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Assessing Waste Sunflower Oil as a Substrate for Citric Acid Production: The Inhibitory Effect of Triton X-100

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Abstract: In this study, waste sunflower oils were evaluated as substrates for citric acid (CA) production by *Yarrowia lipolytica* IFP29 (ATCC 20460). This strain was selected based on its capacity to produce organic acids in a selective medium. Attempts were made to optimize the process using the Taguchi statistical method in terms of the oil polarity, oil concentration, fermentation time, and Triton X-100 concentration. The results indicated that *Y. lipolytica* IFP29 utilized waste sunflower oil as a substrate and produced a maximum CA of 32.17 ± 1.44 g/L. Additionally, Triton X-100 inhibited the production of CA. For this reason, this process could not be optimized. These results were obtained by periodically adjusting the pH with NaOH during the fermentation period. On the other hand, a new experimental design was created without Triton X-100. As a buffering agent, 2-morpholinoethanesulfonic acid monohydrate (MES) was used to prevent a drop in pH; the maximum concentration of CA was found to be 20.31 ± 2.76. The optimum conditions were as follows: 90 g/L of waste sunflower oil with a polarity of 16 and 12 days of fermentation. According to the analysis of variance results, the effects of factors other than polarity on CA production were found to be significant ($p < 0.05$).

Keywords: citric acid; *Yarrowia lipolytica*; isocitric acid; Taguchi; waste oil; FTIR; fatty acids

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1. Introduction

Approximately 80% of vegetable oils produced are partitioned for human consumption [1]. Therefore, large quantities of waste cooking oil (WCOs) are generated daily from various sources, including the food processing industry, fast-food establishments, restaurants, and households. Bio-refineries offer the potential for achieving "zero waste" and promoting "green chemistry" by encouraging the recycling and utilization of waste and by-products through eco-friendly processes. The removal of WCOs poses an environmental challenge that can be addressed by using them for biodiesel production or as bio-lubricants [2]. Utilizing WCOs directly as feedstock for microbial processes presents an opportunity to reduce the production costs of valuable compounds and enhance the economic value of these wastes, which are hazardous to the environment. Certain species of yeast, fungi, and bacteria can utilize WCOs as carbon and energy sources and convert them into metabolites of added value [3].

Citric acid (CA) is the second-largest fermentation product produced by tonnage after ethanol production [4]. Citric acid is used in the food, pharmaceutical, chemical, and metallurgical industries because of its nontoxic nature and ability to chelate and sequester metal ions [5]. According to the Global Citric Acid Market Outlook (2023), the global CA market reached approximately 2.59 million tons in 2022; approximately 70% of the production is used in the food industry, 12% in pharmacological preparations, and 18% in

technical applications. Furthermore, production is expected to reach 3.29 million tons by 2028 [6]. CA is an intermediate product of the tricarboxylic acid cycle [7]. *Y. lipolytica* is one of the most commonly used yeast species in CA production via fermentation [8]. However, the biggest disadvantage of *Yarrowia lipolytica* production is the production of isocitric acid (ICA) as a by-product. As a strategy, reducing ICA and fatty acid biosynthesis of *Y. lipolytica* increases CA production. One of the most important parameters in production is the excess carbon source and nitrogen limitation in the fermentation medium [9].

Y. lipolytica can use hydrophobic substrates to produce organic acids, single-cell oils, and lipases [10]. The initial step involves the hydrolysis of triacylglycerols by extracellular lipases and the release of glycerol and fatty acids into the culture medium depending on the source of WCO. This hydrolysate can then be used to synthesize CA in the mitochondria of *Y. lipolytica* [11]. Although waste oils are generally preferred for biodiesel production [12–15], various bioproducts, such as biosurfactant [16–23], lipase [24–30], microbial lipids [25,31–38], single-cell protein [39], limonene [40], itaconic acid [41], and succinic acid [42], can also be produced using WCOs in yeast-based processes. Moreover, non-waste sunflower and canola oils have been used as substrates for CA production by *Y. lipolytica* strains, and high concentrations were obtained [7,43–47]. However, only a few studies have been conducted on CA production by *Y. lipolytica* using WCO in the production medium [11,48].

Optimizing and scaling up fermentation processes can significantly improve the performance of high-yield strains, resulting in increased productivity and reduced cost [49]. Among these optimization methods, the Taguchi method utilizes fractional factorial designs known as orthogonal arrays (OAs). This approach helps optimize multiple process variables while minimizing the total number of experiments required. The selection of an appropriate OA depends on the number of control factors and their respective levels [50].

CA is an important organic acid that is frequently used in various industries and is typically produced via fermentation. The fact that demand for CA has increased every year has caused studies on its production to continue. In this study, CA production by *Y. lipolytica* using the waste sunflower oil obtained from potato frying was investigated. The Taguchi experimental design was used to optimize production in terms of oil polarity, oil concentration, fermentation time, and Triton X-100 concentration. This study also characterized waste oil in more detail compared to similar studies in the literature and made a difference through process optimization. In addition, the effects of different buffering agents and Triton X-100 on CA production were revealed for the first time.

