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RESEARCH ARTICLE

Enhanced Lipid Yield from Olive-Mill Wastewater by *Yarrowia lipolytica* NRRL YB-423

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ABSTRACT

Lipid production from olive-mill wastewater (OMW) by *Yarrowia lipolytica* NRRL YB-423 was optimized (biomass concentration and lipid yield based on dry cell weight) using multi-response criteria based on the Taguchi orthogonal array. Sixteen experimental runs were performed using the L16 orthogonal array. Dilution rates of OMW (15, 30, 45, and 60%), Tween 80 (0, 0.2, 0.4, and 0.6%), sodium chloride (NaCl; 0, 1, 2, and 3%), and sterility were selected as factors. The significance of the parameters was determined using analysis of variance (ANOVA). The effects of all factors on the lipid yield were statistically significant (p<0.05). The results showed that sterility had a maximum contribution of 48.12% to lipid yield. The highest lipid yield (40.88 %) was achieved in sterile medium supplemented with 15% diluted OMW, 0.6% Tween 80, and 3% NaCl.

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1. Introduction

The global annual olive oil production is approximately 3.32 million, predominantly concentrated in Europe, accounting for 72% of the total. The primary producers are Spain (42%), Italy (17%), and Greece (11%), whereas other Mediterranean countries such as Türkiye (6%), Syria (6%), Tunisia (6%), Morocco (4%), Jordan (3%), and Lebanon (1.5%) contribute to the remaining production share (Khdair & Abu-Rumman, 2020). Olive oil is obtained from olives using physical methods such as crushing, malaxation, and oil phase separation. These steps are entegrated to three methods: traditional discontinuous pressing, continuous three-phase horizontal centrifuge systems, and continuous two-phase horizontal centrifuge systems. The first two processes necessitate the use of hot water, resulting in the generation of substantial quantities of liquid waste, commonly known as olive mill wastewater (OMW). The two-phase system separates olive pulp into oil and wet solid residue (Abrunhosa et al., 2013). Approximately 20% of the final product is in the oily phase, with the remainder comprising of solid waste (approximately 30%) and OMW (up to 50%) (Alique et al., 2020).

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OMW elevated organic content necessitates considerable oxygen consumption, resulting in surface waters eutrophication. Additionally, OMW diminishes soil quality by inducing water repellence, which inhibits seed germination and plant growth (Ayadi et al., 2022). Direct disposal of OMW into sewage systems or its use as an organic fertilizer in agriculture is not advisable because of its potential to disrupt the efficiency of sewage treatment plants and adversely affect microbial soil properties. On the other hand, the phytotoxic and antibacterial effects of OMW are primarily due to its polyphenolic content. Therefore, biological degradation of OMW phenolic compounds is regarded as a safer, more effective, and less costly approach for pollutant reduction than other methods (Dahmen-Ben Moussa et al., 2021). The phenolic content of OMW varies widely based on the olive variety, maturity, and oil extraction technology (Diamantis et al., 2022).

Biodiesel has attracted increasing attention as an alternative to petroleum-based fuel, driven by concerns over the energy crisis and the environmental consequences of fossil fuel combustion. Biodiesel, which is derived from renewable sources such as vegetable oils, animal fats, and waste oils, is a biodegradable and non-toxic biofuel. Moreover, microorganisms are now recognized as novel sources of lipids, known as "second-generation biodiesel" (Arous et al., 2016). Microbial oils are lipids synthesized by oleaginous microorganisms. Conventionally, bacteria, yeasts, molds, and microalgae capable of accumulating lipids exceeding 20% of their dry weight are classified as oleaginous microorganisms (Huang et al., 2013). Microbial lipids exhibit unique compositions and structures that are useful in the highly interesting to food and pharmaceutical industries (Sarris et al., 2019). Considering the depletion of crude oil, the "food-or-fuel" debate regarding plant oils for biodiesel production, overfishing, and the need to reduce greenhouse gas emissions, microbial lipids offer promising alternatives to crude oil, plant oil, and fish oil. Microbial lipid production is independent of season, climate, and location (Ochsenreither, 2016).

