were incubated at 30 °C under cool-white fluorescent light with an intensity of 12.5 W m^{-2} (2400 lx). During photoautotrophic cultivation, no external sugar source was added to the BG-11 medium. Under photoheterotrophic conditions, microalgal cultures were grown in BG-11 supplemented with either 0.5 g/L glucose or 0.5 g/L carrot pomace (CP)-derived sugars under continous illumination. To assess the effect of CP, cultures supplemented with 0.5 g/L CP-derived sugars were incubated for 12 days under photoheterotrophic conditions at 30 °C and 2400 lx.

Microalgal biomass was harvested after 12 days by centrifugation at 5,000 rpm for 10 min using a Hettich Rotofix 32A centrifuge. The harvested cells were dried overnight at 70 °C. The resulting dried microalgal biomass was then used in fermentation assays (Acebu et al., 2022; Agwa et al., 2017; Wistara et al., 2016).

Pretreatment of CP

CP was supplied by BELSO/Türkiye and dried overnight in an oven at 80 °C. Dried CP (100 g/L) was pretreated with 1% H_2SO_4 at 121 °C for 15 min. Following pretreatment, the liquid fraction was separated by filteration using Whatman No. 1 filter paper.

Fermentation Conditions

C. boidinii was obtained from the culture collection of Ankara University, Department of Biology, Biotåechnology Research Laboratory Culture Collection. For pre-incubation, *C. boidinii* was cultivated for 24 hours in PGY medium, containing 10 g/L peptone, 20 g/L glucose, and 3 g/L yeast extract. Prior to fermentation, the microalgal biomass was subjected to a pretreatment process. Initially, the biomass was homogenized using an IKA T18 Ultra-Turrax at 13,000 rpm for 1 min. The homogenized biomass was then treated with 1% H_2SO_4 and sterilized by autoclaving at 121 °C for 15 min using an ALP/CL-40 M autoclave (ALP/CL-40 M, Germany). The inoculation ratio was adjusted to 10% (v/v). All fermentation experiments were conducted in 100 mL Erlenmeyer flasks with a working volume of 40 mL. Fermentations were carried out at 30 °C and 100 rpm in a shaking incubator (Gerhardt/Thermoshake THO 500/1/Germany). The fermentation pH was mainatined at 5. Initial sugar and ethanol concentrations were measured after 6, 12, and 24 hours of fermentation.

Effect of Pretreatment on Bioethanol Production

Two different pretreatment methods were applied to *C. vulgaris* biomass. The microalgal biomass was treated with either 1% H_2SO_4 or 1% NaOH and autoclaved at 121 °C for 15 min using an autoclave (ALP/CL-40 M/Germany). After pretreatment, the samples were centrifuged at 5,000 rpm for 10 min. The solid pellet was discarded, and the resulting liquid fractions were used for subsequent fermentation experiments.

Effects of Initial Biomass Loading on Bioethanol Production

To determine the effect of initial biomass loading on bioethanol production, three different microalgal biomass concentrations (50, 100, 200 g/L) were examined. *C. vulgaris* biomass was pretreated with 1% H_2SO_4 at 121 °C for 15 min in an autoclave. This pretreated microalgal biomass was subsequently used as the carbon source for fermentation.

Effect of Different Supplements on Bioethanol Production

To assess the effect of nutient supplementation on bioethanol production, two different fermentation media containing *C. vulgaris* biomass were evaluated. Medium 1 consisted of 5 g/L peptone, 3 g/L yeast extract, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L KH_2PO_4, 0.1 g/L $CaCl_2$, and 0.05 $ZnSO_4$ prior to fermentation (Demiray et al., 2020). Medium 2 contained 1 g/L yeast extract, 0.4 g/L KH_2PO_4, and 0.2 g/L NH_4Cl as mineral and nitrogen sources (Yu et al., 2020).