2. Materials and Methods

2.1. Microorganisms

Strains of *Y. lipolytica* NRRL Y-1094 (ATCC 8662), *Y. lipolytica* NRRL YB-423 (ATCC 18942), *Y. lipolytica* IFP29 (ATCC 20460), and *Y. lipolytica* NRRL YB 423-12 (ATCC 18944) were sourced from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were preserved at −80 °C in MEB with 50% (*v*/*v*) glycerol and activated in Malt Extract Broth (MEB).

2.2. Collection of Sunflower Oils

Waste oil samples were obtained by frying potatoes with sunflower oil (a well-known brand in Türkiye) in a kitchen-type deep fryer. The polarities of the samples were adjusted using a Testo 270 frying oil tester (Testo, Lenzkirch, Germany). The samples could not be obtained from small- and medium-sized enterprises because they generally do not use pure sunflower oil and sometimes fry products other than potatoes. In this context, controlled conditions were created to elucidate the mechanism of CA production using waste sunflower oils.

2.3. Characterization of Sunflower Oils

2.3.1. Determination of Fatty Acid Compositions of Oils

The fatty acid compositions of the samples were determined using an Agilent 7890 B gas chromatograph equipped with a Flame Ionization Detector (Agilent Technologies, Palo Alto, CA, USA) and Agilent J&W DB-FastFAME column (30 m × 0.25 mm, 0.25 µm, p/n G3903-63011). The inlet temperature was set to 250 °C and a 1 µL sample injection was performed. Hydrogen gas was used at a flow rate of 40 mL/min. The oven program was set as follows: 0.5 min at 50 °C, 194 °C at a rate of 30 °C/min for 3.5 min, and 240 °C at a rate of 5 °C/min for 1 min. A mixture of fatty acid methyl esters (37 components, C4-C24, ANPEL, Shanghai, China) was used as the external standard.

2.3.2. FTIR Analysis

The Fourier Transform Infrared (FTIR) spectra of oils were obtained utilizing an FTIR spectrometer (Thermo Scientific, Nicolet iS50 Spectrometer, Waltham, MA, USA) equipped with an ATR (Attenuated Total Reflectance) sampling accessory featuring a single bounce diamond crystal. The spectra were measured in absorbance mode over the range of 4000 cm^{-1} to 600 cm^{-1}, with 7 scans accumulated at a spectral resolution of 4 cm^{-1}.

2.4. Screening of Y. lipolytica Strains for Acid Production Capacities

The medium proposed by Hesham et al. [51] was modified and used to select producer microorganisms. The medium components and concentrations (g/L) were as follows: waste oil, 60; NH_4Cl, 0.6; KH_2PO_4, 1; $MgSO_47H_2O$, 1; yeast extract, 1; bromocresol green, 0.2; Triton X-100, 1.5; and agar, 20. Waste sunflower oil (60 g/L) with a polarity of 16 was added to the culture medium instead of glucose but the pH was not adjusted. Wells were created on each agar plate and 150 µL of the active yeast culture was separately added to each well. The yellow-colored zones formed by acid formation of the strains incubated at 28 °C for 48 h were measured.

2.5. Fermentation Conditions

The fermentation medium components and concentrations (g/L) are as follows: yeast extract, 0.8; KH_2PO_4, 1; $MgSO_47H_2O$, 1.5; $FeCl_36H_2O$, 0.2; $ZnSO_47H_2O$, 0.02; and $CuSO_4$, 0.02. Active cultures were transferred to a fermentation medium (at a 2% inoculation rate) and prepared according to the experimental design conditions. Fermentations were performed on a rotary shaker (50 mL medium/250 mL flask/28 °C, 180 rpm).

During fermentation, the pH was adjusted to 5.5–6.5 with 5 N NaOH on days 4 and 8, to prevent a decrease in pH. For the second experimental design, pH was adjusted using 100 mM 2-Morpholinoethanesulfonic acid monohydrate (MES) buffer at pH 6.5.

2.6. Determination of CA, ICA, and Biomass Concentrations

After fermentation, the medium was centrifuged at 9000 rpm for 15 min to separate the cells and the obtained supernatant was passed through a syringe filter (0.45 µm) and mixed with 8% $HClO_4$ in equal volume. Measurements were performed on an HPLC system (Agilent Technologies 1100 Series, Palo Alto, CA, USA) and a reverse-phase column (Inertsil ODS-3, 4.6 × 250 mm) was used. The wavelength was set to 210 nm. The column temperature was 40 °C and the flow rate of the mobile phase was 1 mL/min. The mobile phase was selected as 0.01 M H_2SO_4 [52]. The concentrations of CA and ICA were determined using a calibration curve obtained with standards (Sigma-Aldrich, St. Louis, MO, USA).

Biomass concentration was determined by centrifuging the fermentation liquids to separate the cells, followed by drying at 80 °C for 18–24 h.

The CA production process is presented in Figure S1 (Supplementary Materials).