An estimated 10-30 m^3 million OMW are produced globally annually (Dias et al., 2021). Bacteria typically struggle to break down the complex polyphenols responsible for OMW's dark coloration of OMWs. Filamentous fungi are constrained by the challenges of obtaining a homogeneous culture and long fermentation cycles. In contrast, yeasts are promising because of their adaptability and resistance to high phenolic concentrations and low pH. OMW contains essential carbohydrates, proteins, and minerals, which provide the necessary components for fermentation (Benhoula et al., 2023). *Yarrowia lipolytica*, an oleaginous yeast, has been extensively studied owing to its fully sequenced genome and well-known metabolism (Fabiszewska et al., 2019). *Y. lipolytica* is commonly employed in OMW bioremediation systems because of its high capacity to catabolize polyphenols and produce value-added products (Hamimed et al., 2021). This study focused on optimizing lipid production from OMW using *Y. lipolytica* NRRL YB-423. To achieve this objective, the Taguchi method was employed to systematically investigate the effects of the OMW dilution ratio, amount of Tween 80 and sodium chloride (NaCl), and sterility on the biomass concentration and lipid yield.

2. Materials and Methods

2.1. OMW, Microorganism and Culture Conditions

OMW was supplied by a local olive oil production plant located in İzmir, Türkiye, which has a three-phase olive oil extraction process. The samples were stored at -18°C until analysis. Before analysis, the samples were defrozen, and the solid parts were removed by centrifugation at 5000 rpm for 10 min (Thermo Fisher MR23I, Germany). *Yarrowia lipolytica* NRRL YB-423 (ATCC 18942) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Lipid production experiments were conducted using a modified culture medium with the following composition (g/L): glucose 35, KH_2PO_4 7, Na_2HPO_4 2.5, $CaCl_2.2H_2O$ 0.15, $MnSO_4.H_2O$ 0.06, $ZnSO_4.7H_2O$ 0.02, $FeCl_3.6H_2O$ 0.15, and $MgSO_4.7H_2O$ 1.5 (Sarris et al., 2014). The culture medium was sterilized in an autoclave at 121°C for 15 min. Shake-flask experiments were carried out in 250 mL Erlenmeyer flasks, each containing 50 mL of culture medium. The medium was inoculated with 1 mL of pre-culture containing 10^6 cells. After inoculation, the cultures were incubated on a rotary shaker (JSSI-100; JS Research, Gongju, Korea) at 28°C and 180 rpm. The initial pH value of fermentation medium was 6.5, and the fermentation lasted for 6 days.

2.2. OMW Key Indicators: pH Level, Phenolic Content, Color, and Salt

The pH level of OMW was measured using a pH meter (Mettler Toledo, Switzerland). The phenol concentration in OMW was determined based on the method described by Qwele et al. (2013). Briefly, 0.1 mL of the sample was mixed with 0.2 mL of Folin-Ciocalteu reagent and 3 mL of 5% Na_2CO_3 solution. The reaction mixture was then vortexed and kept at 23°C for 1 h. Finally, the absorbance of the phenolic compounds in 10-fold diluted sample was measured at 765 nm using a UV-visible spectrophotometer (Aquamate 9423 AQA 2000E, Thermo Scientific, England). Phenolic concentrations were quantified using a standard curve prepared with gallic acid and expressed as gallic acid equivalents per liter of OMW. The color of OMW was measured at 395 nm using a UV-visible spectrophotometer. As the absorbance of the undiluted sample exceeded the measurable limit, measurements were conducted using 25-fold diluted samples (Flouri et al., 1996). The NaCl content of the OMW samples was analyzed using the Volhard method (ISO 1841-1, 1996).

2.3. Determination of Biomass Concentration

Dry cell weight was determined to calculate biomass production. For this purpose, the culture broths were centrifuged (Thermo Fisher MR23I, Germany) at 5000 rpm for 10 min. The cell pellets were washed twice with distilled water, dried to a constant weight at 80°C, and then weighed (Chatzifragkou et al., 2011).

