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Optimization of single-cell oil production by Mortierella isabellina using whey as a substrate

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Abstract

Essential fatty acids are indispensable components of a balanced diet, and their deficiency has been associated with various metabolic and physiological disorders. γ-Linolenic acid (GLA), which belongs to the omega-6 family of polyunsaturated fatty acids, has attracted considerable attention due to its therapeutic potential. GLA has been reported to exhibit beneficial effects such as alleviating rheumatoid arthritis, reducing cardiovascular risk factors, and normalizing nerve conduction velocity. Traditionally, GLA has been obtained from plant sources including evening primrose, borage, and blackcurrant seeds. However, increasing demand for GLA has stimulated interest in its production from microbial sources.

In the present study, microbial oil production rich in GLA was investigated using the filamentous fungus Mortierella isabellina DSM 1414 with whey as a substrate. The effects of several fermentation parameters on GLA production, including initial sugar concentration of the medium, pH, shaking speed, nitrogen source, temperature, and inoculum size, were evaluated. In the first stage of the optimization process, the significance of each parameter was determined using the one-factor-at-a-time approach. Among the tested conditions, the highest GLA production was obtained at an initial pH of 7 (0.55 g/L). Yeast extract was identified as the most suitable nitrogen source, yielding 0.70 g/L GLA. Furthermore, an inoculum size of 9% resulted in a higher GLA production of 1.08 g/L.

In the second stage, the combined effects of initial sugar concentration, shaking speed, and fermentation temperature were evaluated using a Box–Behnken response surface design. Statistical models describing the effects of the independent variables on GLA production were developed and validated by analysis of variance (ANOVA). According to the model predictions, the optimal fermentation conditions were determined as 81.87 g/L initial sugar concentration, 120 rpm shaking speed, and a fermentation temperature of 22°C. Validation experiments performed under these conditions resulted in a GLA production of 1.15 g/L, corresponding to a 2.1-fold increase compared with the initial conditions.

The results demonstrate the potential of whey, an agro-industrial by-product of cheese manufacturing, as an effective substrate for the production of microbial oil rich in γ-linolenic acid. Utilization of whey not only enables the production of a high-value bioactive compound but also contributes to the valorization of dairy industry waste streams and the reduction of their environmental impact.

Introduction

The rapid growth of the global population has increased the demand for food and nutritional resources, prompting the search for sustainable alternatives to conventional production systems. In this context, microbial fermentation has emerged as an efficient approach for producing value-added compounds, including proteins, lipids, organic acids, and bioactive metabolites. Microorganisms are capable of utilizing a wide range of substrates and converting them into nutritionally and industrially important products, making them attractive platforms for biotechnological applications [1–2].

Among these products, microbial lipids, also known as single-cell oils (SCOs), have gained considerable attention. The term SCO was introduced in analogy with single-cell protein to describe lipids produced by microorganisms that can be extracted and used similarly to conventional plant or animal oils in food and industrial applications [3–7]. Certain microorganisms are capable of accumulating large quantities of intracellular lipids under suitable cultivation conditions [6, 8–10].

Although all living microorganisms synthesize structural lipids as components of cellular membranes, only a limited number of species are capable of accumulating lipids at levels exceeding 20% of their dry cell weight. Microorganisms with this capability are referred to as oleaginous microorganisms. Under optimal conditions, some oleaginous microorganisms can accumulate lipids up to 70% of their biomass in the form of triacylglycerols, similar to lipid-accumulating plants [6–11]. These microorganisms include various species of yeasts, fungi, bacteria, and microalgae. Due to their capacity to produce significant quantities of lipids with desirable fatty acid compositions, microbial oils have been investigated extensively for various industrial and nutritional applications [6–7, 11–15].

The economic feasibility of microbial lipid production largely depends on the type of lipid produced and its potential market value. In general, microbial oil production becomes economically attractive when the target product is a high-value lipid that cannot be produced easily by agricultural methods. This condition is particularly applicable to long-chain polyunsaturated fatty acids (LCPUFAs), which have high nutritional and pharmaceutical importance [7, 16–18]. Historically, LCPUFAs were obtained primarily from marine animal oils. However, increasing global demand for these fatty acids has raised concerns regarding the sustainability and availability of traditional marine resources [18–20].

Numerous studies have demonstrated the beneficial effects of PUFAs in human health. These fatty acids have been reported to reduce serum cholesterol and triglyceride levels, inhibit atherosclerosis, prevent coronary heart disease, and exhibit anti-inflammatory properties [21–23]. In addition, omega-3 fatty acids such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) play critical roles in preventing cardiovascular diseases, rheumatoid arthritis, asthma, and certain cancers, Alzheimer as well as supporting neurological and retinal development in infants. Because mammals are unable to synthesize these essential fatty acids, they must obtain them through dietary intake [16, 24–26].

Among polyunsaturated fatty acids, γ-linolenic acid (GLA;18:3 ω-6) has attracted considerable attention due to its important physiological and therapeutic properties. GLA plays an essential role in several metabolic pathways and is involved in the synthesis of biologically active compounds such as prostaglandins, leukotrienes, and other lipid mediators. These compounds participate in the regulation of numerous physiological processes including immune responses, inflammation, and cellular signaling [27–28]. Moreover, GLA and other long-chain polyunsaturated fatty acids are integral components of cell membranes and influence membrane fluidity and biological functions [23].

One of the earliest commercial motivations for microbial lipid production was the synthesis of GLA as an alternative to plant-derived sources such as evening primrose oil (*Oenothera biennis*). Researchers investigated numerous filamentous fungi belonging to the order *Mucorales* to identify potential GLA-

producing strains. Although several strains were found to accumulate significant quantities of lipids, many of them contained relatively low proportions of GLA. Conversely, strains with high GLA content often exhibited relatively low lipid yields [29–30]. Among these microorganisms, species of the genus *Mortierella* have been identified as promising producers of microbial lipids rich in polyunsaturated fatty acids. In particular, *Mortierella isabellina* has been reported to produce lipids containing oleic, palmitic, and linoleic acids, as well as γ-linolenic acid [30–32].

The selection of inexpensive substrates is another crucial factor influencing the economic viability of microbial lipid production. Agro-industrial by-products are considered promising feedstocks because they are widely available and relatively inexpensive. Cheese whey, a major by-product generated during cheese manufacturing, is produced in large quantities worldwide. Whey is a greenish-yellow liquid rich in lactose, proteins, minerals, and vitamins, making it a valuable substrate for microbial fermentation processes. However, when discharged without proper treatment, whey can cause serious environmental problems due to its high biochemical oxygen demand [31, 33–36].

It has been estimated that approximately 165 million tons of whey are produced annually worldwide. Although a portion of this by-product is utilized in the food and feed industries, a significant fraction remains underutilized and is often discarded, resulting in environmental and economic losses [37]. Consequently, the conversion of whey into value-added products through microbial fermentation has attracted considerable interest. Previous studies have demonstrated that whey can be used as a substrate for the production of various microbial products, including carotenoids, polysaccharides, ethanol, lactic acid, single-cell protein, and microbial lipids [30, 35, 38–44].

Improving system performance and increasing process efficiency without raising costs is crucial, and the methods used for this purpose are called optimization. Statistical experimental design techniques are an effective procedure for planning experiments and analyzing outputs in a way that allows for objective conclusions. Defining the objectives of an experiment is the first step in experimental design. Selecting the parameters and responses to be analyzed is the second crucial step in optimizing key variables in a process. Therefore optimization of fermentation conditions plays a crucial role in improving the efficiency and productivity of microbial processes. Process parameters such as substrate concentration, pH, temperature, shaking speed, nitrogen source, and inoculum size can significantly influence microbial growth and metabolite production [45–46]. Traditional optimization studies often employ the one-factor-at-a-time (OFAT) approach, in which one variable is varied while all other parameters are kept constant [47–48]. Most of the improvements provided by OFAT designs are realized in the first half of the experiment [49]. It is a commonly used approach in scientific research to identify the most important factor and investigate its effect while ignoring other factors [50]. Optimizing fermentation conditions is used to significantly increase the yield and productivity of many bioprocesses. Traditionally, the process parameters of fermentations are optimized using OFAT [51]. Although this method is simple and widely used, it does not account for interactions between variables. Therefore, statistical experimental design techniques such as response surface methodology (RSM) have been developed to provide more efficient and systematic optimization strategies. Among these methods, Box–Behnken design is frequently applied because it allows the evaluation of interactions between variables while requiring fewer experimental runs compared with Central Composite Designs [52–53].

In the present study, microbial oil production by *Mortierella isabellina* DSM 1414 using whey as a substrate was investigated with particular emphasis on GLA production. The effects of several fermentation parameters, including initial pH, nitrogen source, inoculum size, substrate concentration, shaking speed, and temperature, were evaluated. Furthermore, statistical optimization using a Box–Behnken response surface design was applied to determine the optimal fermentation conditions for maximizing GLA production.

Materials and Methods

Materials

The whey powder used in this study were supplied by Ekso Süt (Ekiciler Süt Gıda Tar. Hay. ve Tic. A.Ş., Antalya, Türkiye). Among the nitrogen sources used in the preparation of fermentation media; meat extract, soy peptone, and yeast extract were purchased from Condalab (Madrid, Spain), ammonium sulfate from Isolab (Wertheim, Germany), and potassium nitrate from Merck (Darmstadt, Germany).

Microorganisms and medium

The culture *Mortierella isabellina* (DSM 1414) used in this study was prepared from stock cultures previously obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH- DSMZ, Braunschweig, Germany). For the preparation of new stock cultures and the cultivation of the microorganism, potato dextrose agar (PDA) was used as recommended by Kavadia et al. [54]. The prepared PDA medium was sterilized by heat treatment at 121°C for 20 min. All inoculation procedures were carried out under aseptic conditions in a biological safety cabinet (ESCO-Class II BSC). For the transfer of the culture from tube to tube, ten sterile glass beads (3 mm diameter) and 5 mL of sterile distilled water were added onto the stock culture and mixed using a vortex (VELP SCIENTIFICA-Vortex Mixer) at 2000 rpm for 5 min. From the resulting mycelial suspension, 1–2 drops was inoculated onto new sterile slant agars. The inoculated slant agars were incubated at 27°C for 3 days to allow mycelium growth. The cultures were then stored at +4°C. The same procedure was repeated periodically every month to maintain and renew the cultures**.** For long-term storage, stock cultures were maintained in 20% glycerol at −80 °C.

Preparation of deproteinized whey

For the preparation of deproteinized whey (DP-W) used as the substrate in the fermentations, reconstituted whey was first prepared using whey powder and deionized water to obtain a solution containing 24% total solids. The reconstituted whey was then subjected to heat treatment in an autoclave at 121°C for 10 min to denature the proteins, as described by Sienkiewicz and Riedel [55]. The heat-treated reconstituted whey was first filtered through coarse filter paper and subsequently through Whatman No. 1 filter paper to remove the denatured proteins, as reported by Rimada and Abraham [56]. The filtration processes

were carried out at 85–90°C in order to prevent lactose crystallization. Preventing lactose crystallization is important both for maintaining the lactose content and for ensuring that the filtration process proceeds smoothly without clogging. The resulting deproteinized whey permeate (DP-WP) with high dry matter content was stored at −20°C until use as a substrate. This stored concentrated DP-WP was subsequently diluted to appropriate ratios and utilized as substrate.

Preparation of pre-culture

Mortierella isabellina DSM 1414 culture was aseptically (in the biological safety cabinet) inoculated from the slant agar tubes into pre-culture medium. For this purpose; the tube containing the stock culture stored at +4°C was taken, and under aseptic conditions ten sterile glass beads (3 mm in diameter) and 5 mL of sterile distilled water were added. The tube was then mixed using the vortex at 2000 rpm for 5 min to obtain a mycelial suspension [57].

The pre-culture medium was prepared as described by Kavadia et al. [54]. For this purpose, 5 g/L glucose, 1 g/L $(NH_4)_2SO_4$, 7 g/L KH_2PO_4, 2 g/L Na_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.008 g/L $FeCl_3 \cdot 6H_2O$, 0.001 g/L $ZnSO_4 \cdot 7H_2O$, 0.0001 g/L $CuSO_4 \cdot 5H_2O$, 0.0001 g/L $Co(NO_3)_2 \cdot H_2O$, 0.0001 g/L $MnSO_4 \cdot 5H_2O$, and 0.5 g/L yeast extract were weighed and dissolved in distilled water, and the volume was adjusted to 1 L. The pH of the medium was adjusted to 6.0 using 1 N HCl or 1 N NaOH and the medium was sterilized at 121°C for 20 min. After preparation and sterilization, the pre-culture medium was cooled to room temperature, and 1 mL of the mycelial suspension was inoculated aseptically into 250 mL Pyrex baffled shaker flasks with silicone sponge closures containing 100 mL of the pre-culture medium. The inoculated pre-culture medium was incubated in an orbital shaker incubator (New Brunswick Scientific Escella E24 incubator) at 28 ± 1°C and 180 rpm for 2 days [17, 58-60].

Fermentation medium

The concentrated DP-WP previously prepared and stored at −20°C was warmed to room temperature and diluted with demineralized water to adjust the dry matter content so as to provide the initial lactose concentration required by the experimental conditions. Nitrogen source was then added, and the medium was transferred into Erlenmeyer flasks in which the fermentations would be carried out. The flasks were closed with silicone sponge plugs and sterilized at 121°C for 20 min. After sterilization, the fermentation media were cooled to room temperature, and 5 mL of the pre-culture obtained after two days of pre-fermentation was inoculated aseptically into each 95 mL fermentation medium. The cultivation was carried out in 250 mL Pyrex baffled shaker flasks with silicone sponge closures, at 180 rpm and 28 °C for 10 days [60]. For each day of fermentation, a 250 mL flask containing 100 mL of medium was prepared. So, for each trial, 10 flasks were placed into the shaker incubator.

Separation of biomass from the fermentation medium

The 100 mL fermented media (culture medium+wet biomass) contained in 250 mL Erlenmeyer flasks periodically removed every day from the incubator were transferred into centrifuge tubes and centrifuged at 14,000 rpm for 10 min. at room temperature using a centrifuge (Herolab UniGen MR, Wiesloch, Germany), based on the method reported by Papanikolaou et al. [61]. In the aforementioned method, samples were centrifuged at +4°C; however, this caused difficulties during the subsequent filtration step. This problem was resolved by performing the centrifugation at room temperature. The samples were then filtered through Whatman No. 1 filter paper according to the method suggested by Wynn and Ratledge [57]. The clear filtrate (spent culture medium) obtained after filtration was transferred into storage tubes and stored at −20°C for subsequent sugar analyses. The wet biomass remaining on the filter paper was washed thoroughly with distilled water until completely free of residual fermentation medium.