Analytical Methods

Ethanol concentration was determined by gas chromatography using a Shimadzu GC-2010 system. For sample preparation, 1.5 mL of fermentation broth was centrifuged at 10,000 rpm and 4 °C for 10 min using a Hettich centrifuge. The resulting supernatant was filtered through a 0.22 µm membrane filter and subjected to gas chromatography analysis. Ethanol was quantified using a flame ionization detector equipped with an RTX-Wax capillary column (60 m length, 0.25 mm internal diameter). A sample volume of 1 µL was injected into the injection port. The injection and detector temperatures were maintained at 140 °C and 160 °C, respectively. The initial column temperature was set at 50 °C and increased to 150 °C over 19 min. The column flow rate was 1.86 mL/min, with

a total carrier gas flow of 190.4 mL/min; nitrogen was used as the carrier gas (Wistara et al., 2016).

Total reducing sugar concentrations were determined using the DNS method (Miller, 1959). Reducing sugars reacted with the DNS reagent to produce an orange-brown colored compound. Sodium potassium tartrate was used to stabilize the color and prevent precipitation. Absorbance was measured spectrophotometrically at 540 nm, with a color intensity directly proportional to the reducing sugar concentration. Yeast growth was monitored spectrophotometrically at 600 nm.

Theoretical ethanol yield was calculated using Equation (1) (Kim & Lee, 2007).

$$\text{Theoretical ethanol yield (\%)} = \frac{\text{ethanol (g/L)}}{\text{(initial sugar (g/L)} \times 0.511)} \text{ x100} \quad (1)$$

Volumetric ethanol productivity (Q_p) was calculated using Equation (2) (Roca & Olsson, 2003).

$$\text{Volumetric ethanol productivity (g/Lh)} = \frac{\text{ethanol (g/L)}}{h_{max}} \quad (2)$$

Ethanol yield based on substrate consumption ($Y_{P/S}$) was calculated using Equation (3) (Yücel & Aksu, 2015).

$$\text{Ethanol yields (g/g)} = \frac{\text{(maximum ethanol (g/L)}}{\text{consumed sugar (g/L)}} \quad (3)$$

Statistical Analysis

Initially, the dataset was evaluated for compliance with the assumptions of normality and homogeneity of variances using the Shapiro–Wilk and Levene's test, respectively. As the data met the requirements for parametric analysis, statistical comparisons were performed using one-way analysis of variance. When significant differences were detected ($p < 0.05$), Tukey's honestly significant difference test was applied for post hoc pairwise comparisons. Results are reported as mean ± standard deviation. Groups within the same column sharing the same superscript letter was not significantly different among treatments. All statistical analyses were conducted using R software (version 4.5.2).

Results

Effect of Different Cultivation Strategies on Microalgal Growth

Cultivation strategy has a direct influence on microbial growth (Aziz et al., 2020). For this reason, *C. vulgaris* was cultivated under both photoautotrophic and photoheterotrophic conditions. Photoheterotrophic cultivation was further evaluated using two different carbon sources: glucose and CP. CP is an inexpensive and abundant by-product of the food industry and contains a considerable amount of reducing sugars (Yoon et al., 2005; Yu et al., 2013). Therefore, CP was selected as an alternative carbon source for the photoheterotrophic growth of *C. vulgaris*. A synthetic medium containing only glucose was used as a control.

The growth of *C. vulgaris* under different cultivation strategies is presented in Figure 1. According to the results, all cultivation conditions supported microalgal growth. The highest growth was observed under photoheterotrophic conditions with CP as the carbon source. Under this condition, the biomass concentration of *C. vulgaris* reached 0.46 g/L after 12 days. In comparison, growth reached 0.33 g/L under photoheterotrophic conditions with glucose, while the lowest biomass concentration of 0.21 g/L was observed under photoautotrophic cultivation. Interestingly, the initial microbial growth values under photoautotrophic cultivation, glucose containing photoheterotrophic, and CP-containing photoheterotrophic cultivations were 0.02, 0.03 and 0.07 g/L, respectively. The results indicate that photoheterotrophic cultivation with CP, resulted in significantly higher initial growth compared to other strategies. A plausible explanation for this observation is the differences inn pre-adaptation media, as cultivation under distinct conditions for 12 days may have led to variations in growth rates. In all experimental groups, microbial growth accelerated after 8 days and approached its maximum level by day 12. No significant increase in biomass was observed beyond this time point. Consequently, *C. vulgaris* growth experiments were terminated after 12 days.