2.4. Total Lipid Production

In the modified method combined with mechanical disruption, the biomass was treated with 8 mL of 4 M HCl solution and 0.7 mm diameter glass beads, followed by waited in an ultrasonic bath at 60°C for 2 h (Kuttiraja et al., 2016; Yu et al., 2015). The acid-hydrolyzed biomass was stirred in 16 mL of a chloroform/methanol mixture (1:1) for 2-3 h at room temperature. Finally, centrifugation was applied at 5000 rpm for 5 min to separate the aqueous upper phase from the organic lower phase. The lipid-containing sub-phase was then removed, and the solvents were evaporated at 40°C in a vacuum rotary evaporator (Buchi Heating Bath B-490, Switzerland) (Enshaeieh et al., 2014). Lipid content was determined as the weight (g) of lipid produced per liter of medium and lipid yields were calculated based on the dry weight of the cells (El-Fadaly et al., 2009).

2.5. Statistical Analysis

The primary reason for choosing the Taguchi method is its ability to simultaneously examine multiple parameters under a limited number of experimental conditions. The Signal-to-Noise (S/N) ratio is a statistical measure used to determine the optimal levels of the control factors by minimizing variability and maximizing performance. This reflects the robustness of a process by evaluating the relationship between the desired signal (mean performance) and undesired noise (variability). The factors and levels were selected to optimize the biomass concentration and lipid yield, as shown in Table 1. The experimental design was created by using a mixed-level design option in the Taguchi model [$L16(4^3 \times 2^1)$]. Experiments were conducted in duplicate under the conditions outlined in the experimental design. Analysis of variance (ANOVA) was used to determine the statistical significance of the experimental parameters. The experiments were designed using the Taguchi method in Minitab (version 19; State College, PA, USA). The experimental results were presented as the mean ± standard deviation.

Table 1. Factors and levels for the process optimization.

Factors	Level 1	Level 2	Level 3	Level 4
Dilution rate (%)	15	30	45	60
Tween 80 (%)	0	0.2	0.4	0.6
NaCl (%)	0	1	2	3
Sterility	0	1		

3. Results and Discussion

3.1. Physicochemical Analysis of OMW

Physicochemical analyses conducted for the characterization of OMW, along with their corresponding results, are shown in Table 2. The chemical composition of OMW varies significantly based on factors such as the olive variety, growing method, harvest timing, and oil extraction method (Roig et al., 2006). Ochando-Pulido et al. (2017) reported that pH values in three-phase and two-phase extraction processes range between 3.5-5.5 and 3.5-6.0, respectively. These results clearly indicated that the OMWs exhibited acidic pH levels, primarily owing to the presence of organic acids. The pH levels are also influenced by olive ripeness and storage conditions after harvest. In contrast, the standard methods of olive processing do not affect pH levels (Barbera et al., 2013). The phenolic compound concentration in OMW varies from 0.5 to 24 g/L (Paraskeva & Diamadopoulos, 2006). Previous studies have reported significantly higher phenol contents (Buchmann et al., 2015; Dourou et al., 2016; Sarris et al., 2013) and lower pH values (Aggoun et al., 2016; Paredes et al., 1999; Sarris et al., 2023a,b). The mineral salt content of OMW typically ranges from 0.4% to 2.5%, as reported by Fattoum et al. (2023). However, in our sample, the mineral salt content was observed to be lower than this range.

Table 2. Characterization of OMW.

Parameter	Results
pH	6.50±0.05
Salt (%)	0.27±0.01
Color	0.22±0.00
Phenol content (mg/L gallic acid)	557.20±7.06

Data are mean values ± standard deviation.

