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*Research Article*

# Biomass Production by *Yarrowia lipolytica* from Olive Mill Wastewater: Evaluation of Protein Content

Bilge Sayın[1]*
[1] Department of Gastronomy and Culinary Arts, School of Tourism and Hotel Management, Ardahan University, 75002, Ardahan, Türkiye
* Correspondence: bilgesayin@ardahan.edu.tr

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**Abstract:** Olive mill wastewater (OMW), a by-product of olive oil production, is a promising substrate for microbial applications. This agro-industrial waste offers considerable potential for biomass production as it contains carbon and nitrogen sources that support microbial growth. Additionally, utilization of this waste contributes to environmentally sustainable waste management. Utilizing OMW as a growth medium, *Yarrowia lipolytica* offers an alternative to synthetic media, enhancing the economic value of this waste while reducing production costs. In this study, biomass production by *Y. lipolytica* from OMW was optimized using the Taguchi method. The experimental design evaluated the effects of the OMW dilution rate (20, 40, and 60%), nitrogen concentration (0, 1, and 2 g/L), and incubation time (3, 5, and 7 days). Nine experiments were conducted using the L9 orthogonal array, and significant factors were identified using analysis of variance (ANOVA). Among these variables, nitrogen concentration significantly influenced biomass production ($p < 0.05$). The highest biomass concentration of 11.58±0.93 g/L was achieved, and the optimum conditions were found as OMW dilution rate of 60%, 2 g/L nitrogen addition, and 5 days of incubation. Under these conditions, the protein content of the biomass was determined as 25.88±0.63% w/w biomass dry weight.

**Keywords:** Olive mill wastewater, biomass, *Yarrowia*, waste management, Taguchi

*Araştırma Makalesi*

# Zeytinyağı Değirmeni Atıksuyundan *Yarrowia lipolytica* ile Biyokütle Üretimi: Protein İçeriğinin Değerlendirilmesi

**Öz:** Zeytinyağı üretiminin bir yan ürünü olan zeytin değirmeni atık suyu (ZDA), mikrobiyal uygulamalar için umut vadeden bir substrattır. Bu tarımsal-endüstriyel atık, mikrobiyal büyümeyi destekleyen karbon ve azot kaynakları içerdiğinden biyokütle üretimi için önemli bir potansiyel sunmaktadır. Ayrıca, bu atığın kullanımı çevresel olarak sürdürülebilir bir atık yönetimine katkıda bulunmaktadır. ZDA'yı bir büyüme ortamı olarak kullanan *Yarrowia lipolytica*, sentetik ortama bir alternatif sunarak bu atığın ekonomik değerini artırırken üretim maliyetlerini düşürmektedir. Bu çalışmada, *Y. lipolytica* tarafından ZDA'dan biyokütle üretimi Taguchi yöntemi kullanılarak optimize edilmiştir. Deneysel tasarım, OMW seyreltme oranının (%20, 40 ve 60), azot konsantrasyonunun (0, 1 ve 2 g/L) ve inkübasyon süresinin (3, 5 ve 7 gün) etkilerini değerlendirmiştir. L9 ortogonal dizilimi kullanılarak dokuz deney yürütülmüş ve önemli faktörler varyans analizi (ANOVA) kullanılarak belirlenmiştir. Bu değişkenler arasında azot konsantrasyonu biyokütle üretimini önemli ölçüde etkilemiştir ($p < 0,05$). En yüksek biyokütle konsantrasyonu 11,58±0,93 g/L olarak elde edilmiş ve optimum koşullar %60 OMW seyreltme oranı, 2 g/L azot ilavesi ve 5 gün inkübasyon olarak bulunmuştur. Bu koşullar altında, biyokütlenin protein içeriği %25,88±0,63 w/w biyokütle kuru ağırlığı olarak belirlenmiştir.

**Anahtar Kelimeler:** Zeytin değirmeni atık suyu, biyokütle, *Yarrowia*, atık yönetimi, Taguchi

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## 1. Introduction

Olive mill wastewater (OMW) is the primary effluent generated during olive oil processing [1]. Global OMW production is estimated to reach approximately $1 \times 10^7$ m³ annually, with the majority originating in the Mediterranean region [2]. OMW poses an environmental challenge owing to its high organic content, which has a substantial impact on the quality of the natural ecosystems where it is discharged. The elevated organic load of OMW necessitates considerable oxygen consumption, as indicated by its chemical oxygen demand (COD) and biological oxygen demand (BOD), resulting in eutrophication of surface waters [3]. In addition, OMW can affect soil quality and negatively influence the growth of trees, plants, and terrestrial grasses [4].