Effect of Pretreatment on Reducing Sugar and Bioethanol Production

Pretreatment is a critical step for the release of fermentable sugars from microalgal biomass. The reducing sugar concentrations and bioethanol production obtained from *C. vulgaris* biomass subjected to different pretreatment methods (1% H_2SO_4 and 1% NaOH) are presented in Figure 2. According to the results, acid pretreatment yielded higher reducing sugar concentrations than alkali pretreatment. The highest initial reducing sugar

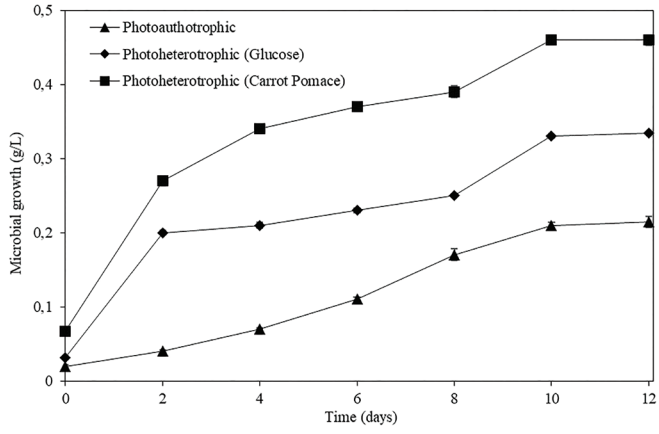

Figure 1. Effects of different carbon sources and cultivation conditions on microalgal growth (BG-11 medium; 0.5 g/L glucose; 0.5 g/L carrot pomace sugar; 2400 lx; 30 °C; pH:7; incubation time, 12 days).

concentration was achieved with 1% H_2SO_4 (9.98 g/L), whereas the lowest concentration was observed following 1% NaOH pretreatment (6.86 g/L).

In parallel with reducing sugar concentrations, the highest bioethanol production was obtained from biomass pretreated with 1% H_2SO_4, reaching 3.29 g/L at 12 h of fermentation. In contrast, bioethanol production decreased to 2.93 g/L when 1% NaOH pretreatment was applied. The kinetic parameters calculated for the different pretreatment methods are shown in Table 1. The highest theoretical bioethanol yield (72.95%) was observed for biomass pretreated with 1% NaOH, which can be attributed to the lower initial reducing sugar concentration compared to acid-pretreated biomass. Conversely, the lowest theoretical yield (65.10%) was obtained following 1% H_2SO_4 pretreatment. However, biomass pretreated with 1% H_2SO_4 exhibited higher volumetric ethanol productivity (Q_p) and ethanol yield (Y_p/s) than biomass treated with 1% NaOH. The maximum Q_p and Y_p/s values for 1% H_2SO_4 were 0.26 g/L·h and 0.49 g/g, respectively, whereas these values decreased to 0.24 g/L·h and 0.45 g/g, respectively, for 1% NaOH pretreatment.

Effects of Initial Biomass Loading on Sugar Concentrations and Ethanol Production

Initial biomass loading is a critical parameter influencing fermentation performance. In this study, the effects of three different initial biomass loadings (50, 100, and 200 g/L) on bioethanol production by *C. boidinii* were evaluated. Prior to fermentation, *C. vulgaris* biomass was pretreated with 1% H_2SO_4 at 121 °C for 15 min.