3.2. Optimization of Lipid Production

Yeasts are capable of degrading phenols, exhibiting resistance to phages, and demonstrating greater tolerance to high osmolarity, pH, and solvents (Singh et al., 2022). OMW is characterized by a high content of phenolic compounds, which have antioxidant and antibacterial activities (Roila et al., 2024). For this reason, one of the simplest methods to reduce the initial phenolic content is to dilute OMW to decrease its antimicrobial effect in the fermentation medium. In this study, different OMW dilution rates were evaluated in terms of biomass and lipid production by *Y. lipolytica* NRRL YB-423. On the other hand, Tween 80 is a widely recognized non-ionic polyoxyethylene detergent and surfactant (Sipiczki et al., 2024). Tween 80 is used as a vehicle for the addition of water-insoluble compounds. It can alter cell membrane permeability, thereby facilitating the uptake of nutrients from the environment into the cells (Taoka et al., 2011). In this study, we investigated the effects of different Tween 80 amounts on biomass and lipid production. Moreover, NaCl was added to the

OMW-based medium to evaluate the formation of biomass and lipids under osmotic stress. Finally, considering the advantages of non-sterile production, particularly on an industrial scale, the effects of this factor on production under various conditions were investigated.

Table 2 shows biomass and lipid concentrations, lipid yields, and S/N ratios. When the biomass level exceeded 1 g/L, it was determined that this occurred under non-sterile conditions, likely due to contamination, which did not cause an increase in lipid yield. The highest lipid yield (40.88 %) was observed in a sterile medium containing 15% diluted OMW, 0.6% Tween 80, and 3% NaCl. The second and third highest lipid yield were 38.40% (with 0.2% Tween 80) and 32.34% (with 1% NaCl), respectively, under sterile conditions, but with 30% diluted OMW. The S/N ratio should be at the maximum level for optimum conditions to minimize the effect of noise. As shown in Table 2, the S/N ratio was the highest under the conditions where the highest biomass concentration and lipid yield were achieved.

Table 2. Experimental design and results for biomass and lipid concentrations, lipid yields, and S/N ratios.

Run	Dilution rate (%)	Tween 80 (%)	NaCl (%)	Sterility	Biomass (g/L)	Lipid (g/L)	Lipid content (w/w dry cell %) ($Y_{L/B}$)	S/N ratio for biomass	S/N ratio for lipid production
1	15	0	0	0	1.23±0.14	0.25±0.02	19.97±0.85	1.79810	26.0076
2	15	0.2	1	0	1.31±0.03	0.32±0.01	24.42±0.39	2.34543	27.7549
3	15	0.4	2	1	0.46±0.00	0.11±0.02	24.65±3.98	-6.74484	27.8363
4	15	0.6	3	1	0.49±0.00	0.20±0.01	40.88±1.02	-6.19608	32.2302
5	30	0	1	1	0.38±0.01	0.12±0.01	32.34±2.66	-8.40433	30.1948
6	30	0.2	0	1	0.53±0.00	0.20±0.02	38.40±3.02	-5.51448	31.6866
7	30	0.4	3	0	1.19±0.11	0.21±0.02	17.73±0.01	1.51094	24.9742
8	30	0.6	2	0	1.27±0.03	0.19±0.01	14.98±0.78	2.07607	23.5102
9	45	0	2	0	0.90±0.02	0.09±0.01	9.97±0.68	-0.91515	19.9739
10	45	0.2	3	0	1.21±0.09	0.20±0.01	16.58±0.54	1.65571	24.3917
11	45	0.4	0	1	0.49±0.03	0.13±0.02	25.77±5.08	-6.19608	28.2223
12	45	0.6	1	1	0.42±0.03	0.14±0.01	31.06±1.31	-7.53501	29.8440
13	60	0	3	1	0.37±0.02	0.10±0.01	26.37±2.88	-8.63597	28.4222
14	60	0.2	2	1	0.44±0.01	0.11±0.00	23.97±0.50	-7.13095	27.5934
15	60	0.4	1	0	0.78±0.02	0.20±0.03	25.42±2,46	-2.15811	28.1035
16	60	0.6	0	0	0.83±0.00	0.21±0.02	24.70±2.37	-1.61844	27.8539