The concept of microbial proteins, commonly referred to as single-cell protein (SCP), has been recognized for a long time. The production of SCP offers distinct advantages over plant- and animal-based sources such as short generation times, the ability to manipulate metabolism and composition, and independence of climatic conditions. Also, it has the potential to significantly reduce the environmental footprint, including land use, water consumption, and greenhouse gas emissions, compared to animal protein production [5]. The global demand for proteins continues to rise, and advancements in the food processing sector are expected to enhance this significance. Notably, the SCP market is projected to surpass $18.5 billion by 2030, underscoring its significance as a sustainable protein source [6]. Reducing the production cost of SCPs largely depends on the selection of inexpensive and suitable substrates. Utilizing biodegradable agro-industrial byproducts as nutrient sources is a cost-effective approach for supporting microbial growth and facilitating large-scale protein production [7].

Microorganisms (algae, yeast, fungi, and bacteria) can produce large amounts of SCP [8], and this production ensures that dried cells serve as a valuable source of protein for both human nutrition and animal feed applications. Moreover, the dry matter of yeast contains protein ranging 5-70%, and is characterized by a favorable amino acid profile [9]. *Yarrowia lipolytica* is a non-conventional dimorphic yeast. Its unique metabolic pathways enable growth on various carbon sources, making it suitable for industrial applications [10]. *Y. lipolytica* is classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA, USA). This classification is important for the use of microorganisms in food and feed applications [11]. *Y. lipolytica* strains are well-suited for OMW resource recovery. These strains can grow on OMW, effectively degrade lipids and polyphenols, consume organic matter, and simultaneously produce biomass and other valuable by-products [12].

Previous studies have explored single-cell biomass [13], [14], [15], [16], [17] and SCP production [18], [19] using OMW. Nonetheless, studies on SCP from OMW, particularly those involving *Y. lipolytica*, are still considered to be relatively limited. Therefore, in this study, considering the diversity of chemical substances used in synthetic media for obtaining biomass by *Y. lipolytica* NRRL YB-423, as well as the economic aspects, the potential for substituting these substances with OMW, a sustainable waste resource, was investigated. For this purpose, the biomass concentration was optimized using the Taguchi statistical method according to the OMW dilution ratio (20, 40, and 60%), nitrogen concentration (0, 2, and 4 g/L), and incubation time (3, 5, and 7 days). Finally, the protein content of the biomass obtained as a result of production under optimum conditions was evaluated.

## 2. Material and methods

### *2.1. Microorganism*

*Yarrowia lipolytica* NRRL YB-423 (ATCC 18942) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The yeast cells were activated in malt extract broth at 28°C for 24-48 h. The strain was stored in 50% (v/v) glycerol at -80°C.

### *2.2. Sample preparation*

The OMW used in this study was sourced from an olive oil production facility in İzmir, Türkiye, which had a three-phase extraction system. The samples were stored at -18°C to preserve their composition. The OMW samples were thawed and solid particulates were separated by centrifugation at 3000 × *g* for 10 min and used for analysis (Hettich, Universal 320 R; Germany).

### *2.3. Key parameters of OMW*

The centrifuged OMW samples were filtered through a 0.45 μm syringe filter (ISOLAB, Germany), and chemical oxygen demand (COD) was measured using Merck Spectroquant kits (Merck, Germany) with a Move 100 colorimeter (Merck, Germany). The absorbance of the samples was measured at a wavelength of 475 nm using a UV–Vis spectrophotometer (UV-1600 PC, VWR) for color determination [20]. The samples were diluted 100-fold for both the analyses. Finally, pH was measured using a pH meter (Mettler Toledo Ion S220; Griefensee, Switzerland).

### *2.4. Culture conditions*

Biomass production experiments were conducted using OMW as the primary substrate, with only nitrogen (according to the experimental design) and glucose supplementation (30 g/L). Ammonium chloride ($NH_4Cl$) was used as nitrogen source. The culture medium was sterilized in an autoclave at 121°C for 15 min to ensure aseptic conditions. Shake-flask fermentation experiments were performed in 250 mL Erlenmeyer flasks, each containing 50 mL of sterilized culture medium. The pH of the culture medium was not adjusted. The medium was inoculated with 1 mL of an exponentially growing pre-culture containing approximately $10^6$ cells/mL. Following inoculation, the cultures were incubated in a rotary shaker (Mikrotest, MSC 55, Türkiye) at 28°C with a shaking speed of 180 rpm.

