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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

# Optimization of extraction conditions for obtaining active compounds of *Ulva* sp.

## *Ulva* sp.'nin aktif bileşiklerinin elde edilmesi için ekstraksiyon koşullarının optimizasyonu

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**Abstract:** *Ulva* sp., a green macroalgae known as sea lettuce, is rich in polysaccharides, proteins, minerals, and bioactive compounds with antimutagenic, anticoagulant, anticancer, anti-inflammatory, antibacterial, and nutraceutical properties. Its abundance along the Aegean Sea coast poses an environmental challenge, as it is often disposed of as waste. However, *Ulva* sp. holds potential for high-value products in cosmetics and dietary supplements. Optimizing the extraction of its bioactive compounds using response surface methodology involved adjusting ethanol concentration, solid/liquid ratio, and extraction time. Key responses evaluated included yield, total polysaccharides, total protein, total phenol, total antioxidant activity, alpha-glucosidase inhibitory activity, and yeast cell glucose uptake. In this study, extraction yields ranged from 0.86% to 22.47% based on variations in extraction conditions. The highest total protein content was 106.88 mg BSA/g dry extract, while the polysaccharide content was determined to be 15.42%. The highest values for total phenol content and antioxidant capacity were found to be 82.15 mg GAE/g dry extract and 63.63 mg Trolox/g dry extract, respectively. The determination of the total amounts of antioxidants and phenolic compounds in extracts may expand their potential applications. In addition, the potential application of *Ulva* sp extracts as inhibitors for the treatment of diabetes has been demonstrated through experiments assessing both alpha-glucosidase enzyme inhibition and glucose uptake in yeast cells. The results support an environmentally friendly approach for the utilization of *Ulva* sp. from waste into valuable antidiabetic products.

**Keywords:** *Ulva* sp., response surface methodology, optimization, bioactivity, characterization

**Öz:** Deniz marulu olarak bilinen yeşil bir makroalg olan *Ulva* sp., antimutajenik, antikoagülan, antikanser, antiinflamatuar, antibakteriyel ve nutrasötik özelliklere sahip polisakkaritler, proteinler, mineraller ve biyoaktif bileşikler açısından zengindir. Ege Denizi kıyılarında bol miktarda bulunması, genellikle atık olarak atıldığı için çevresel bir zorluk oluşturmaktadır. Ancak *Ulva* sp. kozmetiklerde ve diyet takviyelerinde yüksek değerli ürünler için potansiyel taşımaktadır. Tepki yüzey metodolojisi kullanılarak biyoaktif bileşiklerinin ekstraksiyonunun optimize edilmesi, etanol konsantrasyonunun, katı/sıvı oranının ve ekstraksiyon süresinin ayarlanmasını içermektedir. Değerlendirilen temel tepkiler arasında verim, toplam polisakkaritler, toplam protein, toplam fenol, toplam antioksidan aktivite, alfa-glukozidaz inhibitör aktivitesi ve maya hücresi glikoz alımı yer almıştır. Bu çalışmada, ekstraksiyon koşullarındaki değişikliklere bağlı olarak ekstraksiyon verimleri %0,86 ile %22,47 arasında değişmiştir. En yüksek toplam protein içeriği 106.88 mg BSA/g kuru ekstre iken, polisakkarit içeriği %15.42 olarak belirlendi. Toplam fenol içeriği ve antioksidan kapasitesi için en yüksek değerler sırasıyla 82.15 mg GAE/g kuru ekstre ve 63.63 mg Trolox/g kuru ekstre olarak bulundu. Ekstraktlardaki toplam antioksidan ve fenolik bileşik miktarlarının belirlenmesi, potansiyel uygulamalarını genişletebilir. Ayrıca, Ulva sp. ekstraktlarının diyabet tedavisinde inhibitör olarak potansiyel uygulaması, hem alfa-glukozidaz enzim inhibisyonunu hem de maya hücrelerinde glikoz alımını değerlendiren deneyler yoluyla gösterilmiştir. Sonuçlar, *Ulva* sp.'nin atıktan değerli antidiyabetik ürünlere dönüştürülmesi için çevre dostu bir yaklaşımı desteklemektedir.

**Anahtar kelimeler:** *Ulva* sp., tepki yüzey metodolojisi, optimizasyon, biyoaktivite, karakterizasyon

## INTRODUCTION

The oceans, teeming with life, offer a wealth of resources with diverse applications in medicine, cosmetics, and food. Among these resources, macroalgae, classified into Chlorophyta, Rhodophyta, and Ochrophyta, stand out for their rich content of dietary fiber, proteins, vitamins, and minerals, alongside unique secondary metabolites (Milchakova, 2011). The marine environment, boasting vast biological diversity, hosts valuable bioactive compounds, that hold potential therapeutic applications in addressing human diseases like cancer, inflammatory conditions, and viral infections (Nayak et al., 2021). This has fueled a growing interest in harnessing macroalgae as the source of bioactive compounds for various health benefits.

Macroalgae extracts, sourced from various species, exhibit potent pharmacological properties against viral, bacterial, and fungal infections, including common ailments like colds and flu. Notably rich in antioxidants, brown algae and other macroalgae species contain compounds such as sulfated polysaccharides, carotenoids, sterols, peptides, and mycosporine-like amino acids, contributing to their remarkable therapeutic potential (Plaza et al., 2008).

*Ulva* sp., commonly known as sea lettuce, represent a ubiquitous presence worldwide, displaying two main morphologies: tubular monostromatic and leafy distromatic, with some species exhibiting both forms. This diversity arises from genetic variability and environmental conditions, necessitating genetic-based identification methods. Out of

approximately 400 identified *Ulva* sp., only about 40 have been taxonomically recognized through genetic analysis (Alsufyani et al., 2020; Fort et al., 2022).

*Ulva* sp., renowned for their abundant and nutrient-rich biomass, hold immense potential for various industrial applications (Trivedi et al., 2016). Their composition, encompassing proteins, carbohydrates, polysaccharides, minerals, and lipids, exhibits variations influenced by species, populations, and environmental factors like temperature and salinity (Rasyid, 2017).

*Ulva* sp. also contain flavonoid and phenolic compounds, with water-extracted *Ulva lactuca* exhibiting significant phenol and flavonoid content. Notably, phenolic compound levels exhibit seasonal variations, peaking during the summer months.

In addition to their nutritional composition, *Ulva* sp. demonstrate diverse biological activities, including antioxidant (Hassan et al., 2011; Yaich et al., 2017), anti-inflammatory (Wekre et al., 2019), antibacterial (Alghazeer et al., 2013), antitumor (Abd-Ellatef et al., 2017; Arsianti et al., 2016), anti-hyperlipidemic (Pengzhan et al., 2003), hypocholesterolemic (Hassan et al., 2011), hepatoprotective (Devaki et al., 2009), cytotoxic (Alves et al., 2013), antifungal (Tüney et al., 2006), antiviral (Chiu et al., 2012), anti-parasitic (Spavieri et al., 2010), insecticidal (Abbassy et al., 2014), and plant growth activities (Hassan and Ghareib, 2009).

*Ulva* sp. exhibit antibacterial potential, offering a renewable and sustainable source of antibacterial compounds crucial for combating antibiotic resistance (Alghazeer et al., 2013). However, the biological activities of Ulva extracts varies significantly, influenced by extraction methods, solvents, physiological conditions of *Ulva* sp., and harvesting times.