2.7. Process Optimization

An experimental design was created using Minitab statistical software (version 17) by the Taguchi method to optimize CA and ICA production. The experiments were conducted with four replicates (considering possible adverse effects of pH adjustment) and the results were based on two samples. Table 1 lists the factors and levels used in the experiments and Table 2 lists the experimental design.

Table 1. Factors and levels used in the experiments.

Factors	Level 1	Level 2	Level 3
Sunflower oil polarity	8	16	24
Oil concentration (g/L)	60	90	120
Fermentation time (days)	4	8	12
* Triton X-100 concentration (g/L)	0	1	2

* This factor was used only in the first experimental design.

Table 2. Experimental design used for the optimization of CA production.

Run	Polarity	Oil (g/L)	Time (Days)	Triton X-100 (g/L) *
1	8	60	4	0
2	8	90	8	1
3	8	120	12	2
4	16	60	8	2
5	16	90	12	0
6	16	120	4	1
7	24	60	12	1
8	24	90	4	2
9	24	120	8	0

* This factor was used only in the first experimental design. Other factors and levels remained the same in the second experimental design.

Optimal parametric conditions can be determined using a signal-to-noise (S/N) ratio. This ratio reflects the deviation of the experimental results from the desired performance values, indicating closeness to the ideal performance. The S/N ratio values can be analyzed based on three performance characteristics: "larger is better", "nominal is best", and "smaller is better". As we aimed to maximize CA production in our study, the "larger is better" criterion was preferred [53]. The Mean Square Deviation (MSD) represents all variations around the designated target and can be calculated from S/N [54]. The study employing a larger is a better criterion for calculating the S/N ratio, which is defined as

$$S/N = -10 \log MSD$$

$$MSD = [(1/y_1)^2 + (1/y_2)^2 + \cdots + (1/y_n)^2]/n,$$

where n is the representative number of measurements (9 in our case) and y is the experimental value. Combinations of experimental factors were selected from the L9 orthogonal test table to determine the optimum process conditions.

After determining the inhibitory effect of Triton X-100 on CA production, this factor was excluded, and a new experimental design was created (Table 2). Analysis of variance (ANOVA) was used to determine the statistical significance of the factors. Both the S/N ratio and ANOVA were instrumental in predicting the optimal combinations of process parameters.

3. Results and Discussion

3.1. Characterization of Sunflower Oil Samples

The spectra of sunflower oils were obtained using Fourier Transform Infrared (FTIR) spectrometry before and after use as sunflower oils. The wavenumbers corresponding to the oleic (C18:1), linoleic (C18:2), palmitic (C16:0), and stearic acid (C18:0) fatty acids present in sunflower oil were characterized. This method enables the detection of polymerization, oxidation, and esterification reaction products in oils based on the wave numbers and regions where bands occur because of the vibrational energies of the molecules constituting these acids in the oil. Each band observed in the spectrum facilitated the identification of functional groups in the measured sample. The FTIR spectra of the oils are shown in Figure 1. The polarity of the non-waste oil was determined to be eight and the sample was coded as SF-8. This sample was used as the control. Waste oils with polarities of 16 and 24 were coded as SF-16 and SF-24, respectively.

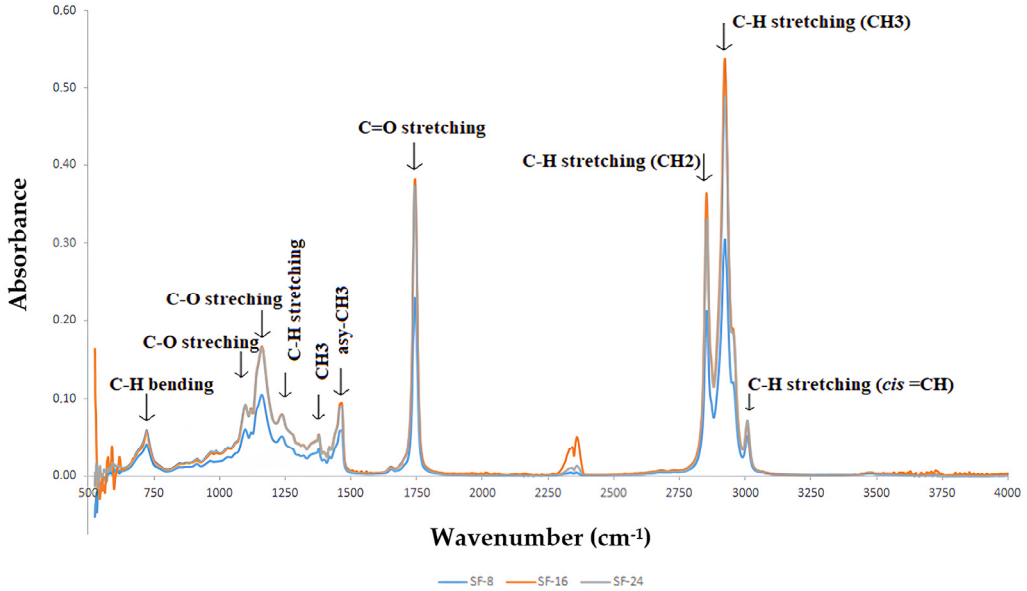


Figure 1. FTIR spectra of sunflower oils and vibration modes of corresponding observed bands.