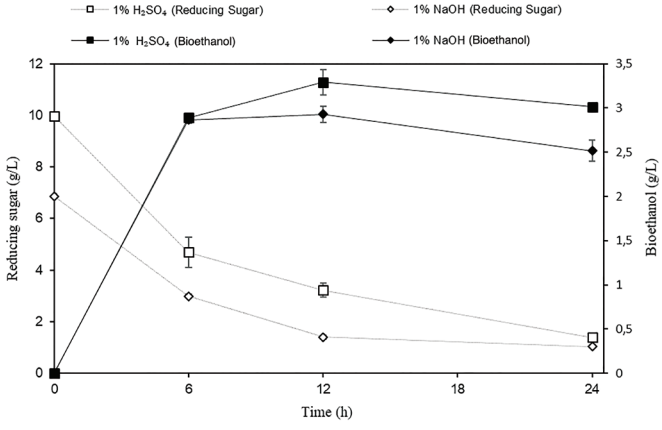

Figure 2. Effects of different pretreatment methods from *C. vulgaris* biomass (initial biomass loading: 50 g/L, pretreatments: 1% H_2SO_4 and 1% NaOH, 121 °C, 15 min, pH: 5, T: 30 °C).

The reducing sugar concentrations obtained from increasing *C. vulgaris* biomass loadings are presented in Table 2. The results indicate that higher biomass loadings led to increased reducing sugar concentrations. The maximum reducing sugar concentration was observed at a biomass loading of 200 g/L biomass (16.23 g/L), whereas the lowest concentration was detected at 50 g/L (9.98 g/L). An intermediate reducing sugar concentration of 13.03 g/L was obtained ata biomass loading of 100 g/L.

In parallel with reducing sugar concentrations, the highest bioethanol concentration was achieved at a biomass loading of 200 g/L biomass (3.89 g/L), while the lowest concentration was obtained at 50 g/L (3.29 g/L) after 12 hours of fermentation (Figure 3). At an initial biomass loading of 100 g/L, *C. boidinii* produced 3.32 g/L bioethanol. The results demonstrate that both reducing sugar and ethanol concentrations increased proportionally with increasing biomass loading. Moreover, high biomass loading (200 g/L) did not inhibit microbial growth or bioethanol production, as the reducing sugar concentrations remained below the tolerance limit of *C. boidinii* (Velazquez-Lucio et al., 2018).

Kinetic parameters associated with the different biomass loadings are summarized in Table 2. Although bioethanol concentrations increased with increasing biomass loading, the maximum theoretical bioethanol yield decreased from 65.10% to 47.39% as the biomass loading increased from 50 g/L to 200 g/L. From the highest biomass loading (200 g/L), the maximum volumetric ethanol productivity (Q_p) and bioethanol yield (Y_p/s) were 0.32 g/L·h and 0.35 g/g, respectively.

Effects of Different Supplements on Sugar Consumption and Bioethanol Production

Mineral salts and nitrogen sources are key factors influencing microbial growth and bioethanol production. In this study, two different fermentation media were evaluated for their effect on *C. boidinii* fermentation. Medium 1 contained 5 g/L peptone, 3 g/L yeast extract, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KH_2PO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, and 0.05 g/L $ZnSO_4 \cdot 7H_2O$ as nitrogen and mineral sources. Medium 2 contained 1 g/L yeast extract, 0.4 g/L KH_2PO_4, and 0.2 g/L NH_4Cl as organic and inorganic nitrogen and mineral sources.

Both media positively influenced the fermentation performance of *C. boidinii*. However, Medium 1 resulted in 1.06 fold higher bioethanol concentrations compared to Medium 2. The maximum bioethanol concentration achieved in this study was 4.54 g/L in Medium 1, whereas a concentration of 4.29 g/L was obtained in Medium 2 (Figure 4).

Table 1. Kinetic parameters of *C. boidinii* fermentation under different pretreatment conditions (initial *C. vulgaris* biomass: 50 g/L; pretreatments: 1% H_2SO_4 and 1% NaOH, 121 °C for 15 min; pH 5; incubation temperature: 30 °C).

Pretreatment	Maximum bioethanol (g/L)$_{12h}$	Theoretical yield (%)$_{12h}$	Q_P (g/L.h)$_{12h}$	$Y_{P/S}$ (g/g)$_{12h}$
1% H_2SO_4	$3.29^a \pm 0.14$	$65.10^a \pm 2.80$	$0.26^a \pm 0.01$	$0.49^a \pm 0.01$
1% NaOH	$2.93^b \pm 0.09$	$72.95^b \pm 2.29$	$0.24^b \pm 0.01$	$0.45^b \pm 0.02$

*Different superscript letters within the same column indicates statistically significant difference ($p < 0.05$).