In the realm of antidiabetic activity, *Ulva* sp. demonstrate promise as natural antidiabetic agents, with studies highlighting their antioxidant properties and ability to inhibit carbohydrate-hydrolyzing enzymes. Polysaccharides from *Ulva lactuca*, for instance, have been shown to effectively lower blood glucose levels and inhibit enzyme activity in diabetic animal models (Belhadj et al., 2021), while Ulva extract-loaded nanoparticles exhibit anti-inflammatory and hypoglycemic effects (Al-Malki et al., 2019). Moreover, Ulva water-ethanol extracts have demonstrated efficacy in reducing insulin resistance and cholesterol levels, showcasing anti-diabetic and anti-atherosclerotic effects (Labbaci and Boukortt, 2020).

The selection of extraction parameters is based on the most critical conditions which affect the biological activities of *Ulva* sp. The most important parameters for extraction optimization were identified as solid/liquid ratio, time, and solvent type based on a systematic literature review and our preliminary experimental results in extraction processes. In this study, different biological activities were reported for extracts obtained by optimization of each of these conditions. The effective extraction of bioactive compounds from Ulva depends on developing suitable extraction methods. Extraction optimization is crucial to maximize the yield of these substances, as improper conditions can lead to their loss or degradation. Therefore, the quality and bioavailability of Ulva extracts are directly tied to the careful optimization of extraction parameters, ensuring the full utilization of their benefits and facilitating their industrial application.

In this study, the extraction conditions for obtaining chemical and bioactive compounds from *Ulva* sp. were optimized using response surface methodology, with ethanol concentration, solid/liquid ratio, and extraction time as independent variables. Various responses including yield, total polysaccharides, total protein, total phenol, total antioxidant activity, alpha-glucosidase enzyme inhibitory activity, and yeast cell glucose uptake for the obtained extracts were evaluated during the optimization process.

## MATERIALS AND METHODS

### Materials

In the study, sea lettuce was collected from the Izmir Gulf on Saturday, October 1, 2022, departing from the Karşıyaka/Izmir coast by boat, at the coordinates 38°27'48.0"N 27°04'03.4"E (Figure 1).

**Figure 1.** Images of sea lettuces collected with nets from seawater

The collected sea lettuce was quickly brought to the laboratory and washed first with tap water and then with distilled water. Afterward, it was spread onto filter papers for drying. The longest intact sea lettuce, found in a single piece, was 71 cm in length. The shortest one was around 30 cm. The examination revealed that all samples exhibited morphological similarities. However, genetic tests are required for species identification.

### Design of experiments

The Box-Behnken Design (BBD) was used to perform Response Surface Methodology (RSM) by using Design Expert 13.0.5.0 software from Stat-Ease Inc. Three independent variables (A: Extraction time (hours), B: Solid/liquid ratio, C: Ethanol (%)) at three levels each were investigated for the optimization of the extraction process (Table 1). Following the extraction, the yield (%) (R1), total polysaccharide content (%) (R2), total phenol content (mg GAE/g dry extract) (R3), total protein (mg BSA/g dry extract) (R4), total antioxidant capacity (mg Trolox/g dry extract) (R5), alpha-glucosidase inhibition activity (mg/ml) (R6), and yeast cell glucose uptake inhibition activity (at constant concentration of 10 mg/ml) (R7) were evaluated as responses.

**Table 1.** Experimental ranges and levels of independent extraction variables

| Factor | Factor Levels | | |
|---|---|---|---|
| | - | 0 | + |
| A: Extraction Time (hours) | 8 | 16 | 24 |
| B: Solid/Liquid Ratio | 20 | 30 | 40 |
| C: Ethanol Percentage | 0 | 50 | 100 |

### Extraction of *Ulva*

The study involved processing dried *Ulva* sp. for grinding to reduce particle size. Extraction experiments, designed via Design-Expert®, varied extraction time, solid-liquid ratio, and ethanol-water ratio while keeping other factors constant. Extraction was performed in a cooled stirred incubator (NB-T205LF, N-BIOTEK), followed by concentration and alcohol removal using a rotary vacuum evaporator (LabTech) before freeze-drying (Telstar Lyoquest). The resulting powder extracts were weighed to calculate yield. Response Surface Methodology (RSM) with statistical analysis determined the suitable extraction model based on significance levels, lack of fit, regression coefficients, and predicted values within confidence intervals, affirming the model's adequacy for the study (Ummat et al., 2021).

### Total phenol content

The phenolic content was analyzed using the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965) with gallic acid as the standard. Samples were prepared and added to 96-well plates, with a control group using methanol. Folin-Ciocalteu reagent and sodium carbonate solution were added sequentially, followed by incubation and absorbance measurement at 725 nm. The phenolic content was quantified in gallic acid equivalents (GAE), using a gallic acid standard calibration curve ($y=5.436x + 0.0095$; $R^2 = 0.9992$).

### Total antioxidant capacity

The Trolox equivalent antioxidant capacity was determined following the method described by Miller et al. (1993). An ABTS-potassium persulfate solution was incubated to trigger the reaction, and its absorbance was adjusted to 0.7 using a spectrophotometer. Extracts at the same concentration were added to 96-well plates along with ABTS solution, incubated in darkness, and absorbance was measured at 734 nm after 30 minutes. Trolox Equivalent Antioxidant Capacity (TEAC) was calculated based on the absorbance of a Trolox standard curve ($y =8578.8x - 1.5223$; $R^2 = 0.9935$).

### Total protein content

The total protein content was determined using the Bicinchoninic Acid Protein based on the assay kit instruction (BCA Assay kit, Sigma-Aldrich, Přerovská et al., 2022). The protein content was calculated using a BSA calibration curve ($y = 0.0011x + 0.0314$; $R^2 = 0.9938$).

### Total polysaccharide content

The determination of total polysaccharide content was performed using the method proposed by He et al. (2018). After creating a standard curve with a reference glucose solution (103 µg/mL) ($y = 11.478x + 0.0449$; $R^2 = 0.9986$), samples were prepared. The samples were treated with a 5% phenol solution and sulfuric acid and then kept in boiling water for 20 minutes. They were subsequently cooled in an ice bath for 5 minutes. Absorbance was then measured at 488 nm.

### Alpha-glucosidase enzyme inhibition assay

Samples were dissolved in 5% DMSO, while alpha-glucosidase enzyme and 4-Nitrophenyl-alpha-D-glucopyranoside substrate were prepared in 0.1 M phosphate buffer (pH 6.9). Acarbose and samples were added to a 96-well plate in triplicates, followed by enzyme addition and incubation at 37°C for 15 minutes. The substrate was then added and further incubated for 10 minutes before stopping the reaction with sodium carbonate solution. Absorbance at 405 nm was measured using a microplate reader (Lankatillake et al., 2021). Results were given in terms of $IC_{50}$ values (mg/ml).

### Yeast cell glucose uptake test

The experiment involved suspending washed yeast cells in pure water to a 10% v/v final volume. Glucose solution and yeast solution were added to Falcon tubes for standard concentration and positive control, with acarbose solutions added for different concentrations. The tubes were then incubated at 37°C in a shaking incubator, followed by centrifugation and transfer of supernatant to test tubes. Acetate buffer and DNS solution were added to the test tubes, which were then boiled and cooled before measuring absorbance at 540 nm using a spectrophotometer. The results were calculated based on the acarbose standart curve ($y= 0.0597 – 0.0033$; $R^2 = 0.9831$).

## RESULTS

Table 2 shows the final version of the experimental matrix with 17 runs obtained by implementing the Box-Behnken method, where 3 independent variables and 5 center points.