The high cost of microbial lipid production is attributed to the low productivity of oleaginous microorganisms and significant energy requirements for medium sterilization. One solution that can decrease the cost of microbial lipid production is to use a non-sterile culture technique, as it can reduce energy consumption, save time, and reduce the required workload (Polburee & Limtong, 2020). Lipid production is typically performed under sterile conditions to prevent microbial contamination. However, non-sterile lipid production can be successfully achieved through careful optimization of various factors. These include adjusting inoculum size and pH, employing carbon source-only or nutrient-starvation media, supplementing with antimicrobial agents, and utilizing metabolic engineering in oleaginous species (He et al., 2025). In a related study, raw glycerol was converted into microbial lipids by *Zygorhynchus moelleri* under non-aseptic conditions, using nitrogen-limited media containing essential oils and/or antibiotics. The findings revealed that lipid accumulation was not affected by the presence of bacteria in the growth medium compared with aseptic conditions (Moustogianni et al., 2014). As highlighted in the aforementioned study, the successful implementation of a lipid-producing bioprocess under non-aseptic conditions requires the presence of inhibitory factors such as high concentrations of NaCl, essential oils, antibiotics, or phenolic compounds (Filippousi et al., 2022). Although OMW was utilized in this study, maintaining sterility proved to be important. This was likely due to the low phenolic and salt content of the OMW and its further dilution, which diminished its ability to inhibit contamination effectively.

Sarris et al. (2011) found that the presence of OMWs in the medium promoted the accumulation of storage lipids in *Y. lipolytica* W29 strain. In another study, mixtures of OMW and crude glycerol were used for lipid production of *Y. lipolytica* LMBF Y-46 and *Y. lipolytica* ACA-YC 5033 and it was reported that $Y_{L/B}$ did not exceed 16.6% (Sarris et al., 2023b). Dourou et al. (2016) reported that *Lipomyces starkeyi* NRRL Y-11557 and *Y. lipolytica* strains accumulated lipids in OMW-based media ($Y_{L/B}$= 15-25%, w/w). Sarris et al. (2019) explored the potential of *Y. lipolytica* ACA-DC 5029 to grow and produce metabolites in crude glycerol and OMW blends with nitrogen-limited submerged shake-flask cultures. The pH of the culture medium was maintained between at 5.0 and 6.0. They

found that the accumulation of microbial oil increased with the addition of OMW (L_{max} ~2.0 g/L, $Y_{L/X}$ ~20% w/w). The $Y_{L/X}$ values remained below 20% despite nitrogen-limited conditions in the growth medium. This result can be attributed to the fact that citric acid production increases at neutral pH levels, whereas lipid production is enhanced under acidic conditions (Zhang et al., 2019). Tzirita et al. (2019) reported that the highest lipid yield (35.1%) was achieved with *Y. lipolytica* ACA-YC 5031 in medium containing glycerol blended with OMW and 5% NaCl, after 308 h. In addition, other studies have reported lipid yields of 60% and above but did not use *Y. lipolytica* (Bellou et al., 2014; Herrero et al., 2018).

The ANOVA results for the biomass concentrations are presented in Table 3. The table shows that among the selected factors, sterility had a stronger influence (87.95%) on the biomass concentration, whereas the amount of NaCl showed the least influence (1.09%). The effects of the dilution rate and sterility were statistically significant (p<0.05). Papanikolaou et al. (2008) enriched OMW-based media with commercial glucose for citric acid and lipid production by *Y. lipolytica* ACA-DC 50109. In contrast, the presence of OMWs in the growth medium had almost no effect on the maximum biomass produced, maximum specific growth rate, and biomass yield on consumed glucose.

Table 3. ANOVA for biomass concentrations.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F	P
Dilution rate (%)	3	16.892	6.38%	16.892	5.631	5.85	0.043
Tween 80 (%)	3	7.333	2.77%	7.333	2.444	2.54	0.170
NaCl (%)	3	2.892	1.09%	2.892	0.964	1.00	0.464
Sterility	1	232.961	87.95%	232.961	232.961	242.11	0.000
Error	5	4.811	1.82%	4.811	0.962		
Total	15	264.890	100%				

DF: Degrees of freedom, Seq SS: Sequential sums of squares, Adj SS: Adjusted sum of square, Adj MS: Adjusted mean square (R^2:98.18%, R^2adj:94.55%).