As the polarization degree increased (from SF-8 to SF-24), there was a noticeable change in the absorbance of the bands. Especially, the C=O stretching band around 1740 cm^{-1} showed a significant increase, indicating higher levels of saturated aldehyde functional groups or other secondary oxidation and polymerization products. The C-H stretching bands around 2850–2925 cm^{-1} also showed changes in absorbance, reflecting the alteration in the alkyl chain structure due to frying. An increase in the absorbance of the C=O stretching band and changes in the C-H stretching band were direct indicators of polymerization and oxidation. These changes were consistent with the chemical transformations that occurred during prolonged frying, which led to the formation of larger and more complex molecules. Figure 1 shows the spectra of sunflower oils and the band vibration assignments are listed in Table 3.

Table 3. FTIR band vibration assignments with fatty acid correlation.

Wavenumber (cm^{-1})	Assignment	Functional Group	Fatty Acid Containing
720	C-H bending [55]	methylene (CH_2) groups and alkanes	long-chain fatty acids (e.g., palmitic acid and stearic acid)
1100–1200	C-O stretching [56]	esters	triglycerides formed from fatty acids (e.g., oleic acid, linoleic acid, palmitic acid, and stearic acid)
1375–1385	CH_3 bending (symmetric) [57]	methyl (CH_3) groups in alkanes	fatty acids with methyl groups (e.g., palmitic acid and stearic acid)
1465	CH_2 bending [58]	alkanes	long-chain fatty acids (e.g., palmitic acid and stearic acid)
1740	C=O stretching [59]	esters	triglycerides and fatty acids (e.g., oleic acid, linoleic acid, palmitic acid, and stearic acid)
2850	C-H stretching (CH_2) [60]	methylene (CH_2) groups and alkanes	long-chain fatty acids (e.g., palmitic acid and stearic acid)
2925	C-H stretching (CH_3) [58,61]	methyl (CH_3) groups in alkanes	fatty acids with methyl groups (e.g., palmitic acid and stearic acid)
3008	C-H stretching (*cis*=CH) [62]	aromatic rings and alkanes (unsaturated)	unsaturated fatty acids (e.g., oleic acid and linoleic acid)

Ester groups were observed in the carbonyl stretching band (C=O ~ 1740 cm^{-1}). The increase in the absorbance of this band might be related to polymerization-induced creation of new ester molecules and oxidation products. We observed an increase in the absorbance of this band because the oil was fried for longer periods (greater degrees of polarization). The stretching vibrations of the methylene (CH_2) and methyl (CH_3) groups were correlated with the C-H stretching (2850–2925 cm^{-1}) bands. Larger polymeric structures and cross-linking of fatty acids were indicated by changes in these bands, particularly their broadening or increase in absorbance.

The fatty acid compositions of the samples are recorded in Table S1 (Supplementary Materials). Linoleic, oleic, palmitic, and stearic acids were the most abundant fatty acids in sunflower oils. It is thought that the lack of a regular increase or decrease in the amount of fatty acids as the polarity increased was due to the samples being obtained at different times.

3.2. Screening of Y. lipolytica Strains for CA Production

At the end of the incubation period, *Y. lipolytica* IFP29, which had the largest zone diameter (2.00 ± 0.00 cm), was selected as the potential producer strain (Table 4). Figure 2 shows the zone formation of *Y. lipolytica* IFP29 in a selective growth medium.

Table 4. The zone diameters in the selective medium for *Y. lipolytica* strains.

Microorganisms	Diameter (cm)	
	24 h	48 h
Y. lipolytica NRRLY-1094	0.60 ± 0.07	1.00 ± 0.14
Y. lipolytica NRRL YB-423	0.05 ± 0.07	0.50 ± 0.00
Y. lipolytica IFP29	0.75 ± 0.07	2.00 ± 0.00
Y. lipolytica NRRL YB 423-12	0.40 ± 0.14	1.25 ± 0.07

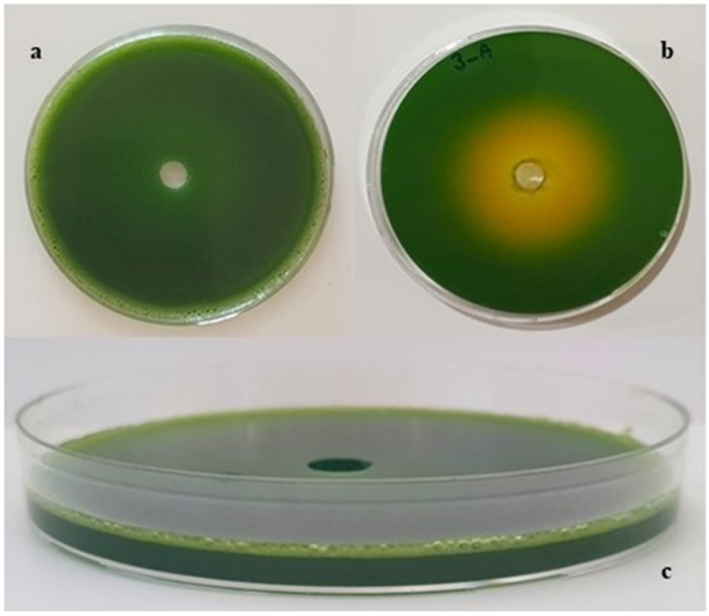


Figure 2. (**a**) Selective medium, (**b**) Zone image of *Y. lipolytica* IFP29 after 48 h, (**c**) The oil layer on the selective medium.