Drying of biomass

The wet biomass remaining on the filter paper was washed with excess of distilled water and dried together with the filter paper (pre-dried and pre-weighed) used during filtration by placing it in an oven at 70°C overnight [60]. After drying, the biomass was weighed together with the filter paper, and the dry biomass content was calculated by subtracting the previously recorded weight of the dry filter paper from the total weight. The dried biomass samples obtained in this manner were transferred into tubes, flushed with nitrogen gas, and tightly sealed. The samples were then stored at −20°C until lipid analyses (lipid content and fatty acid composition) were performed.

Lipid extraction from biomass

The dried biomass samples were transferred into centrifuge tubes, and 20 mL of hexane was added. The dried biomass treated with hexane was homogenized using homogenizer (IKA LABORTECHNIK–T25 Basic Ultra Turrax, Staufen, Germany) at 24,000 rpm for 5 min and subsequently kept in a hot water bath at 55°C for 10 min. The mixture was then centrifuged at 3000 rpm for 10 min at room temperature using centrifuge (Herolab UniGen MR, Wiesloch, Germany), allowing the solids to precipitate in the solvent. The supernatant was separated by filtration using Whatman No. 1 filter paper and then filter paper washed with pure hexane several times. The filtrate containing microbial lipids dissolved in hexane was collected in a pre-weighed ground-glass flask. The extraction procedure was repeated two additional times using 10 mL of hexane. The solvent in the combined filtrates was removed under vacuum (150 mbar) at 50°C using a rotary evaporator (Hei Vap Precision, Heidolph Instruments, Schwabach, Germany). The ground-glass flasks were then kept at 70°C overnight, and the lipid content was determined gravimetrically [60].

Fatty acid composition analysis

To determine the fatty acid composition, the extracted lipids were first subjected to a methyl esterification reaction. For this purpose, approximately 0.01 g of the extracted lipid sample was weighed into test tubes, and 2 mL of 10% methanolic HCl (10 mL HCl diluted to 100 mL with methanol) and 1 mL of methylene chloride were added. The tubes were tightly sealed with screw caps and kept in a water bath at 60°C for 3 h. After cooling to room temperature, the reaction was terminated by adding 4 mL of saturated NaCl solution and 2 mL of hexane. The tubes were closed and mixed using a vortex at 2000 rpm for 2 min, and then allowed to stand for phase separation [62]. The upper hexane phase containing the fatty acid methyl esters was transferred into vials and

analyzed by GC–MS (GC Trace 1300 MS ISQ 7000 Single Quadropole, Termo Fisher Scientific, USA). For the identification of fatty acid methyl ester (FAME) peaks in the chromatograms, a FAME C8–C24 mixture standard (Supelco 37 component FAME mix, Sigma-Aldrich, Laramie, USA) injected into the GC–MS under the same analytical conditions was used. The GC-MS conditions were stated as below;

Column: 100 m×0.25 mm×0.20 µm (TR-CN100)
Oven temperature program: 140°C (6 min), then increased to 240°C at a rate of 3°C/min and held for 14 min
Detector temperature (Interface): 260°C
Carrier gas: He
Carrier gas flow: 30.0 mL/min
Injection block temperature: 260°C
Split ratio: 1
Injection mode: splitless
Ionization source: FID1
Scan rate: 40 mass/s
Combustion gas: H_2
H_2 flow: 40.0 mL/min
Air flow: 400.0 mL/min

For the determination of GLA concentration, a calibration curve was constructed using standards by plotting the peak areas corresponding to different concentrations.

Determination of total carbohydrate content

The Dubois (phenol–sulfuric acid) method [63] was used to determine the sugar content in the spent fermentation media remaining after the separation of wet biomass, the initial sugar content of the fermentation media, and the sugar content of DP-WP. Absorbance measurements were carried out at 492 nm using a UV–Visible spectrophotometer (Thermo Scientific, Evolution Array, United States). Lactose constitutes approximately 75% of the dry matter of whey and is the major component after water [37, 64]. Throughout the study, the sugar content of the media was determined by total carbohydrate analysis, and therefore the term “sugar” will be used interchangeably with “total carbohydrate” in the remainder of the study.

Determination of nitrogen content

The nitrogen content of DP-WP and the initial nitrogen content of the fermentation media were determined according to the Kjeldahl method [65].

Determination of kinetic parameters

After determining the residual sugar, dry biomass, and lipid contents in the samples taken from the Erlenmeyer flasks, the following parameters were calculated based on the obtained data:

• Sugar consumption (g/L): $\Delta S = S_0 - S$
• Biomass production (g/L): $X = X - X_0$
• Lipid production (g/L): P_L
• GLA production (g/L): P_{GLA}
• Biomass lipid content (%): $(P_L / X) \times 100$
• GLA/lipid ratio (%): $(P_{GLA} / P_L) \times 100$
• GLA/biomass ratio (%): $(P_{GLA} / X) \times 100$

• Yield (%)

- Biomass yield ($Y_{X/S}$): $(X / \Delta S) \times 100$
- Lipid yield ($Y_{L/S}$): $(P_L / \Delta S) \times 100$
- GLA yield ($Y_{GLA/S}$): $(P_{GLA} / \Delta S) \times 100$

• Maximum consumption rate (g/L/day)
• Maximum volumetric productivity (g/L/day)
• Specific growth rate (day^{-1})
• Doubling time of viable cells (day)

Maximum volumetric productivity, maximum consumption rate, and specific growth rate were calculated as follows:

• Growth rate (g/L/day): Slope of the steepest part of the biomass curve
• Maximum volumetric productivity (g/L/day): Slope of the steepest part of the lipid curve
• Consumption rate (g/L/day): Slope of the steepest part of the sugar curve
• Specific growth rate (day^{-1}): Calculated from the slope of the plot of lnX versus time [66]

• S_0: Initial substrate concentration (g/L)
• S𝕏: Substrate concentration in the medium at the end of fermentation (g/L)
• X_0: Biomass concentration inoculated into the medium at the beginning of fermentation (g/L)
• X𝕏: Biomass concentration obtained at the end of fermentation (g/L).

Experimental design of the study

The initial fermentation pH, nitrogen source, and inoculation ratio were first determined using the one-factor-at-a-time method. The conditions yielding the highest GLA production were then selected, and the study proceeded to the second stage. In the second stage, the initial sugar concentration, shaking speed, and temperature of the fermentation were optimized using response surface methodology (RSM). A total of 15 fermentations were carried out using the one-factor-at-a-time method and 15 fermentations using response surface methodology. Together with the validation experiment conducted under the optimal conditions determined from the response surface methodology data, a total of 31 experiments were performed. Throughout the study, fermentations were carried out for 10 days under the specified conditions [67].

One-factor-at-a-time experimental design

Fermentation experiments in which parameters were optimized using the one-factor-at-a-time method were carried out at 28 ± 1°C and an shaking speed of 180 rpm, with the initial sugar concentration of the medium adjusted to 30 g/L [54]. In the first stage, the initial pH of the fermentation medium was optimized. The medium used in these fermentations was prepared by diluting concentrated DP-WP to obtain a sugar concentration of 30 g/L. In addition, ammonium sulfate (1 g/L) and yeast extract (0.5 g/L) were added to the DP-WP. After the sterilization, cooling to room temperature, and inoculation steps as mentioned before, the pH values of the media were adjusted aseptically. Previous studies on the optimization of pH for GLA production by *Mortierella isabellina* using different substrates reported experiments within the pH range of 5–9 and indicated that GLA production decreased when the pH exceeded 8 [68-70]. Based on these findings, the pH values of the fermentation media were adjusted to pH 4, 5, 6, 7, and 8 using previously prepared and sterilized 1 M NaOH and 1 M HCl under aseptic conditions. The subsequent stages of the study were continued using the initial pH value that resulted in the highest GLA production.

In the second stage, all fermentation conditions were kept constant while the effect of nitrogen source on GLA production was investigated. For this purpose, separate fermentations were conducted using different nitrogen sources. Ammonium sulfate, meat extract, yeast extract, potassium nitrate, and soy peptone were added individually to the fermentation media at a concentration of 1.5 g/L. After sterilization of the prepared media, the initial pH of the fermentation medium determined in the first stage was adjusted to its optimum value and inoculations were performed at a rate of 5%. At the end of the fermentations, the nitrogen source that resulted in the highest GLA production was selected and used in all subsequent fermentation experiments.

In the final stage of the one-factor-at-a-time optimization, the optimum inoculation ratio was determined. Fermentation media prepared using DP-WP and the nitrogen source determined in the second stage were transferred in volumes of 91, 93, 95, 97, and 99 mL into separate 250 mL baffled Erlenmeyer flasks and sterilized. After cooling to room temperature, 9, 7, 5, 3, and 1 mL of pre-culture were added to the respective flasks to obtain a final fermentation volume of 100 mL. The pH values of the media were then adjusted to the optimum level determined in the first stage, and fermentations were initiated. After 10 days of fermentation, the inoculation ratio resulting in the highest GLA production was selected. The subsequent stages of the study were conducted using the fermentation medium pH, nitrogen source, and inoculation ratio determined by the one-factor-at-a-time method.

Response surface methodology experimental design

In the second stage of the study, the initial sugar concentration of the fermentation medium, shaking speed applied during fermentation, and temperature parameters were optimized using response surface methodology (RSM) in order to achieve the highest level of GLA production. The maximum and minimum parameter values used in the Box–Behnken design to determine the effects of initial sugar concentration, temperature, and shaking speed are presented in Table 1.

Table 1. Parameters and corresponding values used in response surface methodology for the optimization of fermentations

Variable	Minimum	Center point	Maximum	Reference
Initial sugar concentration (g/L)				
	30	80	130	Kavadia vd. 2001; Dyal vd. 2005; Xing vd. 2012
Shaking speed (rpm)	120	180	240	Kavadia vd. 2001; Gu vd. 2018
Temperature (ºC)	22	28	34	Kavadia vd. 2001; Hansson ve Dostalek 1988; Gu vd. 2018

Fermentations were carried out in media with different initial sugar concentrations at various shaking speeds and temperatures, and these three parameters were optimized using response surface methodology. The experimental design of the fermentation trials conducted for this purpose is presented in detail in the table (Table 2).

Table 2. Fermentation conditions generated by the Box–Behnken design

Standart order	Initial sugar concentration (g/L)	Shaking speed (rpm)	Temperature (°C)
1	30	120	28
2	30	180	22
3	30	180	34
4	30	240	28
5	80	120	22
6	80	120	34
7	80	180	28
8	80	180	28
9	80	180	28
10	80	240	22
11	80	240	34
12	130	120	28
13	130	180	22
14	130	180	34
15	130	240	28

Statistical analysis

The first stage of the experiment was designed according to the one-factor-at-a-time method, and the second stage was optimized using response surface methodology. All analyses were performed in duplicate for each sample, and the experiments were conducted with three replicates. The results of this study were assessed with variance analysis using SAS Statistical Software (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to compare differences among treatments. In the second stage, GLA production was selected as the response variable for optimization, and the statistical analysis of the optimization data was performed using Design Expert software (Version 10.0.1; Minneapolis, MN). The variance analysis (ANOVA) of the model developed for the optimization process and the associated factors was evaluated at a 95% confidence level.

Results and Discussion

In the first stage of this study, several fermentation parameters were optimized using the one-factor-at-a-time method. In this context, the initial pH values of the fermentation medium were first optimized, followed by the determination of the nitrogen source and inoculation ratio using the initial pH value at which the highest GLA production was obtained. The conditions under which the highest GLA content was obtained from the produced microbial oils were determined, and the study proceeded to the next stage. In the second stage of the study, the parameters optimized in the first stage using the one-factor-at-a-time method were applied, and the initial sugar concentration of the fermentation medium (g/L), shaking speed (rpm), and fermentation temperature (°C) were optimized for maximum GLA production using the Box–Behnken design.

Effect of initial medium pH on biomass, lipid, and GLA production

The fermentation media were prepared with an initial lactose concentration of 30 g/L, $(NH_4)_2SO_4$ at 1.0 g/L, and yeast extract at 0.5 g/L. The fermentation media were individually adjusted to pH values of 4, 5, 6, 7, and 8, and fermentations were carried out at 28°C and 180 rpm for 10 days.

Table 3 presents mean values (X ± SE) of responses (the biomass and lipid production values, biomass lipid content, sugar consumption, biomass yield, GLA production, GLA yield, lipid yield, GLA/lipid ratio, and GLA/biomass ratio) at different initial pH values and Duncan's multiple comparison results. The differences among biomass and lipid production (g/L), lipid content of dry biomass (%), and sugar consumption (g/L) for each experiment were found to be statistically significant ($P < 0.05$). When the data for biomass yield ($Y_{X/S}$) were compared, it was determined that only the difference between the values obtained at pH 4 and pH 8 was statistically significant ($P < 0.05$). The highest biomass and lipid production (11.41 g/L and 3.35 g/L, respectively) were achieved in the fermentations carried out at an initial medium pH of 4. When the values related to GLA production were examined, it was observed that the lowest GLA production and yield were obtained at an initial medium pH of 5 (0.35 g/L; 1.24%), whereas the highest GLA production and yield (0.55 g/L; 2.07%) were obtained from fermentations with an initial medium pH of 7. The differences among lipid yield values were found to be statistically insignificant ($P > 0.05$). However, the differences among GLA yield (%), GLA/lipid (%), and GLA/biomass (%) ratios were determined to be statistically significant ($P < 0.05$).