Theoretical ethanol yields were higher for both supplemted media compared to the unsupplemented 200 g/L biomass condition (47.39%), reaching 54.74% and 51.73% for Medium 1 and Medium 2, respectively (Table 3). A similar trend was observed for reducing sugar consumption. In the unsupplemented 200 g/L biomass, the reducing sugar concentration was 5.04 g/L after 12 hours of fermentation, whereas concentrations decreased to 4.26 g/L and 4.24 g/L in the presence of Medium 1 and Medium 2, respectively.

Discussion

Experiments were conducted under different cultivation conditions, including photoautotrophic cultivation, photoheterotrophic cultivation with 0.5 g/L glucose, and photoheterotrophic cultivation with 0.5 g/L CP. The growth performance of *C. vulgaris* varied significantly among these conditions, with the highest biomass accumulation observed under photoheterotrophic cultivation supplemented with CP-derived sugars.

Previous studies have reported that the presence of organic carbon sources in growth media can increase microalgae growth (Abreu et al., 2012; Park et al., 2014). In the present study, microalgal growth under photoheterotrophic conditions was approximately 2.2-fold higher than that under photoautotrophic conditions after 12 days of incubation. Similarly, Grama et al. (2016) reported that *Dactylococcus* sp. cultivated under photoheterotrophic conditions exhibited 43% higher growth under photoautotrophic conditions. Although both CP- and glucose-supplemented photoheterotrophic media contained the same initial reducing sugar concentrations (0.5 g/L), higher microbial growth was observed in the CP-containing medium. This difference may be attributed to additional growth-promoting components present in CP, such as minerals and vitamins, which may stimulate *C. vulgaris* growth. Based on these findings, CP was selected as a cost-effective raw material for further studies.

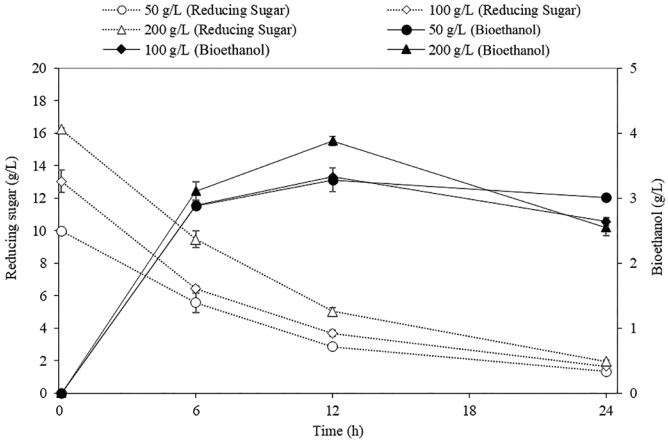

Figure 3. Effects of different *C. vulgaris* biomass loadings on bioethanol production and reducing sugar consumption by *C. boidinii* (pretreatment: 1% H_2SO_4 at 121 °C for 15 min).