The ANOVA results (Table 4) showed that among the selected factors, sterility had a stronger influence (48.12%) on lipid yield, whereas the amount of Tween 80 showed the least influence (7.44%). As an important finding, the effects of all selected factors on the process were statistically significant (p<0.05). In contrast to the results obtained in our study, Sarris et al. (2017) found that the addition of OMWs to the medium resulted in the accumulation of lipid reserves of *Y. lipolytica* ACA-YC 5033 and no significant differences were observed between aseptic and pasteurized cultures in terms of biomass and total cellular lipid production.

Table 4. ANOVA for lipid yields.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F	P
Dilution rate (%)	3	18.868	12.98%	18.868	6.2895	10.85	0.013
Tween 80 (%)	3	10.823	7.44%	10.823	3.6075	6.22	0.038
NaCl (%)	3	42.852	29.47%	42.852	14.2839	24.64	0.002
Sterility	1	69.973	48.12%	69.973	69.9729	120.70	0.000
Error	5	2.899	1.99%	2.899	0.5797		
Total	15	145.414	100%				

DF: Degrees of freedom, Seq SS: Sequential sums of squares, Adj SS: Adjusted sum of square, Adj MS: Adjusted mean square (R^2:98.01%, R^2adj:94.02%).

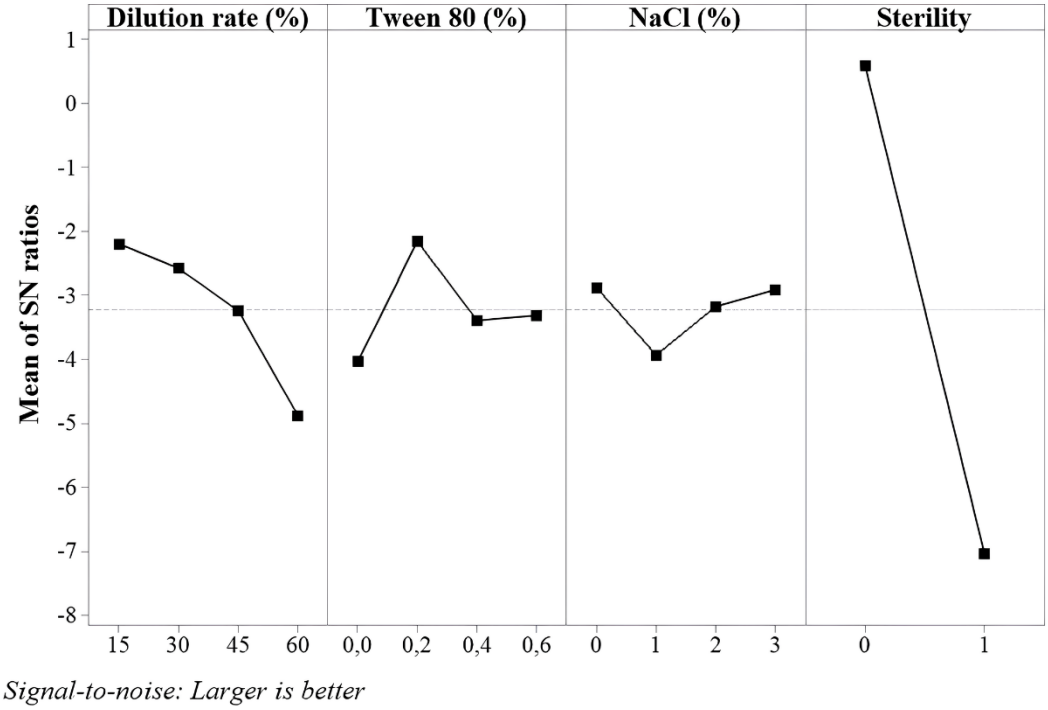

Figure 1. Main effect plot for S/N ratios for biomass concentration.