### Yield of extraction

A quadratic model was selected for optimizing yield (%) based on the sequential sum of squares and lack of fit tests. The ANOVA results highlight the model's high significance with a Model F-value of 58.38 and a low probability of occurrence at 0.01%. Key variables like A-Time, C-Ethanol, $A^2$, and $C^2$ are deemed important for yield prediction. The equations created with the quadratic model, which was used to determine the independent variables affecting the extraction yield (%) through the sequential model sum of squares and lack of fit test, are provided below in encoded variables:

$$Yield\ \% = 6.03 + 0.2955*A + 0.5973*B – 9.35*C – 0.242*AB + 0.208*AC – 0.8685*BC + 2.74*A^2 + 3.09*C^2$$

The three-dimensional surface plots of yield (%) have been generated using the obtained model from the analysis. These

graphs (Figure 2a-b-c) demonstrate the effect of combinations of the two variables on yield while keeping one factor at the center point (0) of the experimental design constant. Upon examining Figure 2a-c, although the changes in solid/liquid ratio and time are similar, it is evident that the percentage of ethanol is the most influential factor on yield.

**Table 2.** Final version of the experimental matrix with obtained results

| | Factors* | | | Responses** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Run | F1 | F2 | F3 | R1 | R2 | R3 | R4 | R5 | R6 | R7 |
| 1 | 16 | 30 | 50 | 6.074 | 7.3 | 52.51 | 59.76 | 52.82 | 1.2 | 39.68 |
| 2 | 24 | 30 | 100 | 1.68 | 4.24 | 58.2 | 106.88 | 37.25 | 0.82 | 36.84 |
| 3 | 8 | 30 | 100 | 1.064 | 1.03 | 70.14 | 74.82 | 28.22 | 0.61 | 34.05 |
| 4 | 24 | 20 | 50 | 9.2 | 7.025 | 53.38 | 90.42 | 57.27 | 0.8 | 42.13 |
| 5 | 16 | 40 | 0 | 19.09 | 13.2 | 24.91 | 53.1 | 55.46 | 1.28 | 12.47 |
| 6 | 8 | 30 | 0 | 22.47 | 11.09 | 18.4 | 75.67 | 42.6 | 0.65 | 14.02 |
| 7 | 16 | 40 | 100 | 0.94 | 6.13 | 70.39 | 103.628 | 34.1 | 0.64 | 23.65 |
| 8 | 24 | 30 | 0 | 22.254 | 12.54 | 24.38 | 80.25 | 56.36 | 1.6 | 25.81 |
| 9 | 16 | 30 | 50 | 6.36 | 7.35 | 47.81 | 68.42 | 47.88 | 1.67 | 35.81 |
| 10 | 8 | 20 | 50 | 7.734 | 6.88 | 53.72 | 62.78 | 45.07 | 0.45 | 28 |
| 11 | 16 | 30 | 50 | 6.658 | 7.1 | 44.52 | 56.77 | 46.13 | 1.1 | 38.3 |
| 12 | 16 | 20 | 100 | 0.856 | 6.93 | 82.15 | 69.1 | 51.96 | 1.2 | 38.12 |
| 13 | 16 | 20 | 0 | 15.532 | 15.42 | 29.17 | 94.07 | 63.63 | 1.46 | 18.91 |
| 14 | 16 | 30 | 50 | 6.208 | 7.5 | 48.61 | 56.82 | 49.98 | 1.3 | 36.55 |
| 15 | 8 | 40 | 50 | 8.786 | 6.34 | 47.37 | 49.19 | 42.63 | 0.17 | 26.12 |
| 16 | 16 | 30 | 50 | 4.889 | 5.08 | 45.63 | 60.82 | 44.12 | 1.18 | 35.3 |
| 17 | 24 | 40 | 50 | 9.284 | 9.96 | 58.6 | 90.16 | 49.71 | 0.35 | 18.58 |

*F1: A: Extraction Time (hours); F2: B: Solid/Liquid Ratio; F3: C: Ethanol %
**R1: Yield %; R2 Total Polysaccharide Content %; R3: Total Phenol Content (mg GAE/g Dry Extract); R4: Total Protein Content (mg BSA/g Dry Extract); R5: Total Antioxidant Capacity (mg Trolox/g Dry Extract); R6: Alpha-glucosidase Inhibition IC50 (mg/ml); R7: Yeast Cell Glucose Uptake %

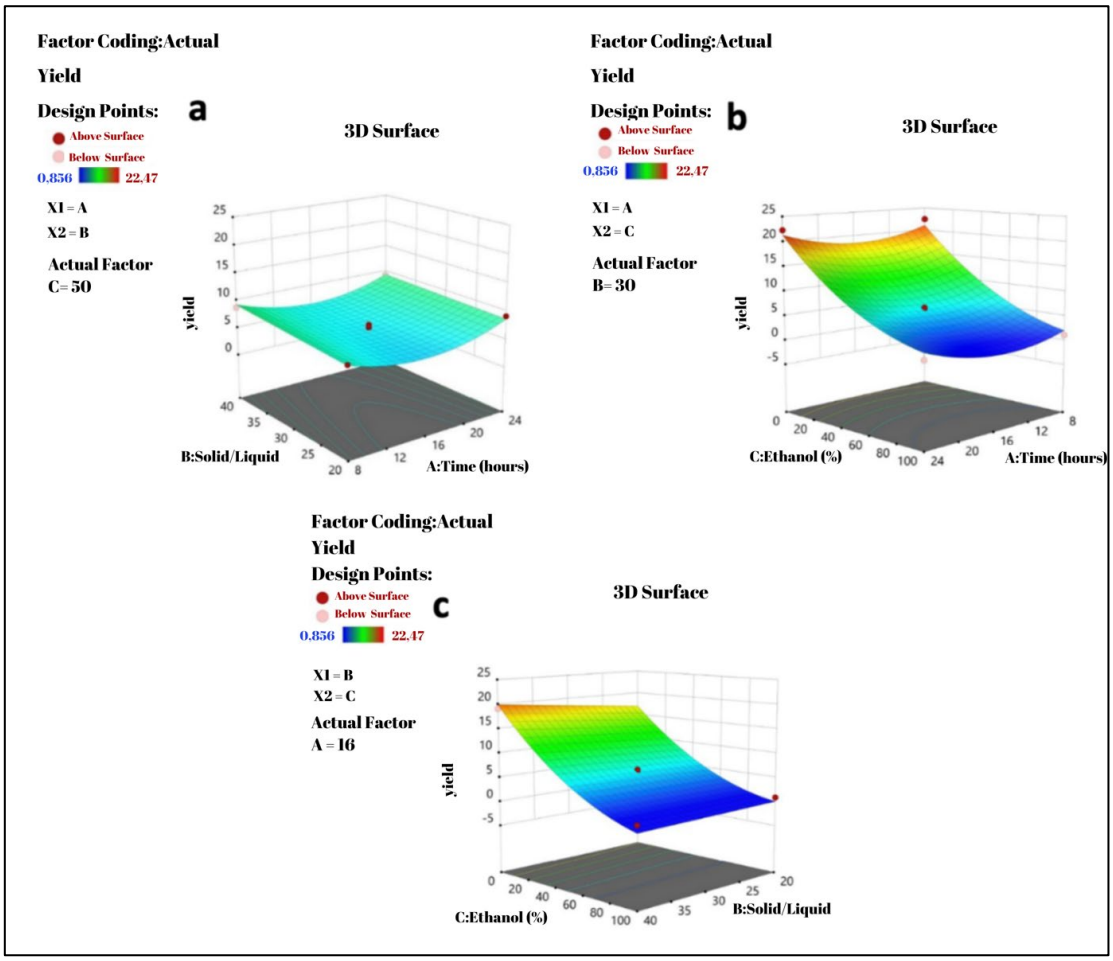


**Figure 2.** Response surface plots for the effects of independent variables on extraction yield. (a) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50; (b) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio):30; (c) B (Solid/Liquid Ratio) vs C (Ethanol, %), A (Time, hours): 16.