### *2.5. Determination of biomass concentration*

After incubation, the samples were centrifuged at 3000 × *g* for 10 min. Cell pellets were collected, washed twice with distilled water to remove residual medium components, and subsequently dried at 80°C until a constant weight was achieved. The dried pellets were weighed to obtain the final dry cell weight [21].

### *2.6. Protein content of biomass*

Protein content was determined according to the Kjeldahl method by digestion, distillation, and acid-base titration using a Behrotest digestor and distiller (Behr Labor-Technik, Germany). The protein amount (%) was determined by multiplying the measured nitrogen content by a nitrogen-to-protein conversion factor of 6.25.

### *2.7. Optimization of biomass concentration*

The experimental results are expressed as signal-to-noise (S/N) ratios, where the “signal” represents the mean for the output and “noise” indicates the variability or deviations from the target values. Taguchi’s S/N analysis classifies response characteristics into three main categories: “smaller-the-better”, “larger-the-better”, and, “nominal-the-best” ensuring flexibility across various optimization scenarios. The factor levels with the highest S/N ratio are considered optimal [22]. In this study, experimental combinations were selected using an L9 orthogonal test table to identify optimal pretreatment conditions. The factors and their levels are listed in Table 1.

**Table 1.** Factors and levels for biomass concentration

| Factors | Level 1 | Level 2 | Level 3 |
|---|---|---|---|
| Dilution rate (%) | 20 | 40 | 60 |
| Nitrogen concentration (g/L) | 0 | 1 | 2 |
| Incubation time (days) | 3 | 5 | 7 |

All experiments were conducted in duplicates. Analysis of variance (ANOVA), was performed to evaluate the significance of the tested parameters. The combined use of S/N ratios and ANOVA facilitated the identification of the optimal process conditions and provided insights into the impact of each variable on the outcome.

The Taguchi method is widely used in biotechnological applications owing to its effectiveness in optimizing complex processes with a minimal number of experiments. Through the application of orthogonal array designs, this method facilitates the analysis of multiple factors and their interactions, without the need for comprehensive full-factorial experimentation. Its strength lies in minimizing process variability and

determining optimal conditions using S/N ratio analysis, making it highly suitable for reducing inconsistencies, enhancing output, and achieving specific target parameters.

## 3. Results and discussion

### *3.1.Characterization of OMW*

The pH of the undiluted OMW was 4.50±0.01. The COD concentration was measured as 635±7.07 mg/L, and the color absorbance was recorded as 0.105±1.41 at 475 nm. Lucas and Peres [23] studied an OMW sample with similar COD value. Lopes et al. [12] used OMW with a much lower COD value (19,584 mg/L). Jamrah et al. [24] stated that COD concentrations depend on the extraction method and the country of origin. Generally, the COD value ranges between 80,000 and 200,000 mg/L in the OMW samples [11]. The COD levels of the OMW sources used in this study were below this range.

### *3.2. Process optimization*

The experimental design, biomass production, and S/N ratio are presented in Table 2. As can be seen from the table, for the samples without nitrogen, the biomass concentration remained low and comparable level, regardless of the OMW dilution rate and incubation time (0.39-0.51 g/L). However, the highest biomass concentration was obtained after 5 days of incubation when the culture medium contained 60% diluted OMW, supplemented with 2 g/L $NH_4Cl$, reached in a value of 11.58±0.93 g/L. Under these conditions, the protein content of the biomass was determined as 25.88±0.63% w/w biomass dry weight.

For effective protein production, the protein content within single-cell organisms typically ranges from to 39-73% [25]. Although *Y. lipolytica* is compatible with these conditions, further optimization is required to improve its productivity and/or protein content for enhanced efficiency. On the other hand, the findings demonstrated the feasibility of employing OMW as a cost-effective and sustainable substrate for SCP production via microbial biomass production.