3.3. Results of Experimental Design 1

The data obtained from the applied experimental design are presented in Table 5. Overall, it can be concluded that Triton X-100 reduced the biomass concentration and inhibited CA production. As is evident from the table, the highest biomass concentration (3.30 ± 0.42) was achieved when the condition of the 1st run was applied. This outcome was expected because of the non-waste oil used and the absence of Triton X-100. The lowest biomass concentration was observed as 1.51 ± 0.10, using the highest polarity and Triton X-100 concentration. The highest CA and ICA production were 32.17 ± 2.04 and 29.44 ± 1.80, respectively. One noteworthy parameter was the considerable concentration of ICA formed as a byproduct (CA/ICA ratio of 1.09). Although the CA concentration remained relatively consistent, the ICA concentration decreased when non-waste oil and 1 g/L Triton X-100 were used. Additionally, it was evident that Triton X-100 adversely affected production compared with condition 2, even though the highest polarity level and oil concentration were used under condition 9 (with the same fermentation time).

Table 5. Results of biomass, CA, and ICA concentrations and the final pH.

Run	Polarity	Oil (g/L)	Time (Days)	Triton X-100 (g/L)	Biomass (g/L)	CA (g/L)	ICA (g/L)	Final pH
1	8	60	4	0	3.30 ± 0.42	4.36 ± 0.24	3.76 ± 0.38	2.74 ± 0.14
2	8	90	8	1	2.83 ± 0.18	4.53 ± 1.44	1.64 ± 0.26	3.60 ± 0.23
3	8	120	12	2	2.34 ± 0.25	-	-	5.15 ± 0.28
4	16	60	8	2	2.11 ± 0.31	-	-	4.78 ± 0.27
5	16	90	12	0	3.10 ± 0.17	32.17 ± 2.04	29.44 ± 1.80	4.65 ± 0.41
6	16	120	4	1	2.52 ± 0.20	-	-	3.42 ± 0.50
7	24	60	12	1	2.22 ± 0.61	-	-	5.50 ± 0.21
8	24	90	4	2	1.51 ± 0.10	-	-	4.48 ± 0.06
9	24	120	8	0	3.00 ± 0.41	11.40 ± 1.27	7.27 ± 1.10	3.68 ± 0.64

Liu et al. [11] showed that 30.3 g/L of CA by *Y. lipolytica* SWJ-1b was obtained after 336 h in a medium containing 80 g/L waste oil. Under these conditions, the concentrations of ICA and biomass were calculated as 6.9 and 6.1 g/L, respectively. Upon increasing the waste oil concentration to 120 and 140 g/L, a decrease in the CA concentration and an increase in the ICA concentration were observed. In our study, achieving a higher CA yield after 288 h was significant; however, a high ICA concentration was disadvantageous. Another important point is that the CA concentration reached was obtained in shake flasks but not in the bioreactor. Therefore, using less than 90 g/L waste oil in the fermentation medium without a surfactant may reduce the concentration of ICA. In another study, 12.2 g/L of CA was achieved in a fermentation medium containing 30 g/L of waste oil when 20 g/L of NaCl (for an initial osmotic pressure of 0.75 osmol/L) was used. Moreover, the synergistic effect of osmotic pressure with pH was investigated and 12.6 g/L of CA was obtained, with a combination of 0.75 osmol/L and pH 6.0 [48].

3.4. Results of Experimental Design 2

The data obtained from the new experimental design are given in Table 6. Except for the conditions in the seventh trial, the pH value did not drop below four. Biomass values ranged between 1.64 ± 0.35 and 3.89 ± 0.47. The highest CA concentration, calculated as 20.31 ± 2.76, was obtained under the fifth condition, similar to the first experimental design. The concentration of ICA produced under these conditions was calculated to be 13.63 ± 1.46. Compared with the experimental designs, the difference in CA concentrations obtained under the fifth condition was attributed solely to pH adjustment. This difference could be due to the substances used for buffering and/or the stabilization of the pH around four with MES buffer, which may decrease or block the rate of CA production. While the use of MES buffer aimed to provide more standard production, it is anticipated that continuous pH control with NaOH would yield better results if a bioreactor were used.

Table 6. The results were obtained by applying the new experimental design.