Table 3
Responses at different initial pH values: mean (X ± SE) and Duncan's test results

Initial pH value	Dry biomass (g/L)	Lipid (g/L)	Biomass lipid content (%)	Sugar consumption (g/L)	Biomass yield (%)	GLA (g/L)	GLA yield (%)	Lipid yield (%)	GLA/lipid (%)	GLA/biom (%)
pH 4	11.41^{a}±0.26	3.35^{a}±0.14	29.32^{bc}±1.09	30.15^{a}±0.62	37.88^{a}±0.89	0.43^{bc}±0.03	1.42^{cd}±0.09	11.12 ± 0.59	12.77^{c}±0.34	3.75^{c}± 0.2
pH 5	10.32^{b}±0.33	2.74^{c}±0.17	26.55^{c}±1.32	28.24^{b}±0.28	36.58^{ab}±1.24	0.35^{c}±0.03	1.24^{d}±0.10	9.72 ± 0.65	12.71^{c}±0.20	3.38^{c}±0.2
pH 6	10.04^{b}±0.36	3.23^{ab}±0.10	32.22^{ba}±0.99	28.67^{b}±0.36	35.04^{ab}±1.45	0.48^{ba}±0.03	1.68^{bc}±0.13	11.27 ± 0.44	14.87^{b}±0.73	4.78^{b}±0.2
pH 7	9.02^{c}±0.15	2.88^{bc}±0.04	31.89^{ba}±0.50	26.63^{c}±0.26	34.93^{ab}±0.60	0.55^{a}±0.02	2.07^{a}±0.09	11.14 ± 0.26	18.52^{a}±0.42	5.91^{a} ±0.1
pH 8	7.64^{d}±0.31	2.59^{c}±0.19	33.75^{a}±1.06	23.23^{d}±0.24	33.00^{b}±1.49	0.42^{bc}±0.02	1.80^{ab}±0.08	11.17 ± 0.83	16.25^{b}±0.50	5.48^{a}±0.0

Values carrying different letters in the same column are statistically significantly different (P < 0.05).

X ± SE: Mean ± standard error

When the data were compared, it was determined that the highest biomass and microbial oil production were obtained in fermentations with an initial medium pH of 4, and that biomass and lipid production decreased as the pH value increased. While the lipid contents of the dry biomass were similar in all fermentations, the highest biomass production occurring in the fermentation with an initial medium pH of 4 resulted in a higher amount of microbial oil being obtained in this fermentation. It was observed that the initial pH value did not have a significant effect on the lipid content of the biomass. The highest GLA production and yield were observed in fermentations with an initial pH value of 7 (Fig. 1). Therefore, in the subsequent stages of the study, fermentations were initiated by adjusting the initial medium pH to 7, the value at which the highest GLA production (g/L) was obtained.

In a study investigating the effects of medium components and culture conditions on the biomass and lipid production of *Trichosporon fermentans*, fermentations were carried out for seven days in media containing 100 g/L glucose at 25°C and a shaking speed of 160 rpm. The effect of the initial pH value was examined within the range of 4–10, and a linear increase in biomass and lipid production as well as biomass lipid content was observed as the pH increased from 4 to 6.5. Biomass and lipid production and biomass lipid content reached their maximum values at pH 6.5 (28.1 g/L, 17.5 g/L, and 62.4%, respectively), while a slight decrease in these values was observed when the pH was increased to 7 (27.9 g/L, 17.3 g/L, and 61.9%). Beyond this point, biomass and lipid production and biomass lipid content continued to decrease as the pH increased [71]. In the present study, however, biomass production decreased as the initial pH value increased. Lipid production and biomass lipid content also varied depending on the initial pH value.

In another study conducted to maximize GLA production using *Mortierella ramanniana* var. *ramanniana*, fermentation initial pH values ranging from 1.00 to 14.00 were evaluated, and the highest yield (lipid content/g dry biomass, %) was reported at an initial fermentation pH value of 5 [72]. In a study conducted by Leman and Brakoniecka-Sikorska [73], the effects of growth conditions on lipid biosynthesis were investigated. The effects of pH, temperature, and incubation time were examined using *Mortierella ramanniana* var. *angulispora* IFO 8187, and the highest GLA content in the lipid was obtained after 48 h of incubation under fermentation conditions of 30°C and pH 5. In the present study, the GLA content in the lipid was found to be 18.52%, whereas the highest GLA content reported in the aforementioned study was 8.2%.

In a study using *Mortierella alpina* CBS 343.66, different pH values, temperatures, and carbon sources were evaluated in order to optimize culture conditions for biomass and lipid production rich in PUFA. Fermentations were carried out at pH values of 5.5, 6.5, 7.5, and 8.5 at 25°C. The highest lipid production (2.6 g/L) was obtained at pH 6.5 after 65 h of incubation, and the GLA content of the lipid was determined to be 7% (w/w). The highest GLA content in the lipid (16%) was obtained at pH 7.5 after 65 h of incubation; however, lipid production at this pH was only 1.1 g/L [74].

In another study examining the effects of various process parameters of submerged culture fermentation on GLA production, experiments were carried out within the pH range of 4.0–8.0 using *Mucor* sp. RRL001. The highest GLA content in the lipid (8.3%) was obtained at pH 5.0, whereas the highest GLA production (0.64 g/L) was achieved in experiments conducted at pH 6.5 [75].

Considering studies conducted with *Mortierella isabellina*, the effect of culture conditions on GLA production was investigated in a study in which hexadecanol was used as the substrate. In this study, using the *Mortierella isabellina* XM-3 strain, fermentations were carried out at 28°C for five days at pH values ranging from 5 to 9. The highest GLA production (0.32 mg/mL, 24.6 mg/g dry biomass) was obtained at pH 6 [68]. In another study, *M. isabellina* was incubated for five days at 28°C in a medium containing 1% yeast extract, 2% octadecanol, and 0.1% Tween 80. It was reported that both the GLA production (g/L) and GLA content (%) increased as the pH increased from 5 to 7, reached a maximum at pH 7, and then began to decrease under slightly alkaline conditions (pH 8–9) [69].

In another study conducted to optimize GLA production using fragmented *M. isabellina* mycelia, cultures were incubated at 28°C for five days in a medium containing 2% hexadecanol (w/v) and 1% yeast extract at pH values ranging from 5.0 to 9.0. It was reported that GLA production increased with increasing pH and reached its highest value (78.6 mg/g dry biomass) at pH 7 [70]. When the results were compared, the GLA amount obtained in that study was higher than the GLA content in the biomass obtained in the present study (59.1 mg/g dry biomass). This difference may be attributed to the use of hexadecanol, a long-chain fatty alcohol containing 16 carbon atoms, which can be converted more efficiently into long-chain fatty acids by microorganisms.

It is evident that the initial fermentation pH generally affects not only the biomass and lipid production of microorganisms but also GLA production. The optimum pH value for lipid, biomass, and GLA production appears to vary depending on the microorganism used. Moreover, previous studies have shown that the optimum pH value for GLA production may also be influenced by the type of substrate on which the same microorganism is cultivated [68–69]. As a result of the present study, the optimum initial fermentation pH for GLA production by *Mortierella isabellina* (DSM 1414) using whey was determined to be pH 7.

Effect of different nitrogen sources used in the medium on biomass, lipid, and GLA production

Ammonium sulfate, meat extract, yeast extract, potassium nitrate, and soy peptone were used in the fermentations carried out to determine the effect of nitrogen sources on GLA production. The pH of each medium was adjusted to pH 7 prior to the initiation of fermentation.

Table 4 presents the results of Duncan's Multiple Comparison Test for responses (the biomass and lipid production values, biomass lipid content, sugar consumption, biomass yield, GLA production, GLA yield, lipid yield, GLA/lipid ratio, and GLA/biomass ratio) obtained from fermentations conducted using different nitrogen sources. The highest biomass production was obtained in fermentations carried out in media containing ammonium sulfate, whereas the lowest biomass production was observed in media prepared with potassium nitrate. When the data were compared, the differences between biomass (g/L) and lipid production (g/L) obtained from each experiment were found to be statistically significant ($P < 0.05$). The highest biomass yield ($Y_{X/S}$) and lipid production (g/L) were obtained from fermentations in which yeast extract was used as the nitrogen source. The fermentation showing the highest biomass production and the lowest lipid content of biomass was the one in which ammonium sulfate was used as the nitrogen source. This may be attributed to the relatively high nitrogen content (0.91 g/L) of the medium prepared with ammonium sulfate, which likely allowed the microorganism to continue cellular growth and delayed the onset of lipid accumulation. Indeed, the lipid accumulation process in microbial cells generally begins after the depletion of nitrogen in the medium. The results obtained from fermentations conducted with potassium nitrate, in which the lipid content of the biomass was the highest (64.39%), support this explanation. In fermentations carried out in media containing potassium nitrate, which had a lower initial nitrogen content (0.61 g/L), biomass production was low, whereas the lipid content of the produced biomass was considerably high. When the data were examined, the differences among GLA production and GLA yield values were found to be statistically significant ($P < 0.05$). When the GLA contents of the microbial oils were compared, it was observed that the values obtained from meat extract, yeast extract, and soy peptone were very similar and did not differ statistically ($P > 0.05$).

Table 4
Responses at different nitrogen sources: mean (X ± SE) and Duncan's test results

Nitrogen source	Dry biomass (g/L)	Lipid (g/L)	Biomass lipid content (%)	Sugar consumption (g/L)	Biomass yield (%)	GLA (g/L)	GLA yield (%)	Lipid yield (%)	GLA/lipid (%)	G (
Ammonium sulfate	9.67^{a}±0.26	3.27^{c}±0.12	33.44^{c}±0.88	27.29^{a}±0.13	35.43^{a}±0.90	0.39^{d}±0.02	1.45^{c}±0.08	12.04^{b}±0.42	12.03^{c}±0.40	4
Meat extract	7.36^{c}±0.16	4.06^{b}±0.09	55.11^{b}±0.42	21.67^{b}±0.75	32.85^{a}±1.67	0.63^{b}±0.01	2.92^{a}±0.10	18.87^{a}±0.93	15.54^{a}±0.34	8
Yeast extract	7.92^{b}±0.09	4.38^{a}±0.11	55.41^{b}±1.46	21.82^{b}±0.40	36.33^{a}±0.70	0.70^{a}±0.020	3.20^{a}±0.13	20.15^{a}±0.78	15.87^{a}±0.34	8
Potassium nitrate	3.00^{e}±0.04	1.93^{d}±0.06	64.39^{a}±2.31	22.51^{b}±0.41	13.37^{c}±0.27	0.26^{e}±0.008	1.16^{c}±0.022	8.59^{c}±0.24	13.63^{b}±0.41	8
Soy peptone	6.55^{d}±0.13	3.52^{c}±0.13	53.85^{b}±1.97	26.31^{a}±1.74	25.63^{b}±2.27	0.56^{c}±0.033	2.18^{b}±0.191	13.70^{b}±1.06	15.91^{a}±0.75	8
Values carrying different letters in the same column are significantly different ($P < 0.05$).										
X ± SE: Mean ± standard error										

Figure 2 presents the GLA and lipid production (g/L) and GLA yield (%) obtained from fermentations carried out using different nitrogen sources. Examination of the figure shows that the highest lipid and GLA production as well as the highest GLA yield were obtained from fermentations in which yeast extract was used as the nitrogen source. Based on these results, yeast extract was used as the nitrogen source in the subsequent stages of the study.

In a study investigating the effects of different nitrogen sources on lipid accumulation in oleaginous yeasts, 17 different yeast species and strains were evaluated. Based on the results obtained, the study was continued with *Rhodosporidium toruloides*. In the first stage, NH_4Cl, asparagine, and glutamate were examined as nitrogen sources. It was reported that organic nitrogen sources significantly increased the lipid content in *Rhodosporidium toruloides* strains. When NH_4Cl was used as the nitrogen source, lipid accumulation in the *Rs. toruloides* CBS 14 strain was 18%, whereas the use of glutamate, urea, or arginine increased lipid accumulation to over 50% [76].

In another study conducted with the yeast *Cryptococcus albidus* var. *albidus*, the effect of culture conditions on the fatty acid composition of the produced lipids was investigated, and in this context the effects of different carbon and nitrogen sources as well as temperature were determined. NH_4Cl, $(NH_4)_2SO_4$, urea, L-glutamate, L-arginine, L-ornithine, and L-proline were used as nitrogen sources. The highest biomass production (10.0 g/L) was obtained in the medium containing L-glutamate, while the highest lipid content (42.0% w/w of biomass) was obtained in the medium containing NH_4Cl [77].

In another study investigating the effects of culture conditions on mycelial growth and GLA production, four different *Mortierella* strains were initially evaluated, and the study was continued with the strain *M. ramanniana* CBS 112.08, which exhibited the best GLA production. KNO_3, NH_4Cl, and $(NH_4)_2SO_4$

were used as nitrogen sources. In the first stage of the nitrogen source experiment, fermentations were carried out in media containing 50 g/L glucose with a C/N ratio of 20. No lipid production was observed in fermentations conducted with ammonium sulfate, whereas the highest lipid production (15.7% lipid/biomass) was obtained in fermentations where KNO_3 was used as the nitrogen source. In the subsequent stage, potassium nitrate concentrations of 1.0 g/L (C/N = 86) and 0.5 g/L (C/N = 173) were investigated. The highest GLA production (16.9% w/w lipid) was obtained from fermentations carried out in media containing 1.0 g/L KNO_3. In these fermentations, biomass production reached 10.8 g/L, and the lipid content of the biomass was reported to be 23% [78]. In the present study, however, fermentations conducted in media containing potassium nitrate yielded biomass with a lipid content of 64.39%, although the GLA content in the lipid remained at 13.63%.

Stredanska and Sajbidor [79] conducted a similar study using *M. alpina* CCF 185. Ammonium sulfate ($(NH_4)_2SO_4$), urea, L-lysine, casein hydrolysate, peptone, $NaNO_3$, and yeast extract were used as nitrogen sources. Yeast extract was found to be the most suitable nitrogen source for both lipid production (47.1% of dry biomass) and ARA production (1.0 g/L). In addition, microbial lipids obtained from biomass grown in media containing casein hydrolysate and peptone were reported to have similar fatty acid compositions, and both lipids contained approximately 5% GLA (of total fatty acids).