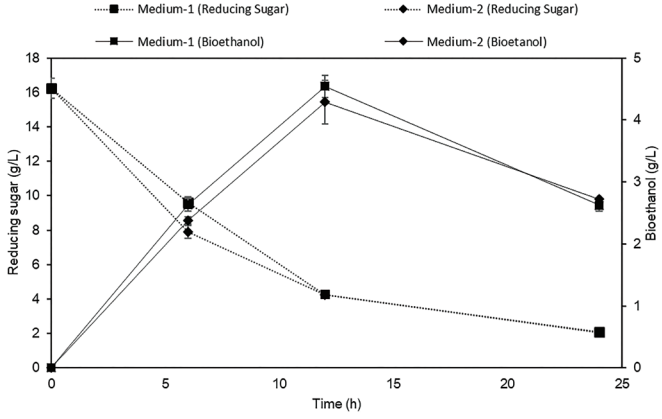

Figure 4. Bioethanol production and reducing sugar consumption by C. boidinii in the presence of different supplement media using 200 g/L initial C. vulgaris biomass (pretreatment: 1% H_2SO_4, 121 °C, 15 min, pH: 5, Medium 1: 5 g/L peptone, 3 g/L yeast extract, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L KH_2PO_4, 0.1 g/L $CaCl_2.2H_2O$, and 0.05 g/L $ZnSO_4.7H_2O$, Medium 2; 1 g/L yeast extract, 0.4 g/L KH_2PO_4, and 0.2 g/L NH_4Cl, T: 30 °C).

Table 2. Bioethanol production by *C. boidinii* at three different initial *C. vulgaris* biomass loadings (pretreatment: 1% H_2SO_4 at 121 °C for 15 min).

Biomass loading	**Reducing sugar (g/L)$_h$**	**Maximum bioethanol (g/L)$_{12h}$**	**Theoretical yield (%)$_{12h}$**	**Q_P (g/L.h)$_{12h}$**	**$Y_{P/S}$ (g/g)$_{12h}$**
50 g/L	$9.98^a \pm 0.12$	$3.29^a \pm 0.14$	$65.10^a \pm 2.80$	$0.26^a \pm 0.01$	$0.49^a \pm 0.01$
100 g/L	$13.03^b \pm 0.70$	$3.32^a \pm 0.01$	$50.31^b \pm 0.42$	$0.28^b \pm 0.002$	$0.35^b \pm 0.005$
200 g/L	$16.23^c \pm 0.10$	$3.89^b \pm 0.05$	$47.39^b \pm 0.34$	$0.32^b \pm 0.002$	$0.35^b \pm 0.01$

*Different superscript letters within the same column indicates statistically significant differences.

Table 3. Effect of different media compositions on bioethanol production by *C. boidinii* (initial biomass loading: 200 g/L *C. vulgaris*, pretreatment: 1% H_2SO_4, 121 °C, 15 min, pH: 5, Medium 1: 5 g/L peptone, 3 g/L yeast extract, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L KH_2PO_4, $CaCl_2.2H_2O$, and 0.05 g/L $ZnSO_4.7H_2O$. Medium 2: 1 g/L yeast extract, 0.4 g/L KH_2PO_4 and 0.2 g/L NH_4Cl, T: 30 °C).

	Reducing sugar (g/L)	**Maximum bioethanol (g/L)$_{12h}$**	**Theoretical yield (%)$_{12h}$**	**Q_P (g/L.h)$_{12h}$**	**$Y_{P/S}$ (g/g)$_{12h}$**
Medium 1	$16.23^a \pm 0.10$	$4.54^a \pm 0.18$	$54.74^a \pm 2.13$	$0.38^a \pm 0.01$	$0.38^a \pm 0.01$
Medium 2	$16.23^a \pm 0.10$	$4.29^a \pm 0.35$	$51.73^a \pm 4.26$	$0.36^a \pm 0.02$	$0.36^a \pm 0.03$

*Different superscript letters within the same column indicate statistically significant differences.

Different pretreatment methods (1% H_2SO_4 and 1% NaOH) were evaluated for their effectiveness in releasing fermentable sugars from *C. vulgaris* biomass cultivated on CP. The results indicated that pretreatment method significantly influenced fermentable sugar release and subsequent bioethanol production. Acid pretreatment more effectively disrupted the microalgal cell wall more by hydrolyzing hemicellulose and cellulose and degrading starch into smaller molecules. In contrast, alkali pretreatment primarily reduced polymer size without comparable sugar release (Kusmiyati et al., 2022; Purewal et al., 2023). The superior performance of acid pretreatment observed in this study is consistent with previous reports. Ngamsirisomsakul et al. (2019) reported a reducing sugar concentration of 6.50 g/L from acid-pretreated *C. vulgaris* biomass, while El-Souod et al. (2021) achieved 24.77 g per 100 g of dried biomass. In the present study, acid pretreatment (1% H_2SO_4) resulted in higher reducing sugar concentrations and bioethanol productivity than 1% NaOH, despite yielding a slightly lower theoretical ethanol yield. These findings suggest that acid pretreatment is more effective for maximizing sugar release and ethanol production. Therefore, *C. vulgaris* biomass pretreated with 1% H_2SO_4 at 121 °C for 15 minutes was selected for further experiments.