Run	Oil (g/L)	Polarity	Time (Days)	Biomass (g/L)	Final pH	CA (g/L)	S/N Ratio	ICA (g/L)	S/N Ratio
1	60	8	4	3.14 ± 0.27	4.48 ± 0.07	8.18 ± 1.22	18.26	6.32 ± 1.15	16.01
2	60	16	8	2.19 ± 0.21	4.03 ± 0.08	10.97 ± 0.49	20.80	7.66 ± 1.10	17.68
3	60	24	12	1.64 ± 0.35	4.01 ± 0.01	13.98 ± 0.18	22.91	10.03 ± 1.54	20.03
4	90	8	8	3.76 ± 0.61	4.01 ± 0.16	13.92 ± 0.47	22.87	10.59 ± 0.93	20.50
5	90	16	12	2.97 ± 0.27	4.36 ± 0.06	20.31 ± 2.76	26.15	13.63 ± 1.46	22.69
6	90	24	4	1.79 ± 0.10	4.95 ± 0.03	6.77 ± 0.66	16.61	5.76 ± 0.71	15.21
7	120	8	12	3.89 ± 0.47	3.88 ± 0.11	14.25 ± 1.09	23.08	10.65 ± 0.89	20.55
8	120	16	4	3.86 ± 0.28	5.00 ± 0.02	5.02 ± 0.20	14.01	4.99 ± 0.34	13.96
9	120	24	8	3.64 ± 0.51	4.51 ± 0.01	7.68 ± 0.69	17.70	6.44 ± 1.20	16.18

The objective of optimizing the process parameters was to enhance the S/N ratio, thereby achieving superior outcomes. The optimal levels for each factor were identified to minimize the variability and maximize the concentrations of CA and ICA. The highest S/N ratios for each factor were observed using 90 g/L of non-waste sunflower oil after 12 days of fermentation. The main effects plots for the S/N ratios are shown in Figures 3 and 4.

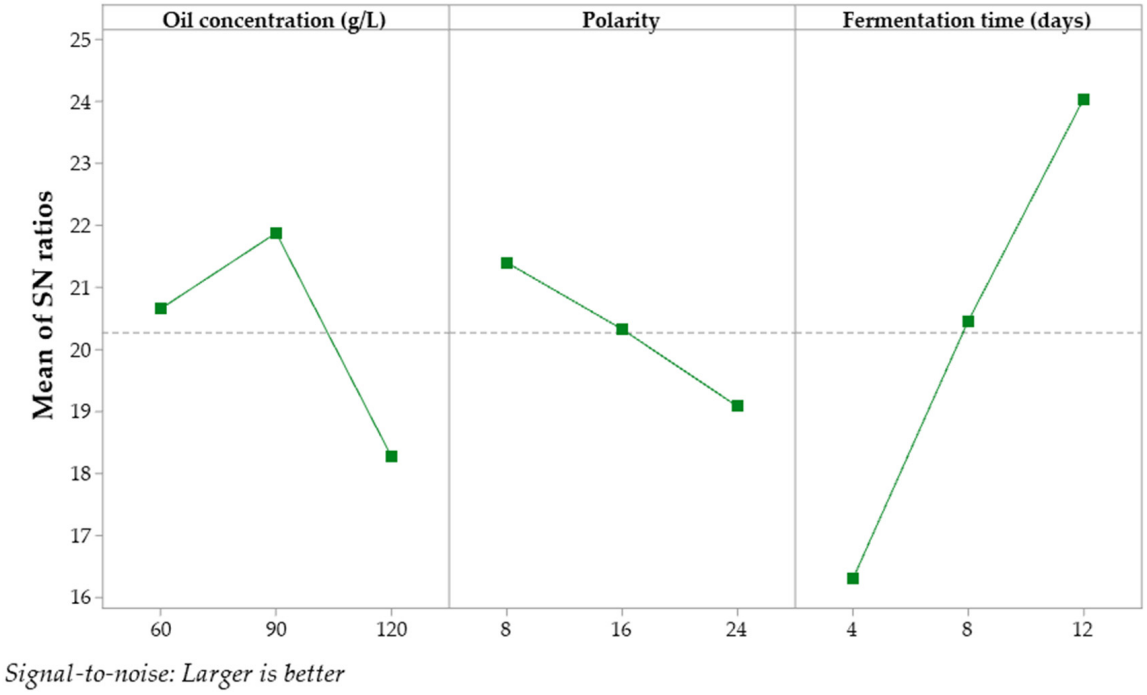


Figure 3. Main effects plot for the S/N ratio (CA concentration).