**Table 2.** Experimental desing and results of biomass concentration and S/N ratio

| Run | Dilution rate (%) | Nitrogen concentration (g/L) | Time (days) | Biomass concentration (g/L) | S/N ratio |
|---|---|---|---|---|---|
| 1 | 20 | 0 | 3 | 0.39±0.10 | -8.1121 |
| 2 | 20 | 1 | 5 | 5.40±0.91 | 14.6479 |
| 3 | 20 | 2 | 7 | 9.93±0.95 | 19.9381 |
| 4 | 40 | 0 | 5 | 0.51±0.11 | -5.9342 |
| 5 | 40 | 1 | 7 | 8.30±1.57 | 18.3816 |
| 6 | 40 | 2 | 3 | 5.52±0.13 | 14.8309 |
| 7 | 60 | 0 | 7 | 0.47±0.09 | -6.5212 |
| 8 | 60 | 1 | 3 | 6.75±0.05 | 16.5899 |
| 9 | 60 | 2 | 5 | 11.58±0.93 | 21.2742 |

Previous studies using OMW supplemented with additional medium components resulted in lower or similar biomass concentrations compared with our study [1], [10], [13], [14], [19]. The microorganisms used for SCP should have rapid growth, minimal nutritional needs, easy processing, nonpathogenic and non-toxic properties, low nucleic acid content, and high biological value [26]. Therefore, the ability of *Y. lipolytica*, recognized as GRAS, to rapidly adapt and grow on OMW supplemented with only 30 g/L glucose and nitrogen concentrations specified in the experimental design, without the addition of any other medium components, was considered significant for this study.

The high cost of synthetic media has limited advancements in microbiology, fermentation, and molecular biology [27]. On the other hand, the treatment of domestic and industrial wastes generated by developed countries requires substantial energy consumption [28]. To effectively address these issues, a circular

economy is designed to maintain a balance between economic growth, resource sustainability, and environmental protection [29]. Therefore, the use of OMW for biomass production is considered a viable option for on-site waste reduction and/or recycling, particularly in Mediterranean countries, because it can serve as a substitute for synthetic media, mitigate environmental issues, and reduce processing costs.

The analysis of variance (ANOVA) results, as presented in Table 3, indicated that among the evaluated factors, nitrogen concentration had the most significant effect on biomass production, with a contribution of 97.36%. In contrast, the dilution rate and incubation time showed minimal contributions, influencing biomass concentration by only 0.37% and 1.08%, respectively. The effect of nitrogen concentration on production was significant ($p < 0.05$), whereas the effects of the other factors were not significant ($p > 0.05$).

**Table 3.** ANOVA for biomass concentration

| Source | DF | Seq SS | Contribution | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|---|
| Dilution rate (%) | 2 | 4.54 | 0.37% | 4.54 | 2.271 | 0.31 | 0.765 |
| Nitrogen concentration (g/L) | 2 | 1204.07 | 97.36% | 1204.07 | 602.036 | 81.52 | 0.012 |
| Time (days) | 2 | 13.33 | 1.08% | 13.33 | 6.665 | 0.90 | 0.526 |
| Error | 2 | 14.77 | 1.19% | 14.77 | 7.385 | | |
| Total | 8 | 1236.71 | 100% | | | | |

DF: Degrees of freedom, Seq SS: Sequential sum of squares, Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean square ($R^2$: 98.81%, $R^2$adj: 95.22%, $R^2$pred: 75.82%)