### Total polysaccharide content

A quadratic model was chosen for optimizing total polysaccharides (%) based on the sequential model sum of squares and lack of fit tests. The ANOVA results highlight a significant Model F value of 20.34 ($p < 0.01$), indicating the model's robustness, while the lack of fit's F value of 1.29 ($p = 40.55\%$) is deemed insignificant. The quadratic model equations created for the independent variables affecting total polysaccharides (%) and formulated in terms of coded variables are as follows:

Total polysaccharides (%) = 6.87 + 1.05A – 0.0781B – 4.24C + 0.8687AB + 0.44AC – 1.25A² + 1.94B² + 1.61C²

Three-dimensional surface curves for total polysaccharides (%) are drawn using the model obtained from the analyses. The graphs obtained from the optimization of the current study are shown in Figure 3. These graphs illustrate the effect of the combination of the other two variables on the total polysaccharides (%) when one factor is kept constant at the center point (0) of the experimental design. Upon examining Figure 3, it can be observed that a decrease in ethanol (%) has a positive effect on the response of total polysaccharides (%). Similarly, an increase in time (hours) also increases the total polysaccharides (%).

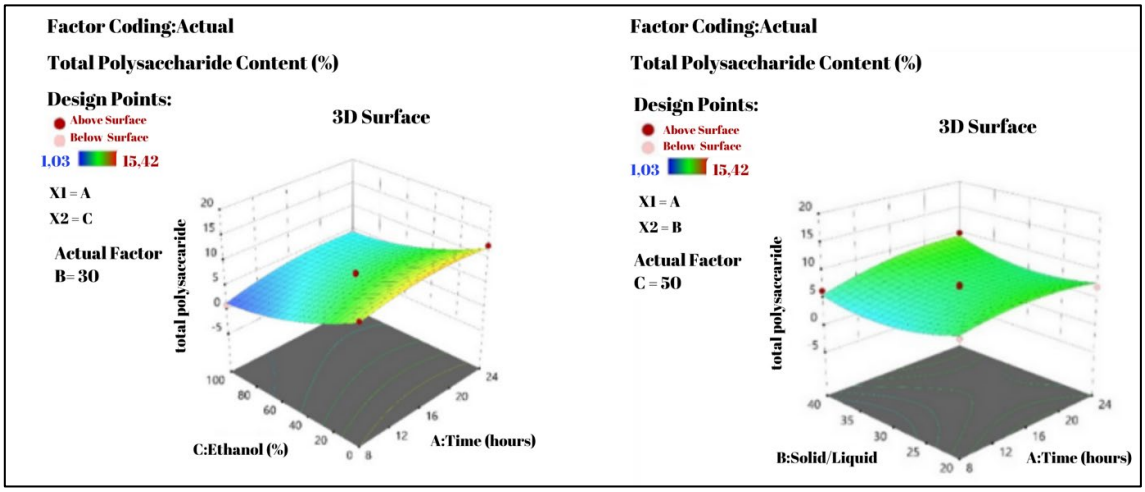


**Figure 3.** Response surface plots for the effects of independent variables on total polysaccharides. (a) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio):30; (b) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50.

### Total phenol content

A quadratic model was used for optimizing total phenol content (mg GAE/g Dry Extract). Significant terms ($p<0.05$) were identified through ANOVA, and insignificant factors were integrated into the model equation. The lack of fit was deemed insignificant at a 95% confidence level, supporting the effectiveness of the simplified model equation due to the significant Model F value (34.54) and insignificant lack of fit (2.52). The optimization study resulted in a model with high $R^2$ (0.9719) and Adj-$R^2$ (0.9437) values, indicating a strong fit and absence of statistically insignificant terms. The "Adeq Precision" value exceeding 4 (20.8348) confirms the model's usability. The quadratic model equations, based on sequential model squares and adequacy tests, are provided in coded variable terms for the independent variables impacting total phenolic content (mg GAE/g Dry Extract).

Total phenol content = 47.1 + 0.6163A – 2.14B + 23C + 2.89AB – 4.48AC – 1.88BC + 7.07B² – 3.41C²

Three-dimensional surface curves for total phenols (%) were created using the model derived from conducted analyses, showcased in Figures 4a-c. These graphs demonstrate how varying combinations of two variables affect total phenols when one factor remains constant at the center point (0) of the experimental design. Figure 4a,b highlights that while solid/liquid ratio and extraction time show similar variations, the percentage of ethanol stands out as the most impactful factor on total phenol content.

### Total protein content

A quadratic model was selected to optimize total protein content (mg BSA/g Dry Extract) based on sequential model squares and lack of fit tests. Significant terms were identified through ANOVA ($p<0.05$), supporting the model's validity with significant F and p-values. Insignificant values were incorporated into the model equation, and the lack of fit was deemed insignificant at a 95% confidence level, with a significant Model F value (18.11) and an insignificant lack of fit value (2.20). The optimized model achieved high $R^2$ (0.9337) and Adj-$R^2$ (0.8821) values, meeting expectations with a small difference between Pred-$R^2$ and Adj-$R^2$ values (<0.2), indicating a lack of statistically significant and insignificant terms.

The quadratic model equations, based on sequential model squares and adequacy tests, are provided in coded variable terms for the independent variables impacting total protein content (mg BSA/g Dry Extract).

The total protein content (mg BSA/g Dry Extract) = 62.24 + 13.16**A* – *2.54**B + 6.42**C* + *6.87**A*C + 18.87**B***C* + *8.74**A² + 15.58*C²