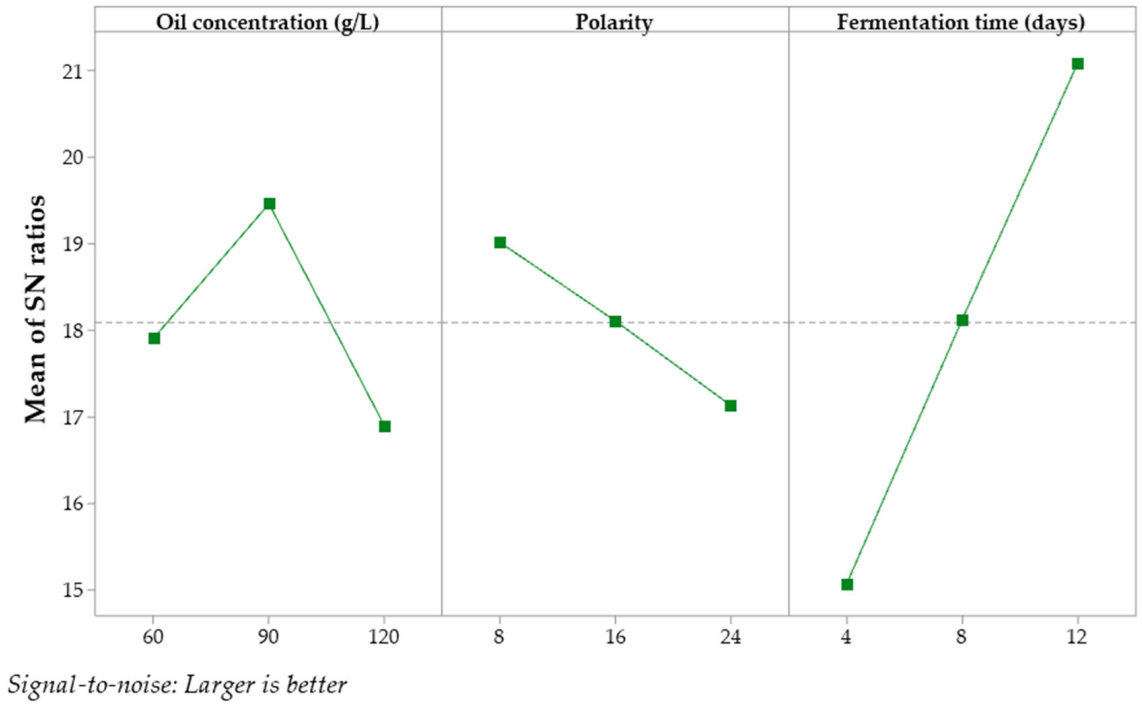


Figure 4. Main effects plot for the S/N ratio (ICA concentration).

The ANOVA results (Tables 7 and 8) revealed that among the selected factors, fermentation time had a stronger influence on CA and ICA concentrations (75.49% and 77.58%, respectively), while polarity had the least influence. The effects of oil concentration and fermentation time were statistically significant for both CA and ICA ($p < 0.05$).

Table 7. Analysis of variance for CA production.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F	P
Oil concentration (g/L)	2	20.27	16.94%	20.27	10.14	21.46	0.05
Polarity	2	8.12	6.79%	8.12	4.06	8.60	0.10
Fermentation time (days)	2	90.34	75.49%	90.34	45.17	95.63	0.01
Error	2	0.95	0.79%	0.94	0.47		
Total	8	119.68	100%				

DF: Degrees of freedom, Seq SS: Sequential sums of squares, Adj SS: Adjusted sum of square, Adj MS: Adjusted mean square (R^2: 99.21%, R^2 (adj): 96.84%).

Table 8. Analysis of variance for ICA production.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F	P
Oil concentration (g/L)	2	10.05	14.32%	10.05	5.03	27.17	0.04
Polarity	2	5.32	7.57%	5.32	2.66	14.37	0.07
Fermentation time (days)	2	54.47	77.58%	54.47	27.24	147.22	0.01
Error	2	0.37	0.53%	0.37	0.19		
Total	8	70.22	100%				

DF: Degrees of freedom, Seq SS: Sequential sums of squares, Adj SS: Adjusted sum of square, Adj MS: Adjusted mean square. (R^2: 99.47%, R^2 (adj): 97.89%).

Kamzolova et al. [44] indicated that plant oils are promising substrates for CA production by *Y. lipolytica* strains. They also emphasized the effectiveness of strain selection for ICA formation. The application of genetic manipulation tools is easier in yeasts because of their less complex genetic background than filamentous fungi. Studies have reported that genetically modified yeast strains can produce more CA, generate less ICA, and reduce residual sugar content [63]. Holz et al. [64] constructed a recombinant *Y. lipolytica* strain containing multiple copies of the aconitase-encoding gene *ACO1*. This high-level expression of aconitase in the *ACO1* multicopy integrative transformant led to a significant decrease in the CA/ICA ratio toward the ICA. ICA formation increased markedly compared to that in the wild-type strain using sunflower oil in the fermentation medium. In another study, two transformants of *Y. lipolytica* A101.1.31, overexpressing either *CIT1* or *CIT2* (encoding proteins with citrate synthase activity), were generated and overexpression of either of these genes was found to increase citrate synthase activity. Moreover, a significant increase in ICA biosynthesis was observed in the overexpressed mutants. Finally, *CIT1* and *CIT2* overexpressing strains produced CA and ICA from vegetable oil at a ratio close to 1. This study is similar to the findings of the present study in terms of the utilization of vegetable oil in production and the CA/ICA ratio [65]. It is noteworthy that the strain used in our study was not a mutant strain but yielded similar results. A major challenge in CA production using yeast is the simultaneous secretion of ICA, which is undesirable and disrupts crystallization. Förster et al. [66] detected that high-level expression of ICL (isocitrate lyase) in *ICL1* (isocitrate lyase-encoding gene) multicopy integrative transformants resulted in an important shift of the CA/ICA ratio in the direction of CA. ICA concentration decreased from 37 to 6% when sunflower oil was used in the fermentation medium. Lastly, in the respective mutant strains, a decrease in aconitase activity and an increase in isocitrate lyase activity resulted in the predominant accumulation of CA. Yuzbasheva et al. [67] stated that the mitochondrial succinate–fumarate carrier YlSfc1 of *Y. lipolytica* controls the ICA efflux from the mitochondria. Overexpression of *YlSFC1* shifted the ICA/CA ratio in favor of ICA. *YlSFC1* expression was repressed in the wild-type strain grown in a glucose-based medium compared to that in an olive oil medium, explaining the preference for CA production when *Y. lipolytica* was grown on carbohydrates.