In another study conducted to optimize GLA production using *Mortierella ramanniana* var. *ramanniana*, the effects of fermentation conditions and medium composition were investigated, and various nitrogen sources were examined. In addition, the effects of different concentrations of each nitrogen source (0.25%, 0.50%, and 1.00%) were also determined. Casein, yeast extract, peptone, KNO_3, and lysine were used as nitrogen sources, and the media were prepared to contain 5% dextrose. The results indicated that the highest biomass production (3.79 ± 0.27 g/100 mL) was obtained in the medium containing 0.5% yeast extract. The highest lipid yield was also obtained from media containing yeast extract. Due to the low lipid yield observed in samples containing KNO_3 and lysine, their fatty acid profiles could not be analyzed. It was determined that increasing the concentration of peptone in the medium reduced the fatty acid yield, whereas increasing the concentration of yeast extract increased it. Consequently, the highest GLA yield (0.0226 ± 0.0052 g/g biomass) was obtained from the medium containing 1% yeast extract [72]. In the present study, the highest GLA yield was also obtained in experiments where yeast extract was used, and the GLA yield achieved was higher than that reported in the aforementioned study.

In a study investigating the effects of various process parameters of submerged culture fermentation on GLA production, fermentations were carried out using yeast extract, corn steep liquor, peptone, baker's yeast, and a mixture of corn steep liquor and baker's yeast (1:1) at a concentration of 1%. Biomass with a GLA content of 8.4% was obtained from fermentations conducted in media containing yeast extract and peptone. However, the total lipid content obtained from the medium containing yeast extract was higher than that obtained from the medium containing peptone. Therefore, it was reported that the total GLA yield was highest in the culture grown in the medium containing yeast extract [75].

In a study conducted with the *M. isabellina* M2 strain, the effects of different nitrogen sources on lipid accumulation were investigated using $NaNO_3$, $(NH_4)_2SO_4$, NH_4NO_3, and urea as sole nitrogen sources. The experiments were carried out at 28°C and a shaking speed of 80 rpm in media containing 60 g/L sugar with an initial pH of 6. It was reported that the nitrogen sources were added to the media to provide a nitrogen concentration of 0.42 g/L. Growth under different nitrogen sources was found to affect both biomass and lipid production. The highest microbial lipid production (8.30 g/L) was obtained in the experiment where ammonium sulfate was used as the nitrogen source [12]. The highest lipid production reported in that study was considerably higher than the maximum lipid production obtained in the present study (4.38 g/L). This difference may be attributed to the higher initial substrate concentration (60 g/L) used in the aforementioned study, whereas the substrate concentration in the present study was 30 g/L, which likely resulted in lower lipid production.

In another study, the effects of culture conditions including nitrogen source and concentration, xylose concentration, and inoculation level on cell growth and lipid production were evaluated using *M. isabellina*. The microorganism was cultivated for six days at 28°C and 160 rpm in a medium containing 30 g/L xylose and 7.58 mM nitrogen. Initially, different organic and inorganic nitrogen sources including yeast extract, urea, peptone, glycine, KNO_3, NH_4NO_3, and $(NH_4)_2SO_4$ were used as the sole nitrogen source. The results showed that all nitrogen sources except KNO_3 were able to support cell growth. Different nitrogen sources resulted in varying amounts of biomass and lipid production. Among the organic nitrogen sources tested, the highest biomass (5.6 g/L) and lipid production (4.3 g/L) were obtained when yeast extract was used as the sole nitrogen source. Among the inorganic nitrogen sources, $(NH_4)_2SO_4$ provided the highest biomass and lipid production, with values of 5.3 g/L and 3.9 g/L, respectively [80]. Similarly, in the present study, the lowest biomass and lipid production were obtained from fermentations where KNO_3 was used as the nitrogen source. The highest microbial lipid production (4.38 g/L) was again obtained when yeast extract was used, whereas the highest biomass production (9.67 g/L) occurred in the medium containing $(NH_4)_2SO_4$.

These studies indicate that carbon and nitrogen sources are utilized differently by various fungal species. The optimum carbon and/or nitrogen source and its concentration for one fungal species cannot be generalized even to other species within the same genus. Nevertheless, it is also evident that yeast extract is consistently utilized efficiently by all mold species examined.

Effect of different inoculation ratios on biomass, lipid, and GLA production

Based on the data obtained in the previous stages of the one-factor-at-a-time method, fermentation media were prepared using DP-WP containing 30 g/L sugar and supplemented with 1.5 g/L yeast extract. The media were inoculated at rates of 1%, 3%, 5%, 7%, and 9%. The initial pH of the media was then adjusted to 7.0, which had previously been determined to be the pH value at which the highest GLA production occurred, and the fermentations were initiated.

Table 5 presents the results of the Duncan Multiple Comparison Test for the effects of different inoculation ratios on responses (the biomass and lipid production values, biomass lipid content, sugar consumption, biomass yield, GLA production, GLA yield, lipid yield, GLA/lipid ratio, and GLA/biomass ratio). Although the highest biomass production (10.41 g/L) was obtained from the fermentation with a 1% inoculation ratio, the highest biomass yield (38.41%) was achieved in the fermentation with a 5% inoculation ratio. The amounts of lipid produced were found to be very similar, and the differences between them were not statistically significant ($P > 0.05$). The highest biomass lipid content (61.04%) was obtained from fermentations with a 9% inoculation ratio, whereas the lowest lipid content (55.87%) was observed in fermentations with a 1% inoculation ratio. When the GLA production, GLA and lipid yields, and GLA/lipid

and GLA/biomass (%) ratios obtained from fermentations at different inoculation ratios were compared, the differences were found to be statistically significant ($P < 0.05$). The highest GLA production (1.08 g/L) and yield (4.25%) were obtained from the fermentation with a 9% inoculation ratio. The inoculation amount or inoculum volume plays an important role in single-cell oil production as the initial preculture in the growth medium. An optimum inoculation level is required to achieve maximum biomass and consequently higher lipid production. It has been reported that low inoculation levels may lead to an extended lag phase [81].

Table 5
Responses at different inoculation ratios: mean (X ± SE) and Duncan's test results

Inoculation ratio	Dry biomass (g/L)	Lipid (g/L)	Biomass lipid content (%)	Sugar consumption (g/L)	Biomass yield (%)	GLA (g/L)	GLA yield (%)	Lipid yield (%)	GLA/lipid (%)
%1	10.41^{a}±0.24	5.82^{a}±0.18	55.87^{b}±0.54	27.41^{a}±0.34	37.98^{ba}±0.55	0.71^{c}±0.01	2.60^{c}±0.06	21.23^{b}±0.47	12.28^{c}±0.51
%3	9.90^{ba}±0.19	5.93^{a}±0.09	59.97^{a}±1.17	27.51^{a}±0.30	36.01^{b}±0.74	0.68^{cd}±0.02	2.46^{c}±0.10	21.55^{b}±0.17	11.41^{c}±0.52
%5	9.42^{bc}±0.17	5.62^{ab}±0.15	59.66^{a}±1.24	24.56^{b}±0.45	38.41^{a}±1.08	0.79^{b}±0.01	3.25^{b}±0.10	22.87^{a}±0.50	14.22^{b}±0.50
%7	9.17^{c}±0.19	5.42^{b}±0.11	59.13^{a}±0.94	25.49^{b}±0.28	35.96^{b}±0.67	0.64^{d}±0.01	2.50^{c}±0.06	21.25^{b}±0.44	11.80^{c}±0.43
%9	9.24^{c}±0.15	5.64^{ab}±0.06	61.04^{a}±0.77	25.47^{b}±0.20	36.28^{ba}±0.51	1.08^{a}±0.02	4.25^{a}±0.11	22.14^{ba}±0.30	19.21^{a}±0.44
Values carrying different letters in the same column are significantly different ($P < 0.05$).									
X ± SE: Mean ± standard error									

Figure 3 presents the GLA and lipid production (g/L) and GLA yield (%) values obtained from fermentations carried out at different inoculation ratios. It can be observed that both the highest GLA yield (%) and the highest GLA production were achieved in fermentations with a 9% inoculation ratio. Based on these results, subsequent experiments were conducted using a 9% inoculation ratio.

In a study investigating the effects of inoculum size on lipid and GLA production using disrupted mycelia of *Cunninghamella echinulata* CCRC 31840, the mycelia were fragmented by vortexing with glass beads for different durations (30, 60, 120, 180, and 240 s). The disrupted mycelia were then inoculated into 25 mL of medium at amounts of 0.2, 0.4, and 1.0 g, and incubated for five days at 25°C and 200 rpm. It was reported that the maximum GLA yield (860 mg/L), obtained under the condition with the longest vortex time (4 min) and highest mycelial weight (1.0 g) was more than twice that obtained under conditions with lower vortex times and mycelial weights [82]. The fact that the highest GLA production was achieved at the highest inoculum level supports the results obtained in the present study.

Microbial lipid production from rice straw acid hydrolysate using *Trichosporon fermentans* has also been reported. In that study, the effect of inoculation ratio on microbial lipid production was investigated under fermentation conditions including a total sugar concentration higher than 100 g/L, peptone concentration of 0.6 g/L, temperature of 25°C, initial pH of 7.0, and a fermentation time of 10 days. Inoculation ratios of 2.5%, 5%, 10%, 15%, and 20% were tested. The highest lipid production and biomass lipid content (12.1 g/L and 39.0%, respectively) were obtained at a 10% inoculation ratio. When the inoculation ratio exceeded 10%, although the biomass of *T. fermentans* increased slightly, the lipid content decreased. It was also determined that the inoculation ratio had little effect on the lipid composition of *T. fermentans* [83]. In contrast to that study, in the present work, the inoculation ratio did not significantly affect biomass and lipid production. However, it was found to be an important factor influencing GLA production.

In a study investigating the topic, microbial lipid production from deproteinized whey was carried out using *Yarrowia lipolytica* B9, and the effects of inoculum size, initial pH, and temperature on lipid production were evaluated for optimization purposes. The experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of deproteinized whey. In the first stage, the effect of inoculum size was examined in the range of 1–6 mL/100 mL. The results indicated that the highest biomass production (5.2 g/L) was obtained at an inoculum size of 5 mL, while the highest lipid production (1.34 g/L) was achieved at an inoculum size of 3 mL [84]. In the present study, the inoculation ratio was not found to have a statistically significant effect on lipid production. However, although the highest lipid production (5.93 g/L) was obtained at a 3% inoculation ratio, the highest biomass production (10.41 g/L) was achieved at a 1% inoculation ratio.

In another study investigating the effect of inoculum size on single-cell oil production from glucose and xylose using the oleaginous yeast *Lipomyces starkeyi*, fermentations were carried out at 30°C and 190 rpm for two days. It was determined that a higher inoculation ratio significantly increased biomass productivity. Furthermore, increasing the inoculation level further was found to enhance both biomass and lipid content [85].

In a study conducted for biodiesel production, optimization of single-cell oil synthesis using slaughterhouse lipid wastes by *Yarrowia lipolytica* MTCC 9520 was performed. The effects of fermentation time, pH, substrate concentration, inoculum size, and C/N ratio were evaluated as optimization parameters. Inoculation studies were carried out at levels of 2.5%, 5%, 7.5%, 10%, 12.5%, and 15%. The highest lipid production (2.35 g/L) and biomass production (3.5 g/L) were obtained at an inoculation level of 5% [86].

These results indicate that inoculation ratio has a significant effect on lipid biosynthesis and cell mass concentration. In general, each oleaginous microorganism has an optimum inoculation level for single-cell oil production, which varies depending on the microorganism and fermentation conditions [14]. In the present study, this optimum value for *Mortierella isabellina* (DSM 1414) cultivated on whey was determined to be 9%.

Determination of optimum operating conditions for maximum GLA production using the Box–Behnken design

In the second stage of the study, the parameters optimized in the first stage using the one-factor-at-a-time method were employed, and the initial sugar concentration of the medium (g/L), shaking speed (rpm), and fermentation temperature (°C) were optimized for maximum GLA production using the Box–Behnken design. The results of the fermentations carried out according to the Box–Behnken design are presented in Table 6. The highest biomass production (18.76 g/L) was obtained in experiment 12 (130 g/L initial sugar concentration, 120 rpm shaking speed, and 28°C temperature). The highest lipid production (7.05 g/L) was observed in experiment 14 (130 g/L initial sugar concentration, 180 rpm shaking speed, and 34°C temperature). However, the biomass with the highest lipid content (50.90%) was obtained from experiment 1. The highest biomass and lipid yields (44.93% and 20.80%, respectively) were achieved under the fermentation conditions of experiment 5, while the highest GLA yield (3.63%) was obtained in experiment 2. The highest GLA production (1.10 g/L) was achieved in experiment 5, where the sugar concentration was 80 g/L, the shaking speed was 120 rpm, and the fermentation temperature was 22°C. The lowest GLA production (0.02 g/L) was observed in experiment 3, where the shaking speed was 180 rpm, the sugar concentration was 30 g/L, and the fermentation temperature was 34°C.