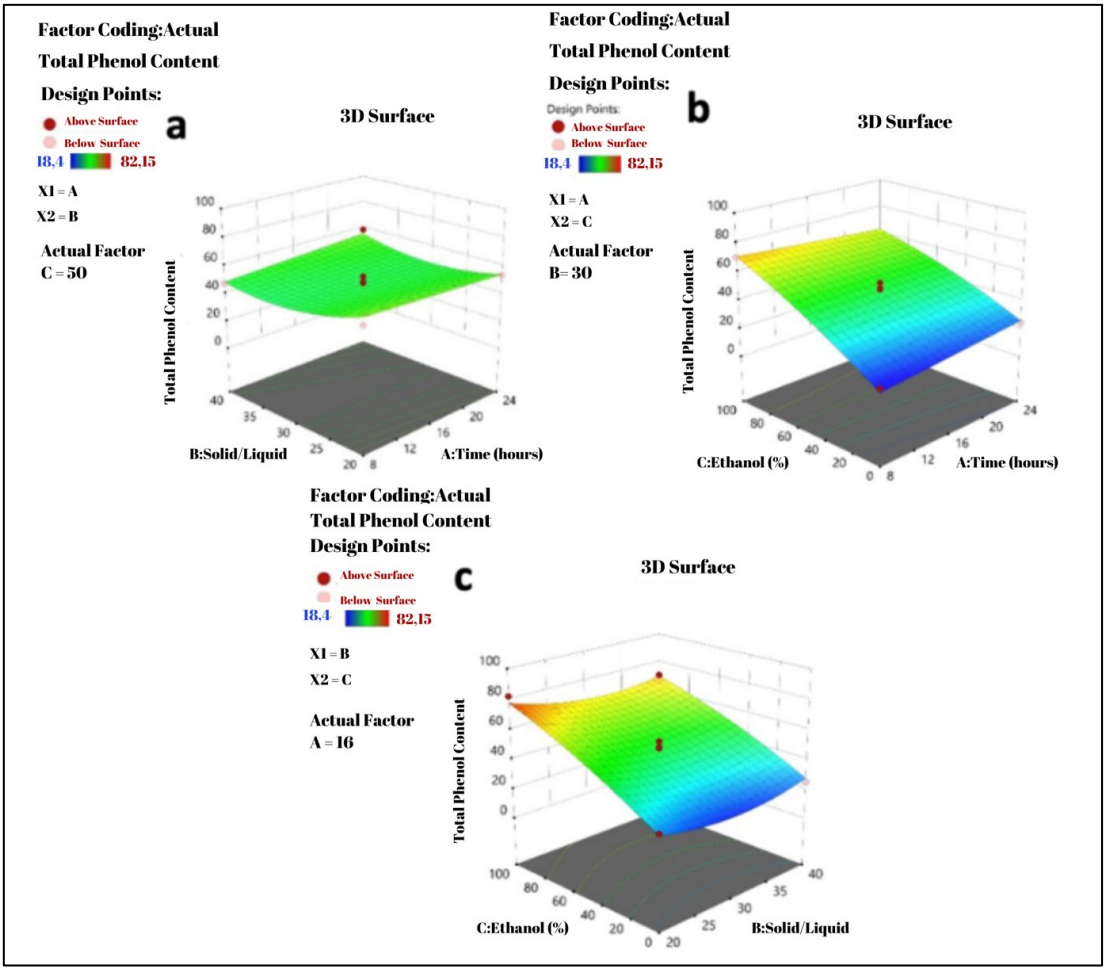


**Figure 4.** Response surface plots for the effects of independent variables on total phenol content. (a) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50; (b) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio):30; (c) B (Solid/Liquid Ratio) vs C (Ethanol, %), A (Time, hours): 16.

Three-dimensional surface curves were created for total protein content (mg BSA/g Dry Extract) using the obtained model, shown in Figure 5a-c. These graphs illustrate how varying combinations of two variables affect total protein content when one factor remains constant at the center point (0) of the experimental design. Figure 5a indicates that total protein content peaks when the solid/liquid ratio is minimal and the ethanol percentage is maximal, while Figure 5c shows that maximum extraction time and ethanol percentage result in the highest total protein content.

**Total antioxidant capacity**

The study on total antioxidant capacity (mg Trolox/g Dry Extract) employed a quadratic model selected based on the sum of squares and lack of fit test results, with significant terms identified through ANOVA ($p<0.05$). Main effects, interactions, and second-order terms were found to be statistically significant, supported by an insignificant lack of fit at a 95% confidence level (F value of 0.883) and a Model F value of 13.92 in ANOVA. The optimized model achieved an $R^2$ value of 0.9330 and an Adj-$R^2$ value of 0.8659, signifying a good fit of the model to the data.

The coefficients obtained for the coded independent variables for the model equation are as follows:

Total antioxidant capacity (mg Trolox/g Dry Extract) = $48.19 + 5.26*A - 4.5*B - 8.32*C - 4.85*A^2 + 5.33*B^2 - 2.23*C^2$

The optimization study resulted in three-dimensional surface plots illustrating the total antioxidant content (mg Trolox/g Dry Extract) using the obtained model. Figure 6a-c showcase the effects of varying combinations of two variables on the response, with one variable held constant at the center point (0) of the experimental design. In Figure 6a, minimizing the percentage of ethanol and the solid/liquid ratio maximizes total antioxidant content, while Figure 6b indicates that increasing extraction time can compensate for a decrease in ethanol percentage. Additionally, Figure 6c demonstrates that reducing the solid/liquid ratio and increasing time lead to increased total antioxidant content.

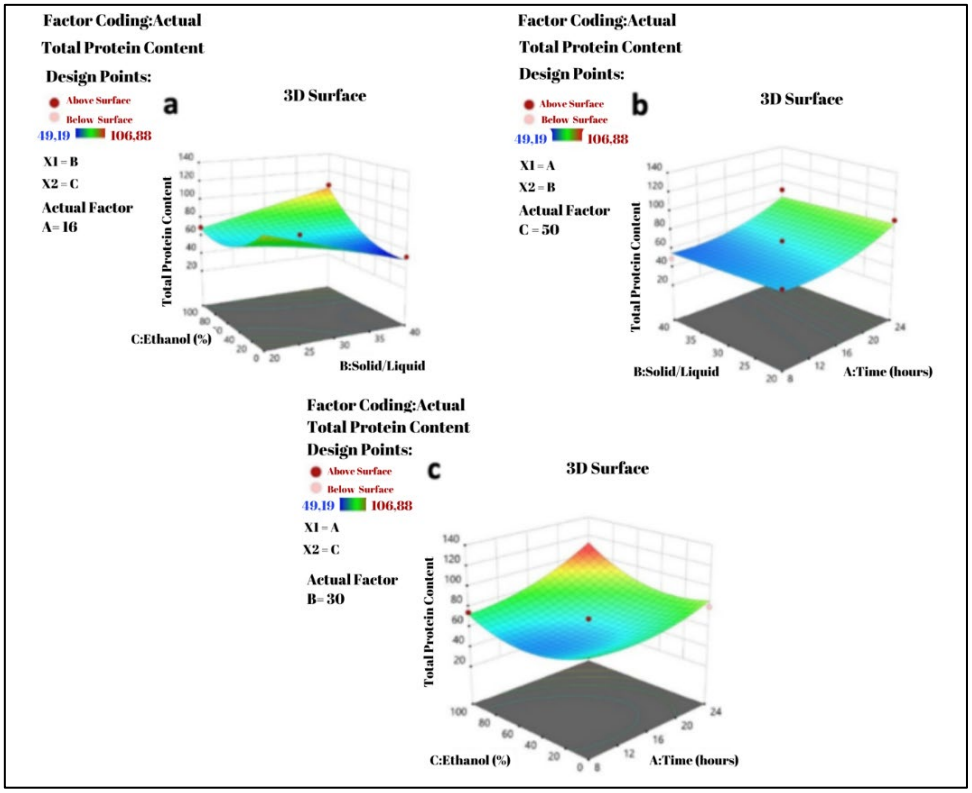


**Figure 5**. Response surface plots for the effects of independent variables on total protein content. (a) B (Solid/Liquid Ratio) vs C (Ethanol, %), A (Time, hours): 16; (b) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50; (c) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio):30.

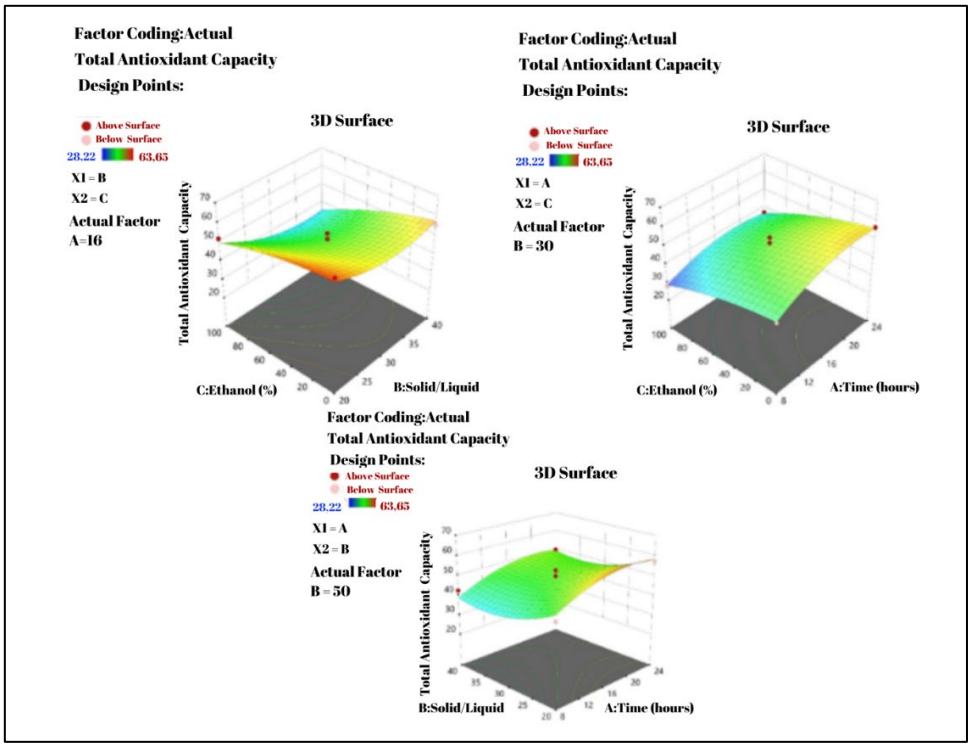


**Figure 6.** Response surface plots for the effects of independent variables on total antioxidant capacity. (a) B (Solid/Liquid Ratio) vs C (Ethanol, %), A (Time, hours): 16; (b) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio):30; (c) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50.