Finogenova et al. [68] reported that the predominant production of CA or ICA was affected by intracellular iron concentration. Kamzolova et al. [69] stated that the limitation of nitrogen, phosphorus, sulfur, or magnesium is required in the fermentation medium for ICA formation by *Y. lipolytica*. Because CA and ICA are chiral compounds [70], separating them is challenging. Therefore, it is essential to reduce the concentration of ICA to improve process efficiency and ease the purification stage.

Venter et al. [46] discovered that adding 10 g/L of acetate in a medium containing 30 g/L of sunflower oil significantly increased CA production by *Y. lipolytica* UOFS Y-1701 while also significantly increasing the CA/ICA ratio. Mitrea et al. [42] used the pure glycerol as a hydrophilic source, stimulating de novo metabolic pathways and waste cooking oil as a hydrophobic source, promoting ex novo metabolic pathways in the yeast cells. Crude glycerol was a mixture of hydrophilic and hydrophobic carbon sources. Importantly, *Y. lipolytica* ATCC 20177 exhibited superior performance when cultivated on waste oil compared with glycerol. After 192 h of fermentation, 3.50 $\pm$ 0.04 g/L CA and 21 $\pm$ 0.16 g/L of succinic acid were obtained.

A significant challenge in using oily substrates is their water insolubility, which leads to insufficient substrate utilization and low mass transfer of air and nutrients into the medium [71]. To address these issues, the nonionic detergent Triton X-100 was added in the present study. Surfactants and their concentrations must be carefully selected; they can be toxic to microorganisms and either promote or inhibit metabolite production [72]. Contrary to our results, Mirbagheri et al. [73] found that the addition of Triton X-100 in the production medium increased the CA concentration of *Y. lipolytica* DSM 3286 and M7. In another study, adding Triton X-100 at 0–1% increased the CA concentration but inhibited mannitol and xylitol production [74]. Ping et al. [75] reported that Tween 80, Tween 20, and Triton X-100 inhibited the lipase activity.

Considering the results of the present study, it was concluded that changes in medium composition and pH values, as well as advanced optimizations and genetic modifications, could effectively reduce ICA concentration and increase CA concentration.

The cost of the culture medium generally accounts for 50–80% of the total cost of the end products. Biological processes can be more economical and sustainable, using low-cost substrates, such as various wastes and by-products from agriculture and other industries [76]. The composition of the culture medium is crucial for CA production by *Y. lipolytica*. Therefore, selecting waste cooking oils produced in large quantities as substrates for fermentation can reduce production costs. Additionally, these oils can be used directly without sterilization or filtering [77]. This approach can also lower the processing costs associated with pretreatment and raw material sterilization. Similarly, in the present study, oil was added to the medium by filtration. It was concluded that the industrial production potential is high because the raw material is cheap, easily accessible, requires no pretreatment, and competes with non-waste oils. However, the concentration of by-products must be reduced.

4. Conclusions

Frying oils used in the food industry have become waste oils because of their prolonged use and loss of physical and chemical properties. The resulting waste oils are harmful to the environment and to human health. One of the greatest advantages of biotechnological production is the ability to use waste as a raw material to produce value-added products. This enables the reduction in waste volume and environmental pollution and saves energy that would otherwise be spent on waste disposal, thus contributing to the country's economy.

In microbial production, different concentrations of detergents can have different effects on yeast cells, such as increasing cell permeability and causing cell membrane lysis and cell death. In the present study, the selection of Triton X-100 in the fermentation medium was aimed at reducing the increased surface tension associated with sunflower oil use and increasing the availability of oil as a substrate for CA production by *Y. lipolytica*

IFP29 (ATCC 20460). However, these results were unexpected. This study is important in terms of characterizing oils in more detail than other studies, revealing the inhibitory effect of Triton X-100 on CA production and determining the effects of different buffers on CA production. In addition, it was found that using waste and non-waste oils for CA production had a statistically insignificant effect ($p > 0.05$), which would increase the interest in preferring waste oils instead of non-waste oils.

Finally, we determined that waste sunflower oil could be a promising substrate for CA production using *Y. lipolytica*. However, to reduce the formation of by-products and further increase the production yield, further optimization studies, changes in some components of the fermentation medium, and genetic engineering approaches can be employed in future studies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10070374/s1, Figure S1: Citric acid production process; Table S1: Fatty acid composition of sunflower oils.

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