Table 6
Results of fermentations carried out under the conditions defined by the Box–Behnken experimental design

Standart order	GLA production (g/L)	Biomass production (g/L)	Lipid production (g/L)	Biomass lipid content (%)	Sugar consumption (g/L)	GLA yield (%)	Lipid yield (%)	Biomass yield (%)	GLA/Lipid (%)	GLA/Biomass (%)
1	0.34 ± 0.02	10.21 ± 0.41	5.20 ± 0.24	50.90 ± 0.93	27.33 ± 0.72	1.22 ± 0.10	19.00 ± 0.54	37.33 ± 0.66	6.41 ± 0.43	3.27 ± 0.25
2	0.77 ± 0.06	6.83 ± 0.30	2.58 ± 0.19	37.83 ± 2.56	21.27 ± 0.46	3.63 ± 0.28	12.13 ± 0.73	32.12 ± 1.53	29.91 ± 1.10	11.30 ± 0.81
3	0.02 ± 0.00	4.17 ± 0.25	0.46 ± 0.10	11.02 ± 2.34	13.50 ± 0.89	0.13 ± 0.02	3.48 ± 0.99	31.07 ± 2.90	4.00 ± 0.25	0.42 ± 0.08
4	0.24 ± 0.01	7.15 ± 0.19	3.16 ± 0.10	44.19 ± 0.67	24.14 ± 0.36	0.99 ± 0.03	13.09 ± 0.36	29.62 ± 0.63	7.54 ± 0.39	3.33 ± 0.15
5	1.10 ± 0.08	13.92 ± 0.31	6.45 ± 0.15	46.29 ± 0.54	31.06 ± 1.43	3.57 ± 0.37	20.80 ± 1.08	44.93 ± 2.19	17.11 ± 1.05	7.92 ± 0.56
6	0.10 ± 0.01	4.43 ± 0.43	1.66 ± 0.11	40.22 ± 2.22	22.96 ± 0.88	0.45 ± 0.05	7.22 ± 0.61	18.04 ± 2.00	6.26 ± 0.40	2.51 ± 0.14
7	0.59 ± 0.02	11.97 ± 0.41	6.01 ± 0.19	50.26 ± 1.55	36.87 ± 1.37	1.61 ± 0.08	16.86 ± 0.78	32.49 ± 1.55	9.55 ± 0.17	4.95 ± 0.10
8	0.60 ± 0.08	11.60 ± 0.61	5.60 ± 0.35	48.29 ± 1.90	36.64 ± 1.82	1.64 ± 0.16	15.29 ± 0.81	31.66 ± 1.05	10.76 ± 0.98	5.20 ± 0.52
9	0.61 ± 0.09	11.95 ± 0.98	5.71 ± 0.77	48.85 ± 1.67	36.80 ± 1.95	1.66 ± 0.18	15.47 ± 1.57	32.45 ± 1.77	10.71 ± 0.50	5.10 ± 0.41
10	0.43 ± 0.00	8.33 ± 0.31	2.66 ± 0.10	32.00 ± 1.54	34.14 ± 1.01	1.26 ± 0.07	7.80 ± 0.29	24.39 ± 0.44	16.18 ± 0.47	5.18 ± 0.30
11	0.15 ± 0.01	9.18 ± 0.42	3.55 ± 0.14	38.67 ± 0.72	37.54 ± 0.46	0.41 ± 0.02	9.45 ± 0.42	24.46 ± 1.28	4.33 ± 0.30	1.68 ± 0.12
12	0.50 ± 0.01	18.76 ± 0.63	5.86 ± 0.29	31.20 ± 0.54	67.54 ± 1.98	0.74 ± 0.02	8.67 ± 0.21	27.78 ± 0.27	8.53 ± 0.39	2.66 ± 0.08
13	0.68 ± 0.07	11.67 ± 1.33	5.39 ± 0.61	46.20 ± 1.73	42.18 ± 2.25	1.62 ± 0.23	12.85 ± 1.94	27.58 ± 1.73	12.65 ± 0.24	5.96 ± 1.21
14	0.21 ± 0.03	14.42 ± 0.76	7.05 ± 0.63	48.83 ± 2.20	58.33 ± 1.97	0.36 ± 0.05	12.09 ± 1.02	24.73 ± 1.31	3.02 ± 0.58	1.46 ± 0.23
15	0.15 ± 0.00	7.68 ± 0.36	1.57 ± 0.03	20.50 ± 0.80	33.74 ± 0.08	0.45 ± 0.01	4.64 ± 0.15	22.67 ± 1.50	9.82 ± 0.40	2.01 ± 0.14

For the optimization of the initial sugar concentration (g/L), shaking speed (rpm), and fermentation temperature (°C) using the Box–Behnken design, the amount of GLA produced was selected as the response variable. The GLA production obtained in each fermentation was considered as the response for that particular experiment. Accordingly, optimization procedures and variance (ANOVA) analyses of the obtained data were performed using the Design Expert 10.0.0 software.

The results of the variance analysis conducted according to the two-factor model proposed by Design Expert (Version 10.0.1) are presented in Table 7. The statistical significance levels of the linear, interaction, and quadratic effects of each independent variable on the response are also provided in the table. The results indicated that the model was statistically significant ($P < 0.0001$), while the lack-of-fit test was not statistically significant ($P > 0.05$). It was observed that the model adequately represented the experimental data with a coefficient of determination (R^2) of 0.996. Furthermore, shaking speed (B), temperature (C), the interactions between all independent variables (A×B, A×C, B×C), and the quadratic effects of variables A and B were found to have statistically significant effects on GLA production ($P < 0.05$).

Table 7
Analysis of variance (ANOVA) results for the effects of initial sugar concentration, shaking speed, and temperature on GLA production

Variance source		**Sum of squares**	**DF**	**Mean square**	***F*-value**	***p*-value**
Model		1.25	9	0.14	151.50	< 0.0001*
A- Initial sugar		0.0039	1	0.0039	4.28	0.0935
B- Shaking speed		0.14	1	0.14	158.53	< 0.0001*
C- Temperature		0.78	1	0.78	857.25	< 0.0001*
AxB		0.015	1	0.015	16.00	0.0103***
AxC		0.020	1	0.020	21.96	0.0054**
BxC		0.13	1	0.13	141.68	< 0.0001*
A^2		0.095	1	0.095	104.24	0.0002*
B^2		0.066	1	0.066	71.94	0.0004*
C^2		0.0018	1	0.0018	1.98	0.2184
Residual error		0.0046	5	0.0009		
Lack of fit		0.0044	3	0.0015	18.76	0.0510
Pure error		0.0002	2	0.0001		
Total		1.25	14			
R^2	Adjusted R^2	Predicted R^2	CV%		Adequate precision	
0.996	0.989	0.943	6.97		42.96	

Note: A: initial sugar concentration (g/L), B: shaking speed (rpm), C: temperature (°C). *$P < 0.001$, **$P < 0.01$, ***$P < 0.05$ represent statistically significant parameter.

It can be understood from both Fig. 4 and Table 7 that the predicted values of the model are in good agreement with the experimental data. It is desirable that the predicted R^2 and adjusted R^2 values are close to each other (with a difference of less than 0.2), which indicates a strong predictive ability of the model. Furthermore, the proximity of R^2 and adjusted R^2 values suggests that the model successfully represents the experimental data. Adequate precision measures the signal-to-noise ratio and should be greater than 4 (> 4). An adequate precision value greater than 4 indicates the presence of an adequate signal. In this study, the adequate precision value of the model was 42.96 (> 4), demonstrating that the model is not affected by noise. Additionally, the coefficient of variation (CV) value (6.97%), being below 10%, indicates an excellent level of model fit [87].

The response surface and contour plots illustrating the effects of sugar concentration (A) and shaking speed (B) on maximum GLA production are presented in Fig. 5. While constructing the plots, the third variable, temperature (C), was kept constant at 28°C. As shown in the figures, the highest GLA production (0.60 g/L) was achieved at a sugar concentration of 80 g/L and a shaking speed of 180 rpm. The lowest GLA production (0.15 g/L) was observed in experiment 15, conducted at 240 rpm and 130 g/L sugar concentration. When the temperature was kept constant (28°C), increases in shaking speed and sugar concentration had a positive effect on GLA production up to a certain point (80 g/L sugar and 180 rpm). However, further increases beyond these levels negatively affected GLA production. At 28°C and 30 g/L sugar concentration, GLA production was 0.34 g/L in experiment 1 conducted at 120 rpm, whereas it decreased to 0.24 g/L in experiment 4 conducted at 240 rpm. Similarly, at 28°C and 130 g/L sugar concentration, GLA production was 0.50 g/L in experiment 12 conducted at 120 rpm, while it decreased to 0.15 g/L in experiment 15 conducted at 240 rpm. Under constant temperature conditions (28°C) and an shaking speed of 240 rpm, GLA production decreased from 0.24 g/L at 30 g/L sugar concentration (experiment 4) to 0.15 g/L when the sugar concentration was increased to 130 g/L.

The response surface and contour plots illustrating the effects of sugar concentration (A) and fermentation temperature (C) on GLA production are presented in Figs. 6. While constructing the plots, the third variable, shaking speed (B) was kept constant at 180 rpm. When the sugar concentration was kept constant at 30 g/L, GLA production decreased from 0.77 g/L at 22°C (experiment 2) to 0.02 g/L when the fermentation temperature was increased to 34°C

(experiment 3). Similarly, when the sugar concentration was maintained at 130 g/L, GLA production decreased from 0.68 g/L at 22°C (experiment 13) to 0.21 g/L at 34°C (experiment 14). Based on these results, it was observed that, at a constant shaking speed, a decrease in fermentation temperature led to an increase in GLA production. When the fermentation temperature was kept constant at 22°C, GLA production decreased from 0.77 g/L at 30 g/L substrate concentration to 0.68 g/L when the substrate concentration was increased to 130 g/L. In contrast, when the temperature was maintained at 34°C, GLA production increased from 0.02 g/L at 30 g/L (experiment 3) to 0.21 g/L at 130 g/L (experiment 14).

The effects of shaking speed (B) and fermentation temperature (C) on GLA production are presented in Fig. 7. While constructing the plots, the sugar concentration (A) was kept constant at 80 g/L. Under these conditions, the highest GLA production (1.10 g/L) was obtained in experiment 5, conducted at a shaking speed of 120 rpm and a temperature of 22°C, as also evident from the figures. When the shaking speed was kept constant at 120 rpm and the fermentation temperature was increased to 34°C, the GLA production decreased to 0.10 g/L. Similarly, when the shaking speed was fixed at 240 rpm, increasing the temperature from 22°C to 34°C resulted in a decrease in GLA production from 0.43 g/L to 0.15 g/L. When the fermentation temperature was maintained at 22°C, increasing the shaking speed from 120 rpm to 240 rpm reduced GLA production from 1.10 g/L to 0.43 g/L. However, when the fermentation temperature was kept constant at 34°C, increasing the shaking speed from 120 rpm (experiment 6) to 240 rpm (experiment 11) led to a slight increase in GLA production from 0.10 g/L to 0.15 g/L. Nevertheless, under these conditions, the lipid content of the biomass decreased from 40.22% (experiment 6) to 38.67% (experiment 11), and the GLA content in the lipid decreased from 6.26% (experiment 6) to 4.33% (experiment 11).

Optimization of fermentation conditions for maximum GLA production by Mortierella isabellina

According to the experimental design generated by the Box–Behnken response surface methodology, 15 different fermentations were conducted under the specified parameter conditions. The GLA amounts determined from these fermentations were used as the response variable for each experimental run. Accordingly, the optimum conditions for maximizing GLA production were determined using the Design-Expert software. The optimum conditions selected among the solutions generated by the program are presented in Table 8. The desirability function value of this solution was determined to be 0.973. The desirability function (D) ranges between 0 and 1, and as the D value approaches 1, the response becomes closer to the desired optimum [88]. Under the conditions presented in Table 8, the predicted GLA production was 1.07 g/L.

Table 8
Optimum conditions determined by the Box–Behnken response surface design and the predicted and experimental GLA production values obtained under these conditions.

Independent variable	Lower limit	Upper limit	Optimum value	Desirability function	Predicted GLA production (g/L)
Sugar concentration (g/L)	30	130	81.87	0.973	1.074
Shaking speed (rpm)	120	240	120		
Temperature (°C)	22	34	22		

Based on all the obtained data, fermentation was carried out under the optimum conditions using a medium prepared with DP-WP containing 81.87 g/L sugar and supplemented with 1.5 g/L yeast extract. The medium was inoculated with 9% preculture, and the initial pH was adjusted to 7. Fermentations were conducted at 22°C and 120 rpm for 10 days. During the fermentation period, samples were taken daily to determine biomass, microbial lipid, GLA production, and residual sugar concentration in the medium. Based on these data, kinetic parameters were calculated.

Validation fermentation under the optimum conditions predicted by the Box–Behnken response surface methodology

The results of the Duncan Multiple Comparison Test for the data obtained during fermentation are presented in Table 9. Accordingly, the changes in biomass, lipid, GLA production, sugar consumption, and biomass lipid content during fermentation were found to be statistically significant ($P < 0.001$). At the end of fermentation, 14.15 g/L biomass, 6.31 g/L microbial lipid, and 1.15 g/L GLA were produced. The highest lipid content of the biomass (44.60%) was reached on day 10.

Table 9
Validation fermentation results: biomass, lipid, GLA, and residual sugar (X ± SE) with Duncan's test

Time (day)	Dry biomass (g/L)	Lipid (g/L)	GLA (g/L)	Biomass lipid content (%)	Total sugar (g/L)
0	0.19^{h}±0.01	0.00^{i}±0.00	0.00^{h}±0.00	0.00^{g}±0.00	82.67^{a}±0.36
1	2.78^{g}±0.05	0.65^{gh}±0.00	0.03^{gh}±0.02	23.54^{e}±0.50	76.45^{b}±0.15
2	3.10^{g}±0.09	0.54^{h}±0.03	0.05^{gh}±0.01	17.28^{f}±0.60	71.58^{c}±0.02
3	4.58^{f}±0.24	0.81^{g}±0.04	0.09^{gf}±0.01	17.60^{f}±0.33	68.38^{d}±0.31
4	7.14^{e}±0.31	1.64^{f}±0.11	0.14^{ef}±0.00	22.97^{e}±1.40	64.08^{e}±0.31
5	8.933^{d}±0.27	2.61^{e}±0.08	0.16^{e}±0.01	29.26^{c}±0.33	59.62^{f}±0.31
6	10.24^{c}±0.31	2.76^{e}±0.09	0.19^{e}±0.01	26.99^{d}±0.55	54.87^{g}±0.36
7	12.27^{b}±0.28	3.81^{d}±0.07	0.44^{d}±0.02	31.06^{c}±0.79	51.26 ± 0.44
8	13.59^{a}±0.24	4.85^{c}±0.15	0.52^{c}±0.03	35.68^{b}±0.86	48.86^{i}±0.45
9	14.16^{a}±0.42	5.12^{b}±0.12	0.65^{b}±0.01	36.18^{b}±0.55	45.30^{j}±0.30
10	14.15^{a}±0.20	6.31^{a}±0.10	1.15^{a}±0.06	44.60^{a}±0.18	41.88^{k}±0.39
Values carrying different letters in the same column are significantly different (P < 0.05)					
X ± SE: Mean ± standard error					

Figure 8 shows the time-dependent changes in residual sugar concentration (g/L) in the medium, as well as GLA (g/L), lipid (g/L), and biomass (g/L) production over a 10-day fermentation period under optimized conditions. Examination of biomass changes indicates that biomass increased rapidly on the first day of fermentation, reached its maximum level on day 9, and then the rate of biomass formation slowed down. Lipid production was very slow during the first three days of fermentation but began to increase rapidly after day 3. This behavior is likely due to the depletion of nitrogen in the medium as it is consumed by the cells, which triggers the transition of cells into the lipid accumulation phase and consequently accelerates lipid production. Lipid production increased throughout the fermentation and reached its maximum level on day 10. GLA production was observed to increase markedly after day 7 and reached its maximum level on day 10. From the graph, it can be seen that as the rate of biomass production began to decline on day 7, the rate of GLA production increased. Furthermore, when biomass production ceased on day 10, both lipid and GLA production rates increased significantly.