The results also demonstrate that increasing the initial biomass loading enhanced both sugar release and bioethanol production. Higher biomass loadings provided a greater amount of fermentable substrate, leading to elevated ethanol titers. Although the theoretical ethanol yield decreased at higher biomass loadings, the highest overall bioethanol concentration was achieved at 200 g/L biomass. This suggests that the sugar concentrations generated remained within the tolerance limits of *C. boidinii*, allowing efficient fermentation (Osawa et al., 2009; Velazquez-Lucio et al., 2018). Previous studies have confirmed the suitability of *C. vulgaris* as a feedstock for bioethanol production. Ngamsirisomsakul et al. (2019) reported that *S. cerevisiae* TISTR 5339 produced 5.62 g/L bioethanol from acid-pretreated *C. vulgaris* biomass containing 18 g/L sugar. Similarly, de Farias Silva and Bertucco (2017) achieved 4.97 g/L bioethanol from 100 g/L *C. vulgaris* biomass. Collectively, these findings indicate that an initial biomass loading of 200 g/L provides the highest ethanol concentration without inhibiting microbial activity, making it optimal for subsequent experiments.

Nitrogen sources and mineral salts were found to positively influence fermentation performance and accelerate sugar consumption. Although both fermentation media contained identical initial sugar concentrations, Medium 1 resulted in slightly higher bioethanol production. This enhancement can be attributed to additional supplements such as peptone, magnesium, calcium, and zinc. Minerals, especially magnesium, function as essential cofactors in key metabolic pathways such as glycolysis (Somda et al., 2011; Stehlik-Tomas et al., 2004). Similar beneficial effects of minerals and nitrogen supplementation on microbial growth and fermentation efficacy have been reported in previous studies (de Souza et al., 2016; Rees & Stewart, 1997). These findings confirm that appropriate supplementation with nitrogen and mineral salts improves ethanol production and accelerates fermentable sugar utilzation during *C. boidinii* fermentation.

Conclusion

In the present study, the effects of different cultivation conditions on *C. vulgaris* growth were optimized, and the influences of pretreatment method, initial biomass loading, and medium composition on bioethanol production by *C. boidinii* were investigated. Photoheterotrophic cultivation uisng CP promoted superior microalgal growth, while acid pretreatment resulted in higher fermentable sugar concentrations compared to alkali pretreatment. Increaing the highest ethanol titer achieved at 200 g/L biomass. Moreover, optimization of the fermentation medium increased bioethanol production by *C. boidinii* to 4.54 g/L. Under these conditions, the bioethanol yield and volumetric productivity reached 0.38 g/g and 0.38 g/L.h, respectively, after 12 h of fermentation. The current work demonstrates that photoheterotrophic cultivation with CP supports microalgal growth. Moreover, *C. vulgaris* is a suitable feedstock for third-generation bioethanol production, and *C. boidinii* was shown to effectively convert microalgal-derived fermentable sugars for ethanol fermentation.

Ethics

Ethics Committee Approval: Not required.

Data Sharing Statement: All data are available within the study.

Footnotes

Authorship Contributions: Conceptualization: S.E.K.; Design methodology: S.E.K.; Execution/investigation: S.E.K.; Resources/materials: S.E.K. and G.D.; Resources/materials: G.D.; Data acquisition: A.K.Y. and M.K.; Data analysis/interpretation: S.E.K.; Writing – original draft: A.K.Y. and S.E.K.; Writing – review & editing/critical revision: A.K.Y., M.K., G.D., and S.E.K.

Conflict of Interest: The author(s) have no conflicts of interest to declare.

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