### Alpha-glucosidase enzyme inhibition activity

The optimization of glucosidase inhibitor activity (mg/ml) involved selecting a quadratic model, with significant terms determined through ANOVA and model adequacy, confirmed at a 95% confidence level. Equations based on coded variables were formulated to represent the influential independent factors affecting glucosidase inhibitor activity.

Equations based on coded variables for influential independent factors affecting glucosidase inhibitor activity were formulated, showcasing the model's suitability for optimization purposes.

Glucosidase inhibitor activity (mg/ml) = 1.29 + 0.2112*A* – *0.1838*B – 0.215**C* – *0.0425**A**B* – *0.185**A**C* – *0.095**B**C* – *0.5363**A$^2$* – 0.3113**B$^2$* + *0.1663**C$^2$*

The analysis led to the generation of 3D surface plots depicting alpha-glucosidase inhibitor activity (mg/ml) using the obtained model, as shown in Figure 7a-c. These graphs demonstrate the impact of variable combinations on the response while maintaining one variable constant at the center point (0) of the experimental design.

The results, represented in terms of $IC_{50}$ values, reveal that increasing the solid-to-liquid ratio and ethanol percentage positively affect the response, as seen in Figure 7a, while Figure 7b shows a positive effect when time and ethanol percentage are minimized, and Figure 7c indicates a positive effect with an increase in the solid-to-liquid ratio and a decrease in extraction time on alpha-glucosidase enzyme inhibitor activity.

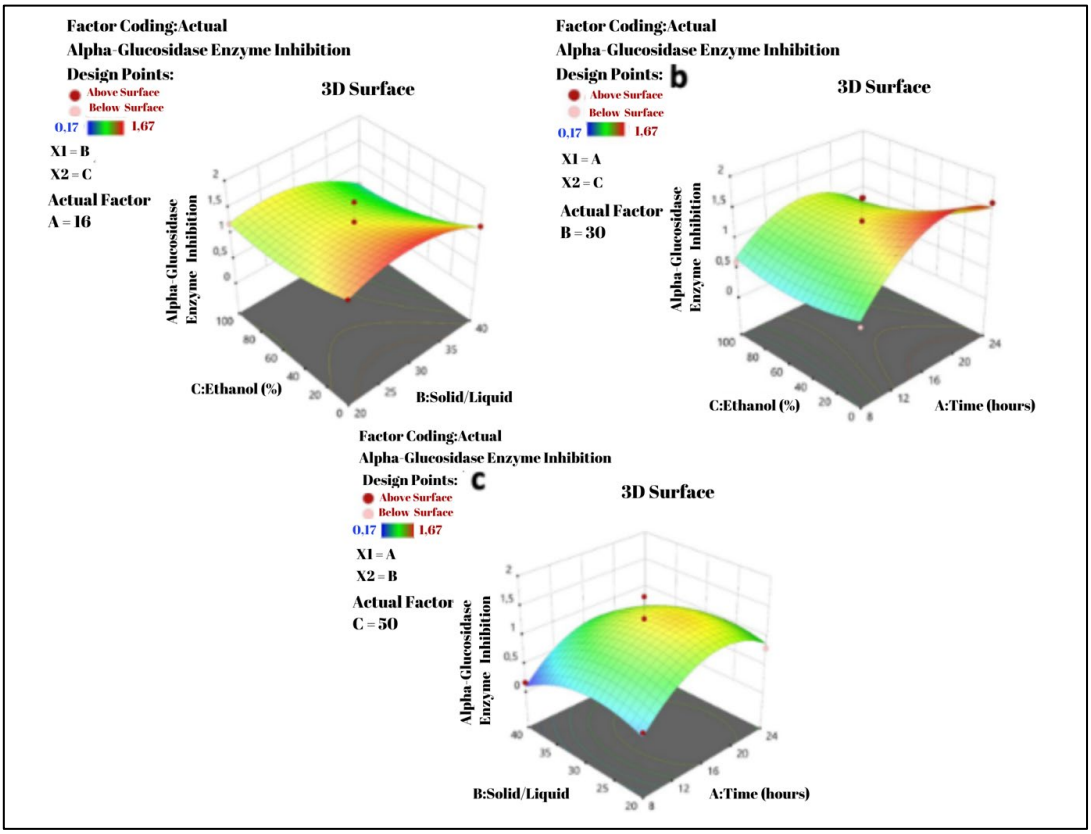


**Figure 7.** Response surface plots for the effects of independent variables on alpha-glucosidase enzyme inhibition activity. (a) B (Solid/Liquid Ratio) vs C (Ethanol, %), A (Time, hours): 16; (b) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio):30; (c) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50.

### Yeast cell glucose uptake

The yeast cell glucose uptake test employed a quadratic model selected based on sequential model sum of squares and model adequacy tests, with significant terms determined via ANOVA ($p<0.05$) for main effects, interactions, and second-degree expressions. Model adequacy was deemed insignificant at a 95% confidence level, indicating a strong fit for the model. The high Model F value (45.93) and insignificant model inadequacy F value (1.06) led to a simplified model equation based on significant effects. Optimization studies resulted in high R-squared (0.9833) and adjusted R-squared (0.9619) values, validating the model's suitability for predictions with a small difference between Pred-$R^2$ and Adj-$R^2$ values (<0.2) and an Adeq Precision value (21.6977) exceeding 4. The quadratic model equations in encoded variable terms accurately predict yeast cell glucose uptake inhibition activity (10 mg/ml). The quadratic model equations were derived for independent variables affecting yeast cell glucose uptake inhibition activity (10 mg/ml) in encoded variable terms

following sequential model sum of squares and model adequacy testing.

Yeast cell glucose uptake inhibition activity (10 mg/ml): 37.13 + 2.65*A – 5.79*B + 7.68*C – 5.42*A*B – 2.25*A*C – 2.01*B*C – 2.01*$A^2$ – 6.41*$B^2$ + 7.43*$C^2$

The obtained model was used to generate three-dimensional surface plots for the yeast cell glucose uptake test, depicted in Figure 8a-c, showcasing how varying two variables while keeping one constant affects the response. Results were calculated as % inhibition of glucose uptake at a specific concentration. Figure 8a indicates a positive impact on the response with increased extraction time and ethanol percentage, while Figure 8b shows a greater positive effect from increased extraction time compared to the solid/liquid ratio. Figure 8c reveals a negative effect on the response with decreased solid/liquid ratio and lower ethanol levels.