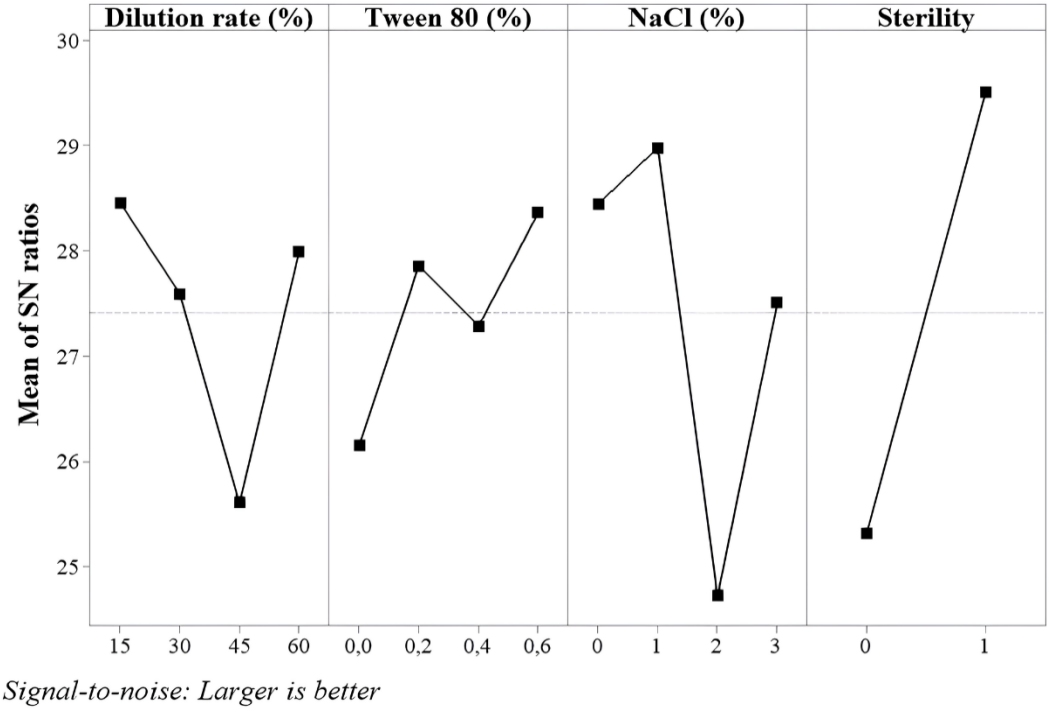

Figure 2. Main effect plot for S/N ratios for lipid yield.

The main effect plots for the S/N ratios of the biomass concentrations and lipid yields are presented in Figure 1 and 2, respectively. The goal of optimizing the process parameters was to improve the S/N ratio for better results. The peak S/N ratio for lipid yield was observed at a 15% dilution rate, 0.6% Tween 80, 1% NaCl, and under sterile process conditions. For biomass concentration, the optimal conditions were 15% dilution rate, 0.2% Tween 80, no NaCl addition, and non-sterile process conditions.

4. Conclusion

The treatment of olive-mill wastewater (OMW), a highly toxic by-product of olive oil production, remains a key environmental challenge, particularly in the Mediterranean

countries. In light of this, the conversion of OMW into value-added bioproducts presents a promising solution for mitigating the environmental impact of its disposal. This study focused on the optimization of biomass and lipid production from OMW using *Yarrowia lipolytica* NRRL YB-423. Through the application of the Taguchi method, the experimental conditions were optimized to maximize the lipid yield, demonstrating the potential of *Y. lipolytica* as an efficient microorganism for biotransformation processes. These results underscore the significance of process optimization in enhancing lipid production from OMW, providing a sustainable approach for both waste management and the generation of valuable bioproducts. Future research could focus scaling up the process and further refining the medium composition to increase efficiency.

Conflict of Interest

The authors have no conflict of interest to declare.

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