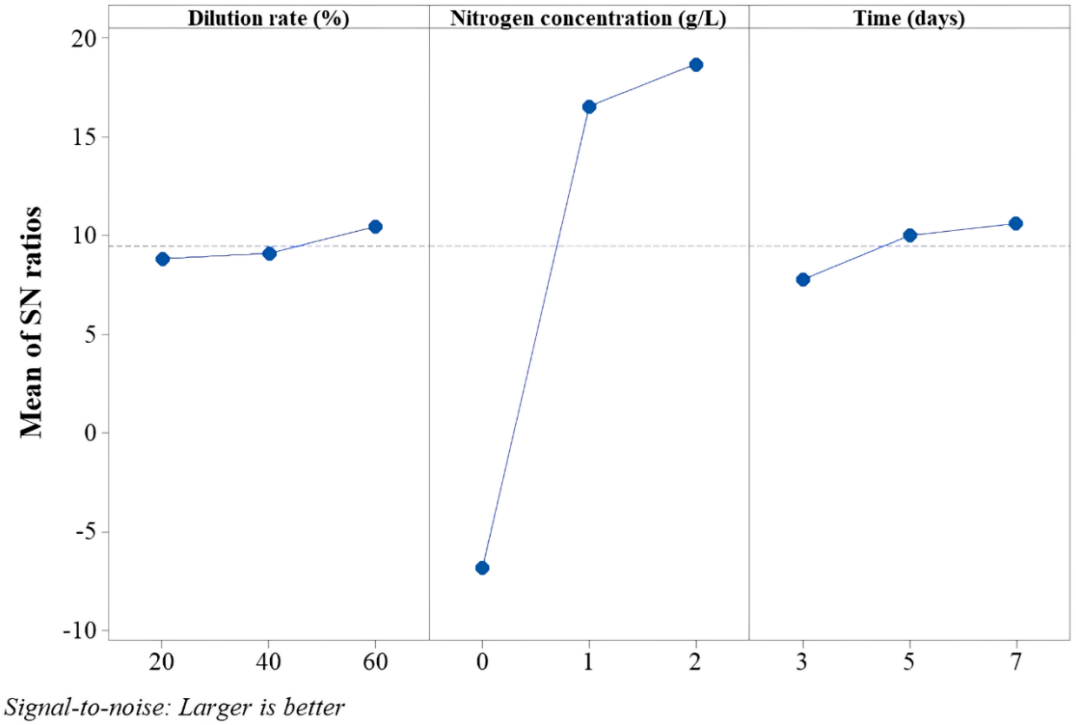


**Figure 1.** The plot of the S/N ratio for biomass concentration

The main effect plots for the mean and signal-to-noise (S/N) ratios are shown in Figure 1. The highest S/N ratio for each control factor was observed at a 60% dilution rate, nitrogen concentration of 2 g/L, and incubation time of 7 days.

Sarris et al. [13] used OMW as a substrate for value-added product production. They determined the highest biomass concentration of 12.7 g/L with *Y. lipolytica* ACA-YC 5028 after 48 h in carbon-limited glucose-based media with various amounts of OMW (initial phenolics 1.23 g/L). Although a similar result was obtained in our study, shorter incubation time was an important factor. Therefore, the composition of the

medium can be further improved. Sarris et al. [30] demonstrated that the addition of OMW in carbon-limited fermentations enhanced biomass production of *Y. lipolytica* ACA-YC 5033. At an initial glucose concentration of approximately 80 g/L, carbon flux was channeled more effectively for biomass synthesis. In this context, different and higher glucose concentrations can be tested in subsequent stages of our study. Carranza-Méndez et al. [31] achieved 15.71 g/L biomass and 6.22% total protein at optimum conditions ($(NH_4)_2SO_4$: 2 g/L, orange peel (% w/v): 10, and 25°C) with *Candida utilis.* In another study, *Y. lipolytica* A-101 was cultured at pH 5.0 and 30°C using biofuel waste as the medium. The protein concentration increased to 8.28 g/L, representing a 44% improvement compared to the initial value of 3.65 g/L [32]. Finally, in a study conducted with *Y. lipolytica* wt A-101 incubated at 28°C, pH 3.5, and 150 rpm for 5 days using shake-flask fermentations, between 30.5-44.5% protein was obtained using rye straw, rye bran, and oat bran as substrates [11].

## 4. Conclusion

The findings of this study emphasize the potential of olive mill wastewater (OMW) as a viable and cost-efficient substrate for microbial biomass production. The optimization process conducted using the Taguchi method revealed that the nitrogen concentration was the most influential factor in biomass yield, with a significant impact on production outcomes. Moreover, the protein content of the biomass can be regarded as significant, both as a contribution to human nutrition and as an animal feed additive. It is believed that more detailed characterization of the OMW will play an important role in determining the production mechanism. Future studies involving scale-up processes, genetic modifications, and further optimization can enhance production. Additionally, investigating the synergistic utilization of OMW in combination with other agricultural by-products could further enhance biomass production and improve process yield. For example, incorporating glucose derived from waste sources into the culture medium would make this process more effective. These results underscore the value of incorporating OMW into biotechnological applications, presenting the dual advantage of resource recovery and waste valorization. This approach not only improves the economic feasibility of microbial biomass production but also contributes to environmental sustainability.

### Conflicts of Interest

The author declares no conflict of interest.

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