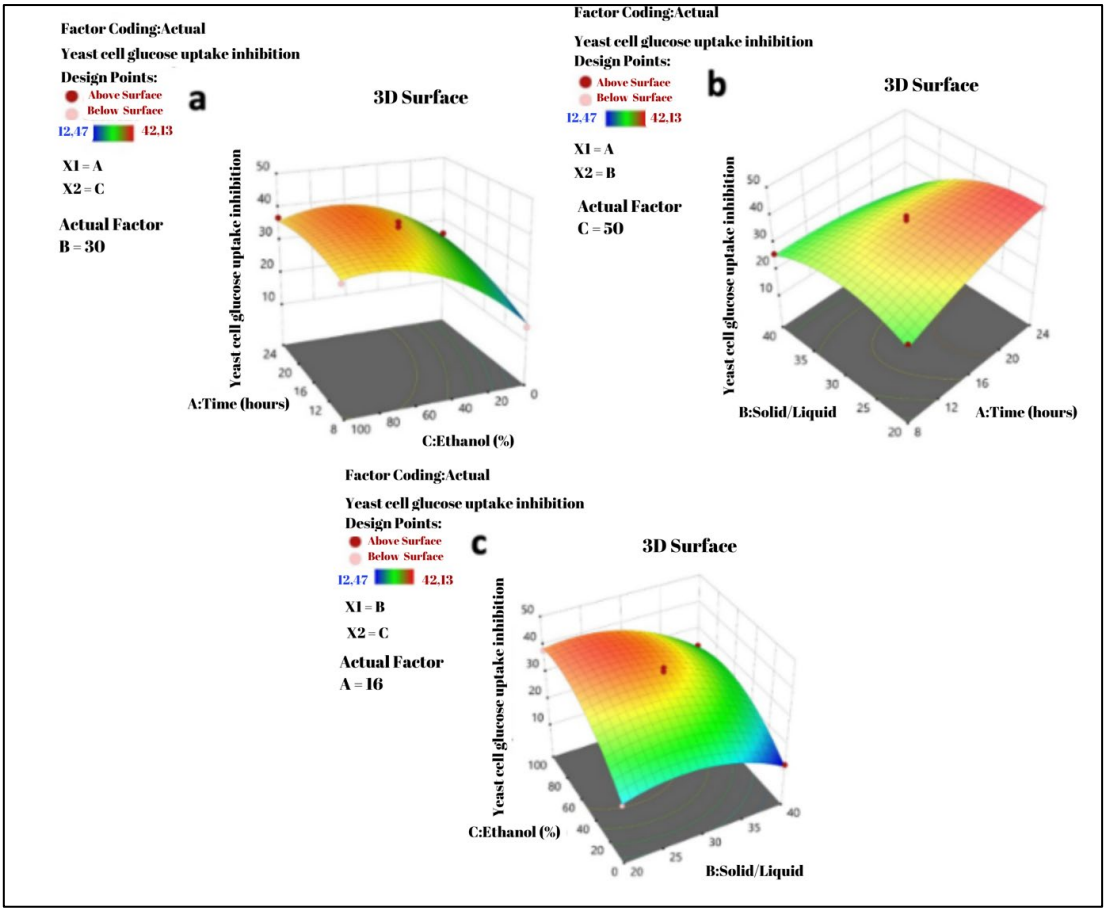


**Figure 8.** Response surface plots for the effects of independent variables on yeast cell glucose uptake activity. (a) A (Time, hours) vs C (Ethanol, %), B (Solid/Liquid Ratio): 30; (b) A (Time, hours) vs B (Solid/Liquid Ratio), C (Ethanol, %):50; (c) B (Solid/Liquid Ratio) vs C (Ethanol, %), A (Time, hours):16.

## DISCUSSION

In the present study, the yield of extraction varied between 0.86% and 22.47% due to changes in extraction process parameters such as ethanol concentration, solid/liquid ratio, and extraction time. Our optimization study resulted in a maximum yield of 22.47% of bioactive compounds from *Ulva* sp. at an ethanol concentration of 0%, a solid/liquid ratio of 1:30, and an extraction time of 24 hours. In the literature, Ulva extraction yield ranging from 13.8% to 26.7% depending on the extraction method and conditions used, was reported. The aqueous mixture of ethanol was found to be the most efficient solvent in the recovery of bioactive compounds, with an extraction yield of 10–15% dry weight (Pappou et al., 2022). The yields obtained by the four extraction methods ranged from 17.88% to 26.77%, and the use of enzymes improved extraction yields, with the maximum yield reaching 26.7% (Juul et al., 2021).

Our results were in accordance with the results reported in the literature. An increase in the yield of extracted protein with the addition of sulfite during protein extraction from *Ulva* sp. was reported (Juul et al., 2021). The extraction yield of Ulva can be optimized by selecting the appropriate extraction method, conditions, and additives such as enzymes or sulfite (Chen et al., 2021).

In the present study, the total polysaccharide content of the extracts varied from 1.03% to 15.42% based on the extraction conditions, with the highest value (15.42%) obtained at an ethanol concentration of 0%, a solid/liquid ratio of 1:20, and an extraction time of 16 hours. Polysaccharides from *Ulva* sp., specifically ulvan, have been extracted using various methods in the literature. Ulvan is a sulfated heteropolysaccharide found in the cell wall of *Ulva* sp. It has been extracted from *Ulva fasciata Delile* collected from the Alexandria coast in Egypt. The ulvan content in *Ulva fasciata Delile* was found to be 43.66% of the total carbohydrate, with a sulfate content of 20.45% (Barakat et al., 2022). *Ulva lactuca* collected from the Alexandria coastline in Egypt also yielded ulvan, with a polysaccharide content of 36.50 g/100 g and a sulfate content of 19.72% (Ibrahim et al., 2022). The extraction of ulvan from *Ulva lactuca* biomass has been optimized using different solvents, with the most efficient solvent being an ethanol/water mixture (Pappou et al., 2022). The extraction parameters, such as time, temperature, and biomass-to-solvent ratio, have been investigated to optimize the extraction yield and antioxidant activity of ulvan (Ning et al., 2022). Ulvan has also been used to synthesize an ulvan/chitosan biomembrane with potential applications in the biomedical field (Ben Amor et al., 2023).

In the present study, the total phenol content of the extracts varied from 18.4 to 82.15 mg GAE/g Dry Extract based on the extraction conditions, with the highest value (82.15 mg GAE/g Dry Extract) obtained at an ethanol concentration of 100%, a solid/liquid ratio of 1:20, and an extraction time of 16 hours. The total phenolic content of the extracts was primarily influenced by the ethanol concentration, with extraction time and solid/liquid ratio having lesser impacts. *Ulva lactuca* and *Ulva linza* macroalgae have been studied for their potential in polyphenol extraction by several researchers. The aqueous mixture of ethanol was found to be the most efficient solvent for extracting bioactive compounds from *Ulva lactuca*, with an extraction yield of 10-15% dw (Pappou et al., 2022). *Ulva linza* extract showed anti-inflammatory effects in TNBS-induced colitis mice, suggesting its potential as a natural therapeutic agent for inflammatory bowel disease (Kim et al., 2018). Another study investigated the protective effect of *Ulva lactuca* polyphenolic extract against heavy metal mixture-induced cardiovascular diseases (Nabil-Adam and Shreadah, 2021). The extract showed antioxidant and anti-inflammatory activities, which were attributed to its high polyphenolic content. A case study explored different methods for quantifying the total phenolic content of *Ulva intestinalis*, including quantitative NMR, HPLC-DAD, and the Folin-Ciocalteu assay (Wekre et al., 2019). Their results showed variations in the quantification of polyphenols, highlighting the challenges in accurately measuring total polyphenolic content (Wekre et al., 2019). Additionally, protein extraction from *Ulva* sp. using double screw pressing and sulfite treatment resulted in improved protein quality, possibly due to inhibited oxidative reactions and improved polyphenol levels (Juul et al., 2021). Finally, pyrolysis of *Ulva lactuca* was investigated for bio-oil production, and the results showed an increase in phenolic compounds with increasing temperature, indicating the potential for producing renewable phenolic resins (Amrullah et al., 2023).