Table 10 presents the kinetic parameters obtained from fermentations carried out under the optimized conditions for maximum GLA production. At the end of fermentation, sugar consumption by the microorganism was determined to be 40.78 g/L, corresponding to a sugar utilization rate of 49.33%. Biomass production, lipid production, and GLA production were found to be 13.96 g/L, 6.31 g/L, and 1.15 g/L, respectively. The yields of biomass, lipid, and GLA based on the consumed sugar were calculated as 34.22%, 15.47%, and 2.81%, respectively. The maximum rates of sugar consumption, lipid production, and GLA production were calculated as 6.21 g/L/day, 1.19 g/L/day, and 0.50 g/L/day, respectively. Under these optimized fermentation conditions for maximum GLA production, the maximum growth rate of *Mortierella isabellina* (DSM 1414) was determined to be 1.45 g/L/day, the specific growth rate was 1.39 day^{-1}, and the doubling time was 0.50 day.

Table 10
Kinetic parameter values obtained from the validation fermentation

Kinetic parameters	
Sugar consumption (g/L)	40.78
Lipid production (g/L)	6.31
Biomass production (g/L)	13.96
GLA production (g/L)	1.15
Yield (Y_{GLA}/s) (%)	2.81
Yield (Y_L/s) (%)	15.47
Yield (Yx/s) (%)	34.22
Maximum consumption rate $(-ds/dt)_{maks}$ (g/L/day)	6.21
Maximum production rate $(d_{GLA}/dt)_{maks}$ (g/L/day)	0.50
Maximum production rate $(d_L/dt)_{maks}$ (g/L/day)	1.19
Maximum growth rate $(dx/dt)_{maks}$ (g/L/day)	1.45
Specific growth rate (day^{-1})	1.39
Doubling time (t_d) (day)	0.50
Sugar utilization rate (%)	49.33

Following production under optimum conditions, the dependent variable values were evaluated at a 95% confidence level using the validation function of the Design Expert® software after analysis. Accordingly, it was observed that the experimental data obtained fell within the maximum and minimum values reported in the table (Table 11).

Table 11
Experimental and predicted values obtained under optimum conditions.

Dependent variable (response)	**Predicted value**	**Experimental value**	**Minimum value**	**Maximum value**
GLA production (g/L)	1.07	1.15	0.97	1.18

It has been reported that microorganisms produce PUFAs under low-temperature conditions in order to maintain membrane fluidity [72, 89]. In a study conducted with psychrophilic, thermotolerant, and thermophilic fungi belonging to the order Mucorales, the effect of growth temperature on fatty acid composition was investigated. It was observed that cultivation of the strains at lower temperatures generally increased the proportion of unsaturated fatty acids in microbial lipids [90]. Based on these findings, the results obtained from the optimization in the present study can be explained, as the optimum temperature for maximum GLA production (22°C) corresponds to the lowest temperature evaluated in this work.

In a study conducted to determine the effects of temperature and cell age on the degree of unsaturation of the fatty acid composition in the fungus *Mucor rouxii*, three different temperatures were investigated. The proportion of GLA in total fatty acids reached a maximum value of 7.9% after 96 h in cells grown at 35°C. When the cells were cultivated at 25°C, the GLA proportion increased to 13% after 72 h of growth. In cells grown at 15°C, the GLA content further increased to 20.1% of total fatty acids. It was observed that the degree of unsaturation increased with decreasing growth temperature [91]. The increase in GLA content with decreasing temperature is consistent with the results obtained in the present study.

In a study conducted using *M. ramanniana* CBS 112.08 in 400 mL culture media containing an initial glucose concentration of 28.7 g/L, the effect of temperature on GLA production was investigated by performing fermentations at 20, 25, and 30°C. The highest biomass concentration (10.2 g/L) was obtained on day 6 of fermentation at 25°C. The lipid content of this biomass was 15.9% (w/w), and the GLA content in the lipid was found to be 17.2% (w/w), corresponding to a GLA production of 0.28 g/L. The highest lipid content in biomass (25.1%) was obtained from cells grown at 30°C for 6 days. However, this condition also resulted in the lowest biomass concentration (5.9 g/L) and the lowest GLA content in the lipid (14.6% w/w). The highest GLA proportion in the lipid (21% w/w) was observed on day 3 of fermentation at 20°C. The maximum GLA production (0.32 g/L) was achieved on day 6 of fermentation at 20°C. It was determined that decreasing fermentation temperature increased both the degree of lipid unsaturation and the amount of GLA produced [78].

Lindberg and Molin [74] investigated the effect of temperature on PUFA production using *Mortierella alpina* CBS 343.66. Fermentations with a duration of 166 h were carried out at 18°C and 12°C, and it was observed that decreasing the temperature resulted in a twofold increase in the proportion of GLA in total fatty acids (5% w/w at 18°C and 10% w/w at 12°C).

Aidil et al. [92] investigated the effects of the C/N (carbon/nitrogen) ratio of the substrate and temperature on lipid production and GLA content using four different oleaginous fungi (*Cunninghamella* sp. 2A1, *Absidia* sp. DR, *Botrytis* sp. K3, and *Geotrichum* sp. SK22). When *Cunninghamella* sp. 2A1, *Absidia* sp. DR,

and *Botrytis* sp. K3 were cultivated at different temperatures (15°C, 20°C, 25°C, and 30°C), it was found that all oleaginous fungi achieved maximum lipid production at 25°C and 30°C. However, different cultivation temperatures did not cause a significant change in GLA content.

In another study, the industrial potential of *Mortierella ramanniana* var. *ramanniana* for GLA production was investigated. Production parameters, including pH, temperature, carbon source, nitrogen source, metal ions, and oil supplementation, were systematically optimized. The results demonstrated that GLA production could be enhanced by incubating the strain at 20°C in a basal medium containing 5% dextrose and 1% yeast extract supplemented with 5 mg/L Mn^{2+}. Under optimum growth conditions, the lipid yield reached 54.2% of total dry biomass, and the unsaturated fatty acid content of the lipid was determined to be 84.3%. The GLA yield per gram of dry biomass was found to be 13.3%, which was slightly higher than the GLA content typically found in evening primrose oil [72].

In another study, the effects of culture medium and conditions on the enhancement of PUFA production using fungi of the genus *Mortierella* were investigated. Eleven different microorganisms were tested, and *M. alpina* ATCC 32222 was selected for further study. To evaluate the effect of temperature, fermentations were carried out in a basal medium at 12–28°C for 7 days with a shaking speed of 200 rpm. The highest biomass production (7.2 g/L) was obtained at 20°C, followed by 15°C and 12°C (6.3 g/L and 6.2 g/L, respectively), while the lowest biomass production (6.0 g/L) was observed at 25°C. It was determined that low temperatures promoted the production of highly unsaturated fatty acids such as ARA, EPA, LA, and GLA. Total PUFA yields were high at 12°C and 15°C but decreased when the incubation temperature exceeded 20°C. In the study conducted with *M. alpina* ATCC 32222, GLA production levels of 164.9 mg/L, 161.0 mg/L, 96.3 mg/L, and 62.2 mg/L were reported at 12°C, 15°C, 20°C, and 25°C, respectively [93]. Consistent with these findings, the present study also demonstrated that decreasing temperature increased GLA production, with a maximum GLA concentration of 1.15 g/L achieved at 22°C.

In a study on the fermentative production of GLA using seven different strains belonging to the order *Mucorales*, *Mucor* sp. RRL001 was selected, and the effects of initial glucose concentration, pH, temperature, and nitrogen source on biomass, lipid, and GLA production were investigated. Initial sugar concentrations of 25, 50, 75, 100, 125, and 150 g/L were evaluated, and it was determined that biomass and lipid production increased proportionally with sugar concentration up to 100 g/L. The maximum dry biomass and lipid production (35.46 g/L and 6.3 g/L, respectively) were achieved at 100 g/L, while higher concentrations resulted in a decline in both parameters. The highest GLA content (8.8% w/w of biomass) was obtained at an initial sugar concentration of 25 g/L [75]. When compared with the results of the present study, although the highest GLA content in biomass (11.30%) was obtained at an initial sugar concentration of 30 g/L, it is evident that initial sugar concentration alone is not the sole determining factor.

The effect of temperature on GLA production was investigated in the range of 20–35°C, and the highest GLA content (8.2%) was obtained at 20°C, with increasing temperature leading to a decrease in GLA content [75]. In the present study, the effect of temperature on GLA production was evaluated at 22, 28, and 34°C, and the highest GLA content was obtained at 22°C. When the results are compared, it is clear that the findings are consistent with each other.

In a study investigating the conversion of waste glycerol into value-added compounds (biomass, single-cell oil, and oxalic acid) using *Aspergillus niger* NRRL 364, the effect of shaking speed on biomass production was evaluated by testing shaking rates of 180, 60, and 0 rpm. The highest biomass production (1.2 g/L) was obtained at 60 rpm on day 12 of fermentation [94].

In another study on the optimization of microbial lipid production from *Rhodotorula* 110, lipid and biomass productions of 7.34 g/L and 13.04 g/L were achieved at 150 rpm, while at 200 rpm, lipid and biomass productions were 6.83 g/L and 12.64 g/L, respectively. It was observed that lower shaking speeds enhanced both lipid and biomass production [95]. This observation is consistent with the results obtained from the optimization fermentations conducted in the present study.

In another study, the effects of physical fermentation parameters on lipid production were investigated using *Yarrowia lipolytica* M7 and *Candida gali*. The influence of medium pH (4–7), shaking speed (100, 150, and 200 rpm), temperature (15, 25, 35, and 45°C), and incubation time (24, 48, 72, and 96 h) on lipid production was evaluated. Initially, the optimum pH was determined by adjusting the medium pH to 4, 5, 6, and 7 while keeping other parameters constant. A similar approach was applied for the optimization of the remaining parameters. For *Candida gali*, the highest lipid and biomass production (3.73 g/L and 16.78 g/L, respectively) were obtained at an initial pH of 6, while the highest biomass lipid content (23.21%) was achieved at pH 7. In the experiments evaluating shaking speed, the highest lipid and biomass production and biomass lipid content (4.15 g/L, 17.11 g/L, and 24.25%, respectively) were obtained at 200 rpm. In the temperature optimization experiments, the highest lipid and biomass production and biomass lipid content (4.25 g/L, 16.89 g/L, and 25.16%, respectively) were achieved at 25°C. For *Y. lipolytica* M7, the highest lipid (4.78 g/L) and biomass production (18.52 g/L) were obtained at an initial pH of 5, while the highest lipid content in biomass (25.95%) was observed at pH 6. In the subsequent optimization step, the highest lipid and biomass production and biomass lipid content (5.25 g/L, 19.23 g/L, and 27.30%, respectively) were achieved at 200 rpm. In the temperature optimization experiments, the highest lipid production and biomass lipid content (5.59 g/L and 28.15%, respectively) were obtained at 25°C, whereas the highest biomass production (19.54 g/L) occurred at 35°C. For both microorganisms, increasing the fermentation temperature from 15°C to 25°C led to an increase in lipid production and biomass lipid content, while temperatures above 25°C resulted in a decrease in these responses [96].

In another study, *Mortierella alpina* LPM-301 was cultivated in a glycerol-containing medium, and the effects of initial pH and fermentation temperature on lipid and ARA synthesis were investigated. The highest biomass production (21.2 g/L) was obtained at an initial pH of 8, while the highest lipid production (6.9 g/L) was achieved at pH 7. When the effect of initial pH on fatty acid composition was examined, GLA was not detected at pH 3, whereas the highest GLA content in the lipid (3.1%) was observed at pH 6. Regarding the effect of temperature, the highest biomass and lipid production (17.4 g/L and 1.9 g/L, respectively) were obtained at 26°C, while the highest GLA content in the lipid (4.8%) was achieved at 28°C [97].

In a study investigating suitable fermentation conditions for the production of fatty acids such as AA, DHA, and EPA using submerged culture fermentation, *Aspergillus oryzae* was cultivated for six days in a medium containing glucose as the carbon source and ammonium sulfate and yeast extract as nitrogen

sources. The optimum shaking speed and temperature for unsaturated fatty acid production by *A. oryzae* were determined to be 120 rpm and 25°C, respectively [98].

In another study, the effects of temperature and shaking speed on the growth of *Mortierella alpina* and the fatty acid composition of the produced lipids were investigated. The fungus was incubated in a liquid medium for 7 days at six different temperatures (10, 15, 20, 25, 30, and 35°C) and seven different shaking speeds (100, 125, 150, 175, 200, 225, and 250 rpm). The results demonstrated that fermentation temperature and shaking speed significantly affected fungal growth and fatty acid composition. The optimum temperature for biomass production, total fatty acid accumulation, and PUFA accumulation was determined to be 20°C, while the optimum shaking speed was 175 rpm. It was reported that cell growth ceased at 30°C or at a shaking speed of 250 rpm [99].

It is evident that the optimum fermentation conditions required for maximum growth, lipid production, and GLA synthesis vary among microorganisms. Based on the results of the present study, in which whey was used as the substrate, the optimum fermentation conditions for maximum GLA production by *Mortierella isabellina* DSM 1414 were determined as follows:

- • Initial medium pH of 7
- • Yeast extract as the nitrogen source
- • 9% inoculation ratio
- • Fermentation temperature of 22°C
- • Shaking speed of 120 rpm
- • Initial sugar concentration of 81.87 g/L

Conclusions

In this study, whey was evaluated as a cost-effective and sustainable substrate for microbial lipid and γ-linolenic acid (GLA) production by *Mortierella isabellina* DSM 1414, and key fermentation parameters were optimized to enhance GLA yield.

In the first stage of the study, fermentations were carried out using the one-factor-at-a-time approach. Within this scope, the effects of initial medium pH (4, 5, 6, 7, and 8), nitrogen source (ammonium sulfate, meat extract, yeast extract, potassium nitrate, and soybean peptone), and inoculation ratio (1%, 3%, 5%, 7%, and 9%) on GLA production were investigated. Throughout these experiments, initial sugar concentration (30 g/L), shaking speed (180 rpm), and fermentation temperature (28°C) were kept constant, and fermentations were conducted by varying only one parameter at a time.