The present study investigated the total protein content of Ulva extracts, ranging from 49.19 to 106.88 mg BSA/g Dry Extract based on the extraction conditions, with the highest value (106.88 mg BSA/g Dry Extract) achieved at an ethanol concentration of 100%, a solid/liquid ratio of 1:30, and an extraction time of 24 hours. Notably, the total protein content of the extracts was significantly influenced by the ethanol concentration, extraction time, and solid/liquid ratio, with ethanol concentration and extraction time showing the most significant effects. In the literatüre various methods have been explored to enhance protein extraction from Ulva macroalgae. For instance, alkaline pretreatment and ultrasonic-assisted extraction improved protein extraction yield from *Ulva rigida* biomass (Pan–Utai et al., 2022). Another study combined enzymatic cell wall degradation with high-voltage Pulsed Electric Fields (PEF), resulting in higher protein extraction yields compared to individual treatments (Steinbruch et al., 2023). Ionic liquids (ILs) assisted mechanical shear followed by two-phase partitioning or ultrafiltration enabled selective extraction of proteins from *Ulva lactuca* (Suarez et al., 2023). Additionally, alkaline extraction at pH 8.5 and double screw pressing with sulfite addition were found to be effective methods for improving protein yield and quality from *Ulva* sp. (Juul et al., 2021).

In the present study, the total antioxidant capacity of Ulva extracts ranged from 28.22 to 63.63 mg Trolox/g Dry Extract, with the highest value (63.63 mg Trolox/g Dry Extract) achieved at an ethanol concentration of 0%, a solid/liquid ratio of 1:20, and an extraction time of 16 hours. Various extraction methods and parameters have been explored to optimize the extraction process and maximize the yield of bioactive compounds from Ulva macroalgae (Pappou et al., 2022). Ultrasonic-assisted extraction (UAE) was found to be highly effective in extracting antioxidants from *Ulva lactuca* biomass (Rashad et al., 2023). Factors such as extraction solvent, time, and temperature significantly affect the extraction process. The extraction of antioxidants from Ulva macroalgae holds promising potential for application in various industries, including food and medicine (Pan-Utai et al., 2022).

Ulva, a green marine seaweed, has been extensively studied for its antioxidant properties. Different extraction methods, such as microwave-assisted extraction, have been explored to optimize the yield and composition of ulvan, a polysaccharide found in *Ulva* sp. Microwave-assisted extraction has shown improved extraction yield and antioxidant activity of ulvan (Chen et al., 2021; Le et al., 2019). Ulva extracts, rich in polyphenols, have demonstrated significant antioxidant and anti-inflammatory activities in various studies, showcasing their potential applications in mitigating oxidative stress-related diseases (Feng et al., 2020; Nabil-Adam and Shreadah, 2021). Furthermore, enzymatic hydrolysis of sulfated polysaccharides extracted from *Ulva lactuca* has resulted in fractions with

promising antioxidant and antitumor activities, suggesting their potential in pharmaceuticals (Abou El Azm et al., 2019).

The Alpha-glucosidase Inhibition activity ($IC_{50}$) of extracts varied from 0.17 to 1.67 mg/ml, with the lowest value (0.17 mg/ml) achieved at specific extraction conditions. *Ulva* sp. demonstrated α-glucosidase inhibitory activity, suggesting their potential as natural inhibitors for controlling blood glucose levels and preventing diabetes (Vega-Gálvez et al., 2022; Nazarudin et al., 2020). *Ulva reticulata* extracts, particularly the methanolic extract, showed significant antidiabetic activity by inhibiting carbohydrate metabolizing enzymes (Unnikrishan et al., 2022; Tong et al., 2020). Additionally, the aqueous extract of *Ulva fasciata* showed hypoglycemic effects and restored hepatic glycogen content in diabetic rats. These findings suggest that *Ulva* sp., including *Ulva reticulata*, *Ulva lactuca*, and *Ulva fasciata*, have potential as antidiabetic agents (Tas et al., 2011). *Ulva lactuca* oligosaccharides were found to have hypoglycemic effects, potentially through a specific pathway (Chen et al., 2022). Additionally, *Ulva australis* exhibited inhibitory effects on α-glucosidase and α-amylase enzymes, indicating its potential as an anti-diabetic agent (Trentin et al., 2020).

In the present study, the Yeast Cell Glucose Uptake Inhibition activity (inhibition %)) of the extracts varied between 12.47 and 42.13 % based on the extraction conditions, with the highest value (42.13 %) obtained at an ethanol concentration of 50%, a solid/liquid ratio of 1:20, and an extraction time of 24 hours. The ethanolic extract of Ulva demonstrated dose-dependent inhibition of glucose uptake in yeast cells, suggesting its potential for treating type 2 diabetes mellitus (Pitchaipillai and Ponniah, 2016). *Ulva reticulata* extracts, including the methanolic extract showed significant antidiabetic activity and were further analyzed to isolate active fractions (Unnikrishnan et al., 2022). Their study focused on identifying and characterizing compounds in the active fraction of *Ulva reticulata* for their antidiabetic potential. Marine algae, such as *Ulva* sp., have been recognized for their medicinal properties and potential as a source of antidiabetic compounds. This study contributes to the search for natural antidiabetic chemicals and emphasizes the importance of investigating marine algae for their therapeutic benefits (Unnikrishnan et al., 2022).

## CONCLUSION

The optimization study on Ulva extraction processes successfully pinpointed specific conditions for achieving the highest yield and optimal levels of bioactive compounds, polysaccharides, proteins, phenols, antioxidants, and alpha-glucosidase enzyme inhibitors. It emphasized the significant influence of ethanol concentration, solid/liquid ratio, and extraction time on the extraction yield variability of bioactive compounds. Notably, the study highlighted Ulva's potential in managing blood glucose levels and preventing diabetes, showcasing its medicinal properties as a natural source of antidiabetic compounds. Marine algae like *Ulva* sp. hold promise in the quest for natural remedies for diabetes, given their recognized medicinal properties and potential as a source of beneficial compounds.

In conclusion, the multifaceted pharmacological properties of *Ulva* sp. underscore their potential as valuable sources of bioactive compounds with diverse applications in health, nutraceuticals, and pharmaceuticals. With ongoing research and development, *Ulva* sp. hold promise in addressing various health challenges and contributing to the advancement of therapeutic interventions. As the algae industry expands, there's a growing need to extract bioactive compounds efficiently. However, phenomena like algal blooms pose environmental challenges, calling for research to transform this waste into valuable products.

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## AUTHOR CONTRIBUTIONS

Oguz Bayraktar contributed to the idea and design of the study. Material preparation and research were carried out by (Gizem Öder) and (Oğuz Bayraktar). The study was the MS Thesis of Gizem Öder, but she switched to another supervisor, and she waived her rights for the publication of findings resulting from the thesis. The writing and editing of the manuscript were done by Prof. Oğuz Bayraktar`s MS student Beyza Tutku Bıçakçı under his supervision. All authors have read and approved the manuscript.

## CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest or competing interests.

## ETHICAL APPROVAL

Ethical approval is not required for this study.

## DATA AVAILABILITY

All relevant data is inside the article. Additional data sets of. the current study will be provided by the corresponding author upon request.

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