Initially, fermentations were performed at different initial pH values of the medium. The highest GLA production (0.55 g/L) was obtained at an initial pH of 7 ($P < 0.05$). Based on these findings, all subsequent fermentations were carried out at an initial pH of 7. At the end of the experiments evaluating the effect of nitrogen source, the highest GLA production (0.70 g/L) was achieved in fermentations where yeast extract was used as the nitrogen source ($P < 0.05$). In the experiments assessing the effect of inoculation ratio, the highest GLA production (1.08 g/L) was obtained at a 9% inoculation level ($P < 0.05$).

Based on the results obtained using the one-factor-at-a-time method, the optimized fermentation conditions for GLA production (initial pH of 7, 1.5 g/L yeast extract as the nitrogen source, and 9% inoculation ratio) were selected for the second stage of the study.

In the second stage, the effects of initial sugar concentration (g/L), shaking speed (rpm), and fermentation temperature (°C) on maximum GLA production were investigated using an experimental design based on the Box–Behnken response surface methodology. For these three factors, a three-level design consisting of 15 experimental runs, including three center points, was constructed. Analysis of variance (ANOVA) results and contour plots of the model indicated that lower fermentation temperature and shaking speed enhanced GLA production. In addition, it was determined that selecting an initial sugar concentration in the range of 72–87 g/L increased GLA production.

The GLA amounts obtained from the fermentation experiments were used as the response variable, and the optimum conditions for maximizing GLA production were determined using Design-Expert software. The optimal conditions were identified as an initial sugar concentration of 81.87 g/L, a shaking speed of 120 rpm, and a fermentation temperature of 22°C, with a desirability function value of 0.973. Under these optimized conditions, validation fermentation resulted in the production of 1.15 g/L GLA and 6.31 g/L microbial lipid. The GLA yield and lipid yield were determined to be 2.81% ($Y_{GLA/S}$) and 15.47% ($Y_{L/S}$), respectively.

The results of this study demonstrated that optimization of fermentation parameters can significantly enhance GLA production. Using *Mortierella isabellina* DSM 1414, microbial oil containing approximately 20% GLA was successfully produced from whey, a by-product of the dairy industry. Thus, a low-cost substrate was effectively converted into GLA, a high-value compound with well-documented health benefits and widespread pharmaceutical applications. These findings can contribute to the development of sustainable bioprocesses for the production of high-value fatty acids and offer a foundation for future scale-up and industrial implementation.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Aysen Güher GÜNDEŞ: Data curation, Formal analysis, Investigation, Writing-original draft. **Ahmet KÜÇÜKÇETİN**: Data curation. **Muammer DEMİR**: Writing-review & editing, Validation, Supervision, Project administration.

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Data Availability

Data will be made available on request.

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Figures

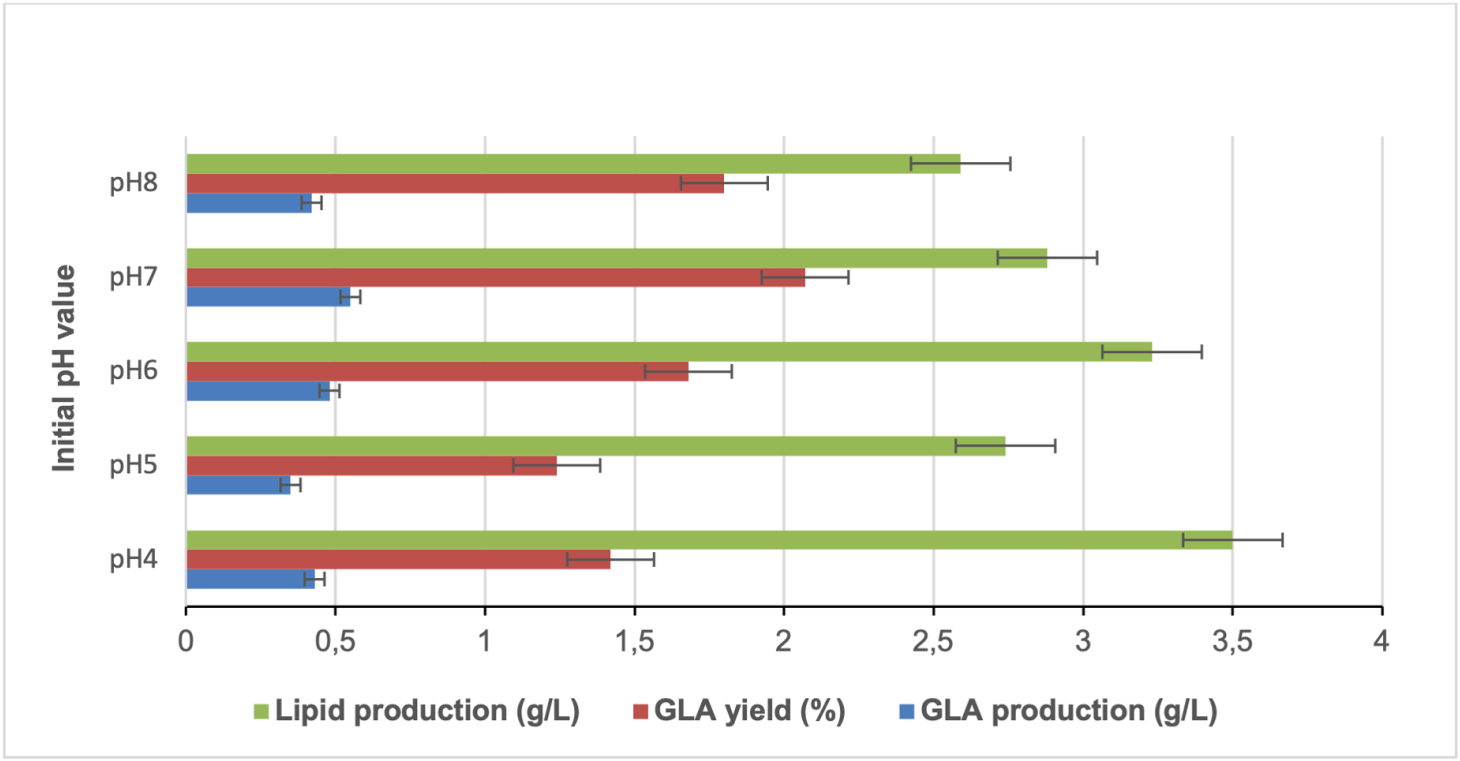


Figure 1

GLA and lipid production (g/L) and GLA yield (%) obtained from fermentations conducted at different initial medium pH values

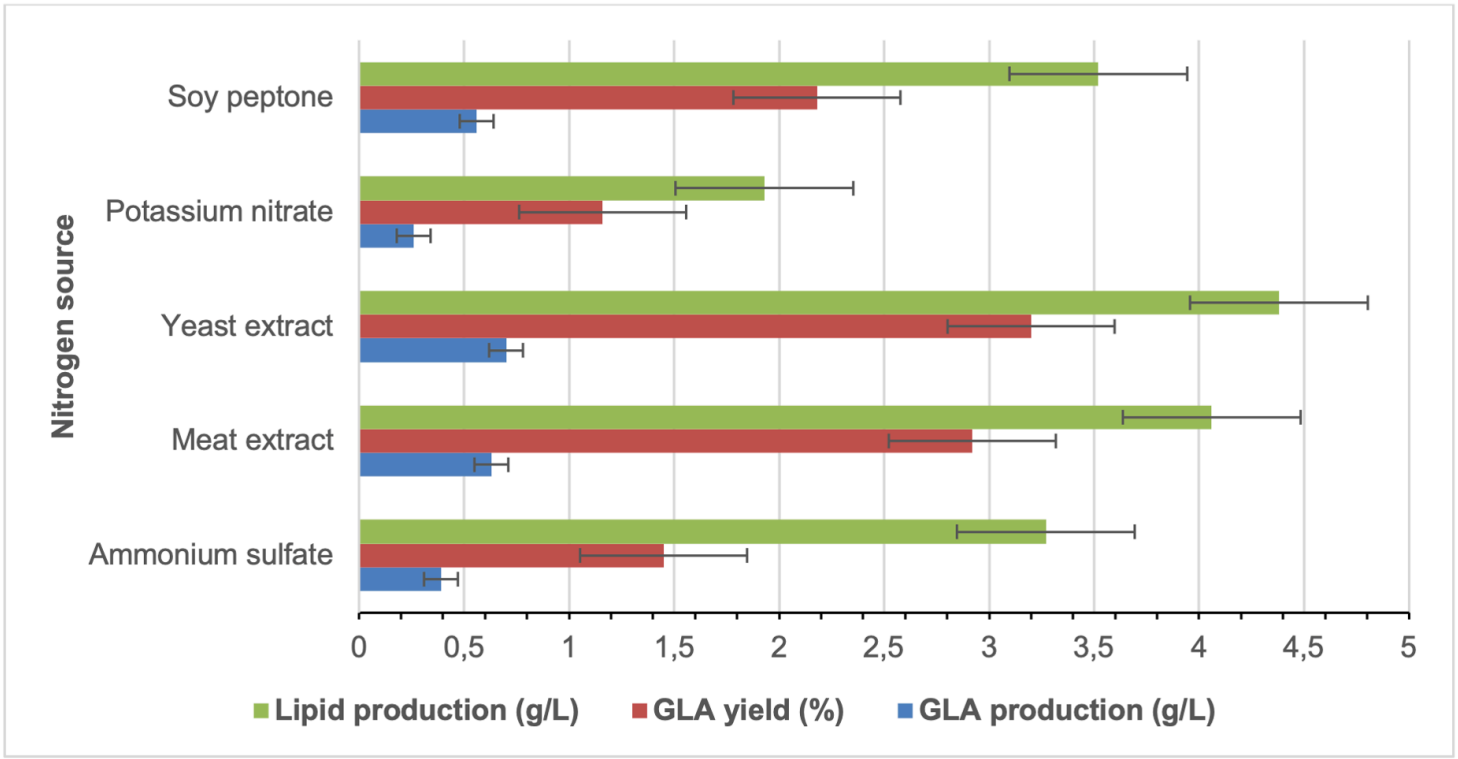


Figure 2

GLA production, lipid production (g/L) and GLA yield (%) obtained from fermentations conducted using different nitrogen sources

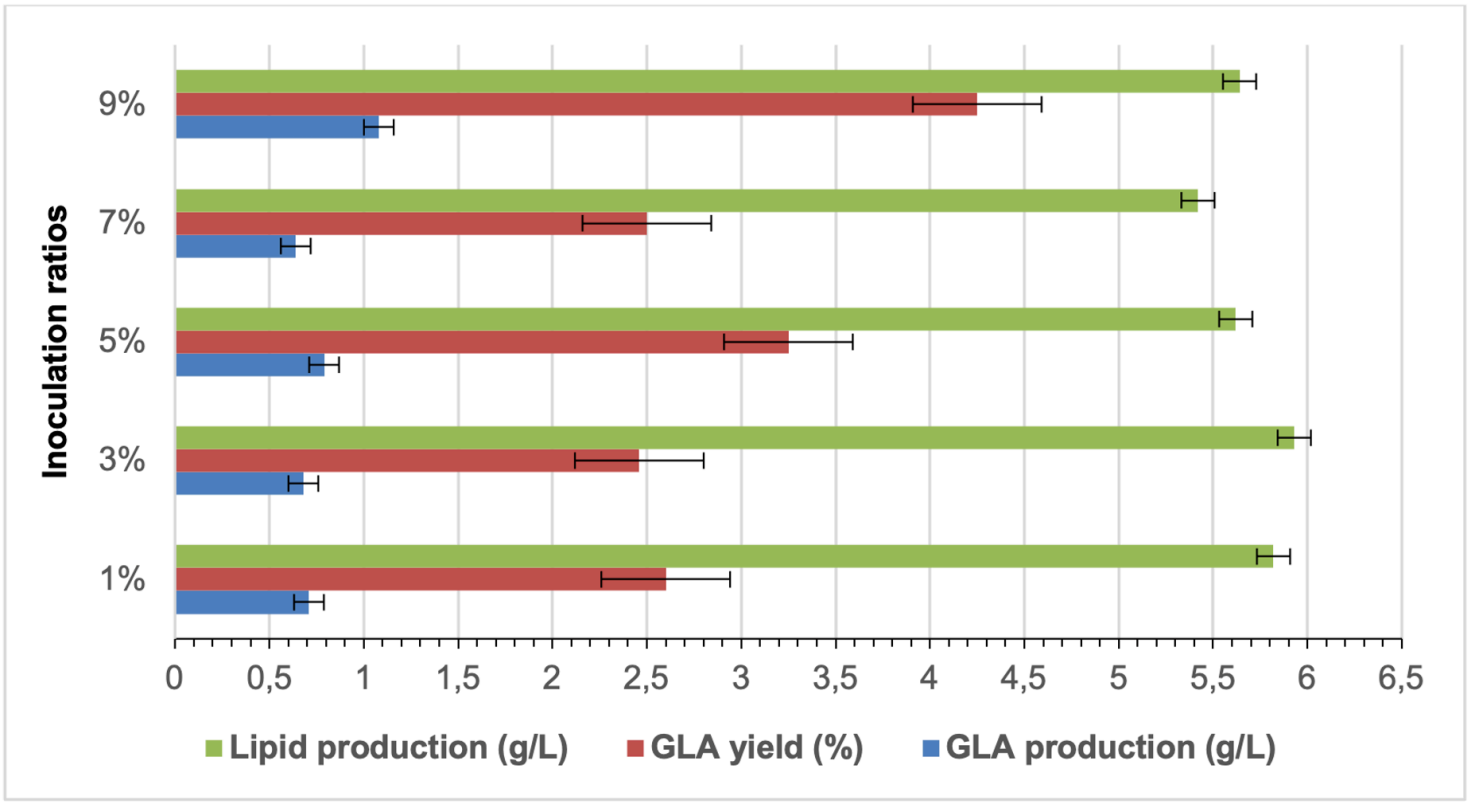


Figure 3

GLA production, lipid production (g/L) and GLA yield (%) obtained from fermentations conducted at different inoculation ratios

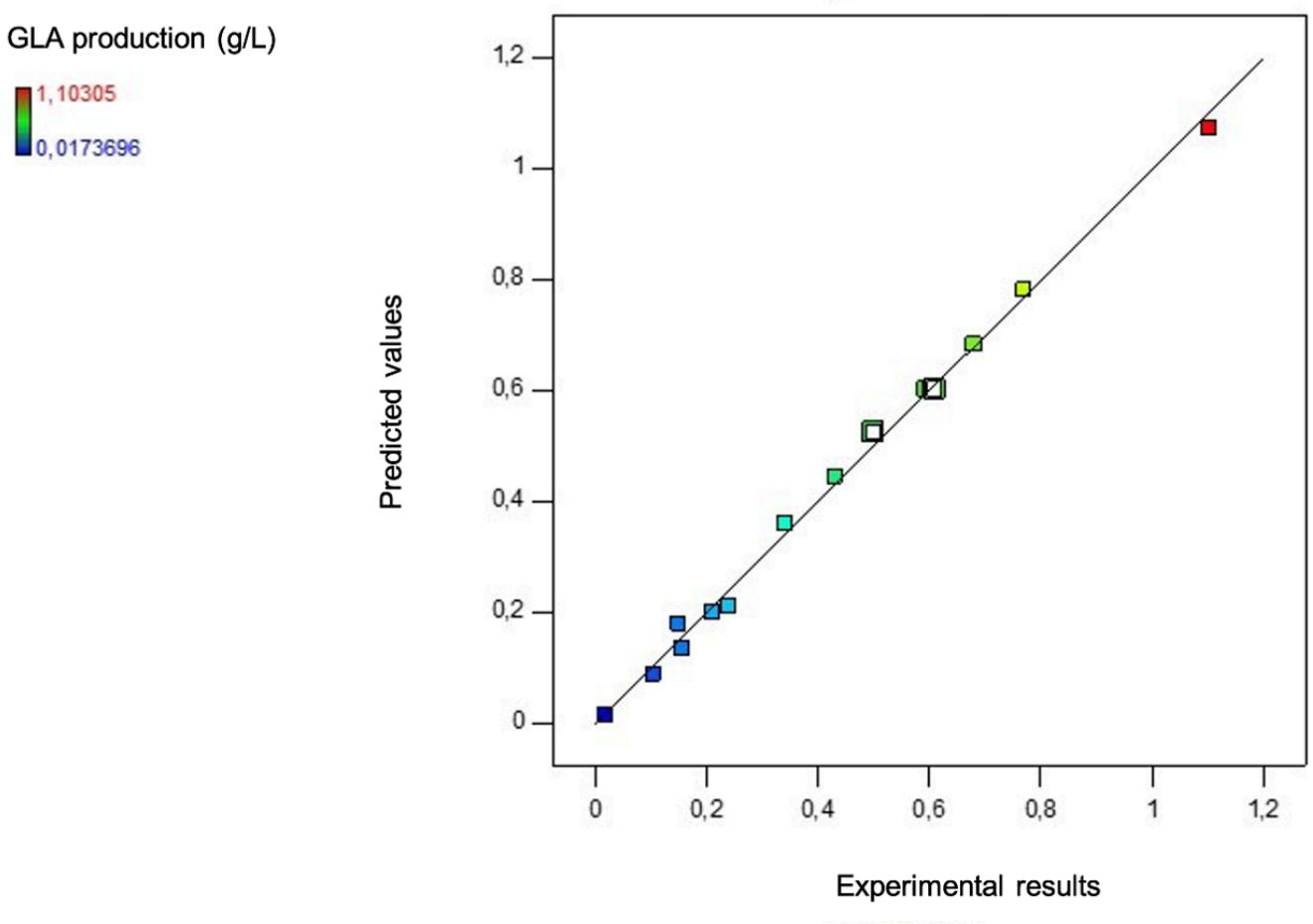


Figure 4

Comparison of the experimental results and the predicted values obtained from fermentations based on the Box–Behnken design for GLA production

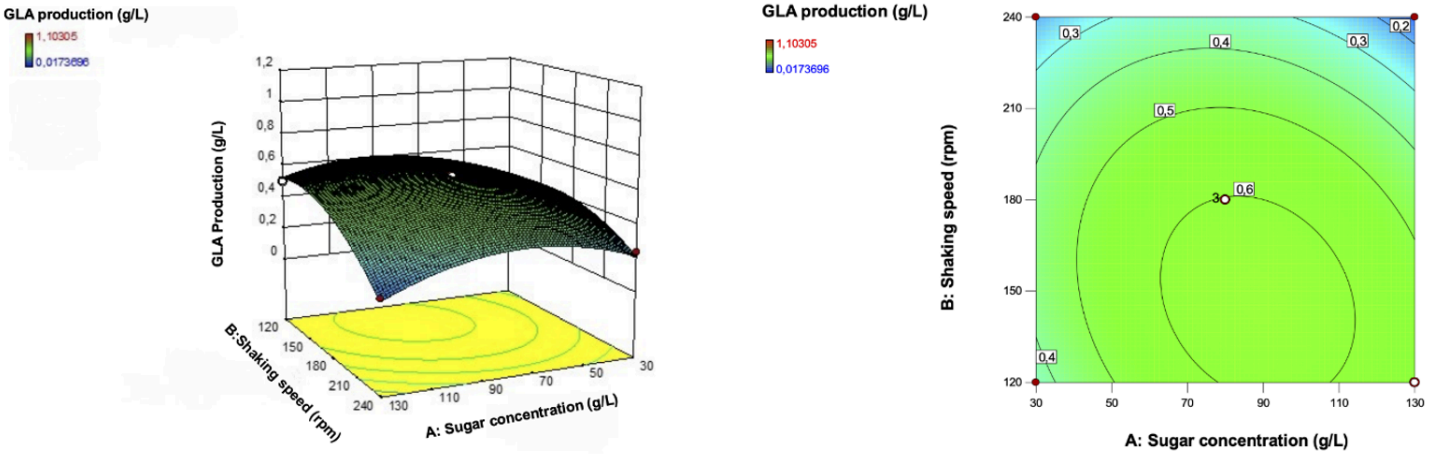


Figure 5

Three-dimensional response surface plot and contour plot showing the effect of sugar concentration and shaking speed on GLA production at 28°C

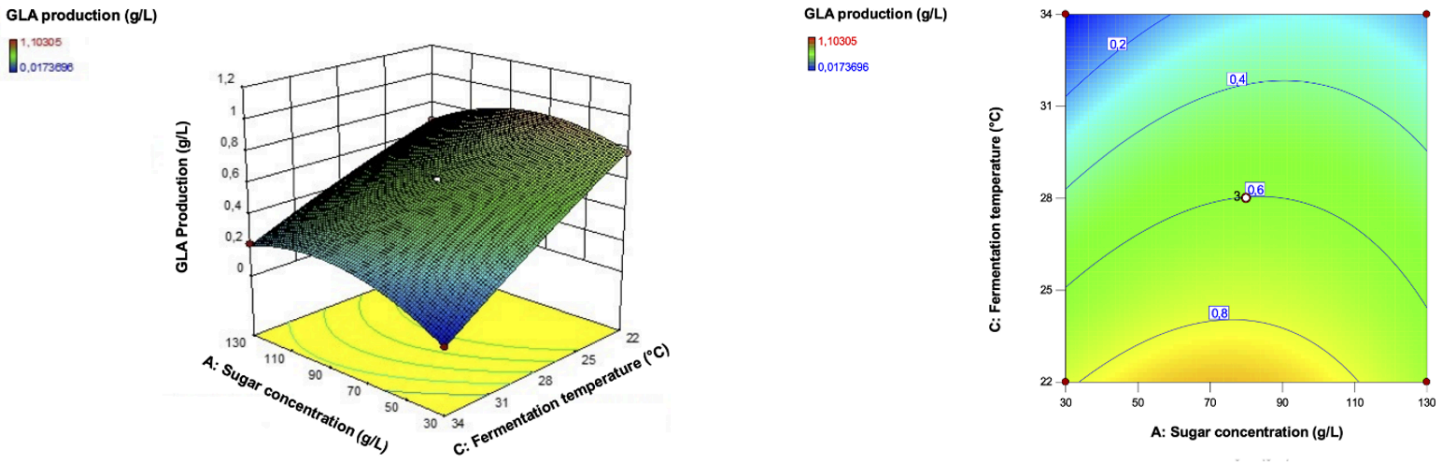


Figure 6

Three-dimensional response surface plot and contour plot showing the effect of initial sugar concentration and fermentation temperature on GLA production at 180 rpm

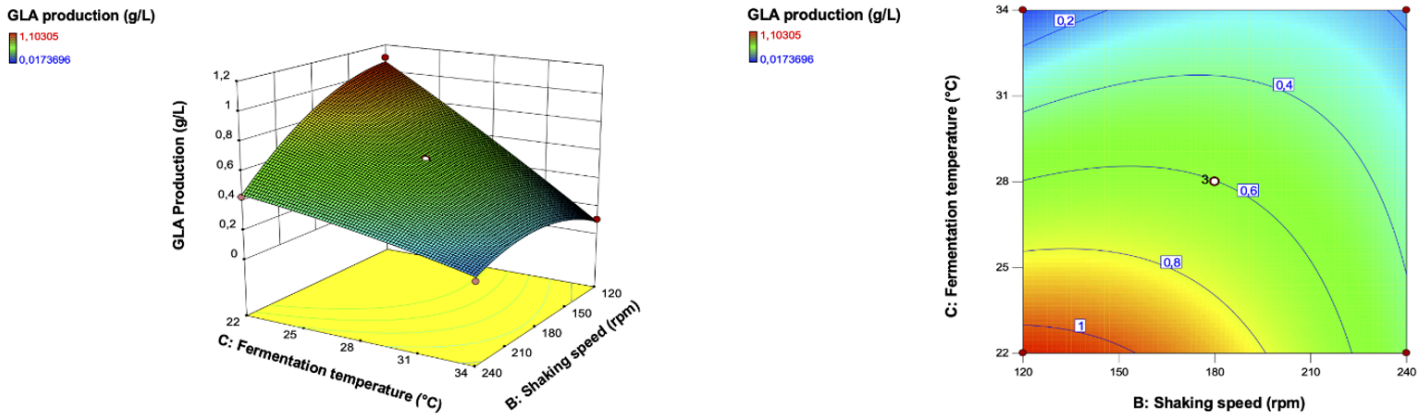


Figure 7

Three-dimensional response surface plot and contour plot showing the effect of shaking speed and fermentation temperature on GLA production at sugar concentration of 80 g/L

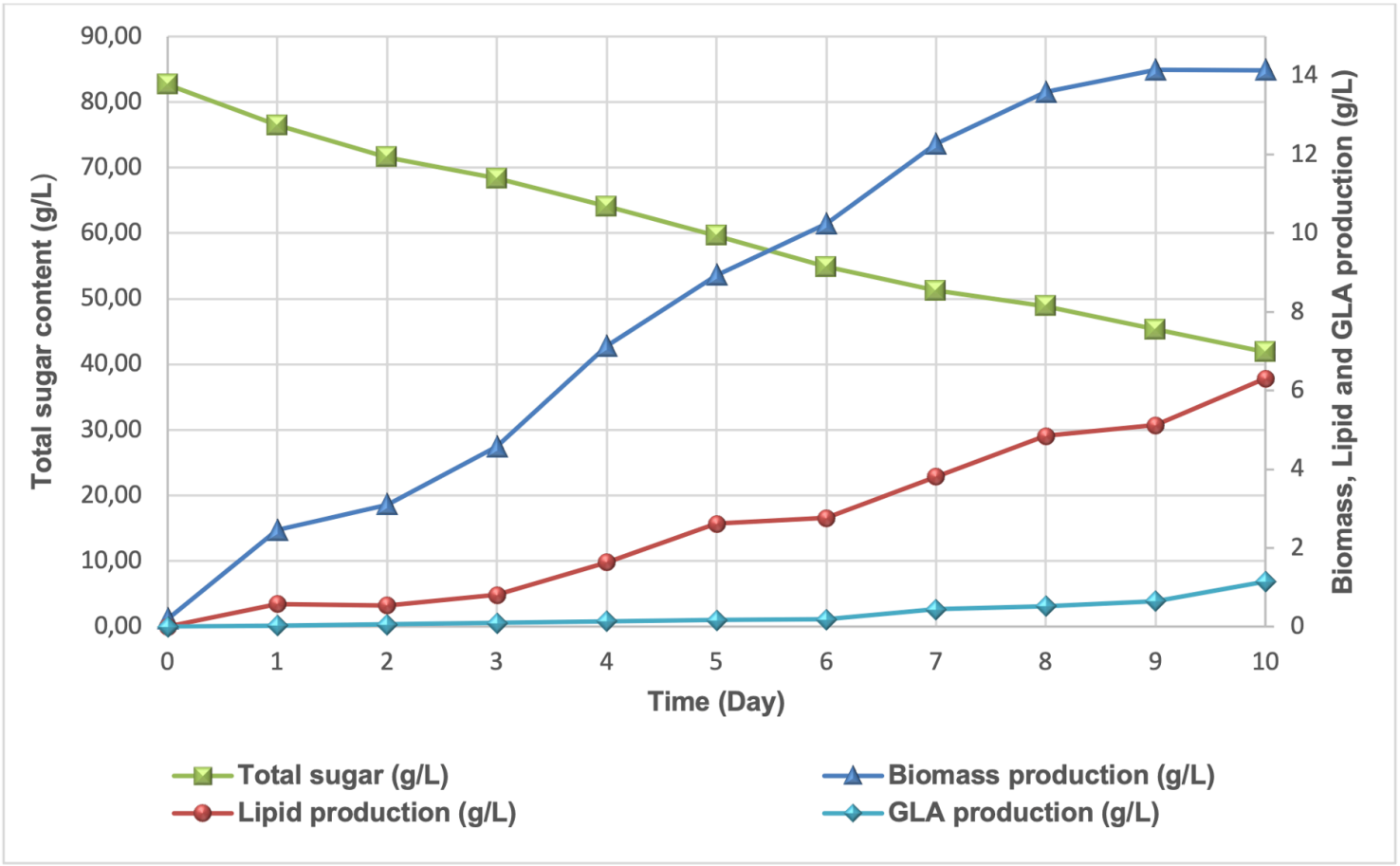


Figure 8

Biomass, lipid, GLA production and residual total sugar during the validation fermentation