



## Article

# Optimization of Medium Constituents for the Production of Citric Acid from Waste Glycerol Using the Central Composite Rotatable Design of Experiments

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**Abstract:** Citric acid is currently produced by submerged fermentation of sucrose with the aid of *Aspergillus niger* mold. Its strains are characterized by a high yield of citric acid biosynthesis and no toxic by-products. Currently, new substrates are sought for production of citric acid by submerged fermentation. Waste materials such as glycerol or pomace could be used as carbon sources in the biosynthesis of citric acid. Due to the complexity of the metabolic state in fungus, there is an obvious need to optimize the important medium constituents to enhance the accumulation of desired product. Potential optimization approach is a statistical method, such as the central composite rotatable design (CCRD). The aim of this study was to increase the yield of citric acid biosynthesis by *Aspergillus niger* PD-66 in media with waste glycerol as the carbon source. A mathematical method was used to optimize the culture medium composition for the biosynthesis of citric acid. In order to maximize the efficiency of the biosynthesis of citric acid the central composite, rotatable design was used. Waste glycerol and ammonium nitrate were identified as significant variables which highly influenced the final concentration of citric acid ( $Y_1$ ), volumetric rate of citric acid biosynthesis ( $Y_2$ ), and yield of citric acid biosynthesis ( $Y_3$ ). These variables were subsequently optimized using a central composite rotatable design. Optimal values of input variables were determined using the method of the utility function. The highest utility value of 0.88 was obtained by the following optimal set of conditions: waste glycerol—114.14 g·L<sup>-1</sup> and NH<sub>4</sub>NO<sub>3</sub>—2.85 g·L<sup>-1</sup>.

**Keywords:** citric acid; *Aspergillus niger*; design of experiments; response surface methodology; optimization; glycerol



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## 1. Introduction

Citric acid is present in plant tissues as well as animal tissues such as blood, bone or muscle. For living organisms, citric acid is one of the essential carboxylic acids of the Krebs cycle, resulting in the oxidation of glucose to carbon dioxide and water with the release of energy [1–3].

Currently, the annual production of citric acid reaches about 1.8 million tons in the world, and the citric acid market is one of the fastest-growing segments of food additives [4,5]. Citric acid has been used in the food, pharmaceutical, chemical and even metallurgical industry due to its harmless nature, chelating and sequestering properties [6]. The reason for the continuous increase in the production of citric acid is its wide range of applications not only in the food and pharmaceutical industry, but also in the production

of biopolymers, environment protection and biomedicine [7]. The industrial production of citric acid is dominated by the method of submerged culture using *Aspergillus niger* strains [6,8]. The main substrates in the production of citric acid are beetroot molasses, sucrose, and glucose syrup [2]. Due to the growing demand for citric acid in the world, scientists are conducting research on its production technology to improve the efficiency of the bioprocess and reduce production costs. Currently, research focuses on the use of unconventional raw materials in the biosynthesis of citric acid. The investigated substrates are mainly fruit and vegetable processing waste, refined fatty acids, bran, and crude glycerol [9–17].

Glycerol is formed as a by-product in the production of biofuels for diesel engines-biodiesel. The recent increase in biodiesel production generated the problem of management of the glycerol phase. The glycerin phase contains from 30 to 80% glycerol, and for every 100 kg of obtained biodiesel, there is about 11 kg of glycerin phase [18–20].

The largest producer of biodiesel is Europe, followed by North America. In 2016, the production of biodiesel in the countries of European Union reached 25 million liters. In 2021, it is estimated that the biodiesel market will reach 41.18 billion dollars in the world [17,21,22].

The increase in biodiesel production has led to the flooding of the market with large amounts of glycerol and search for new methods of its management. However, the use of the glycerin phase is limited because of its contamination with methanol (from 5 to 9%) and alkali. Crude glycerol purification is expensive and the glycerol market is already saturated. Thus, the price of raw glycerol continues to fall and has a direct impact on biodiesel production costs [23]. The solution to this problem may be the use of waste glycerol fraction or partially purified glycerol as a carbon and energy source for the cultivation of microorganisms [24–32]. Glycerol as a reducing carbon source can play an important role as a substrate and source of energy for many microorganisms. In addition, it is a precursor to many cell components and a regulator of such cell processes such as metabolic pathways, redox potential and phosphorus management [33]. Bioconversion of glycerol by microbes allows to overcome the drawbacks associated with its chemical conversion, requiring the use of high temperatures, pressures and high costs [34].

The interest in using glycerol as the main substrate for the biosynthesis of citric acid with the participation of *Aspergillus niger* strains is small, despite the large supply of waste on the market. The literature mainly presents the results of research on citric acid biosynthesis, in which glycerol is an additional carbon source in culture media [35–38]. This is explained by the fact that the substances contained in glycerol have an inhibitory effect on the bioprocess. The next reason is the catabolic repression of carbon in *Aspergillus niger* strains caused by glycerol [39]. Nevertheless, the prospects for using glycerol in the citric acid production with *Aspergillus niger* strains seems to be promising, but this process requires selection of the right strain and optimization of the culture conditions.

The aim of the research was to use the central rotatable plan and utility functions to determine the optimal culture medium composition for the citric acid biosynthesis with *Aspergillus niger* strains using waste glycerol as a main carbon source.

## 2. Results and Discussion

### 2.1. Statistical Analysis

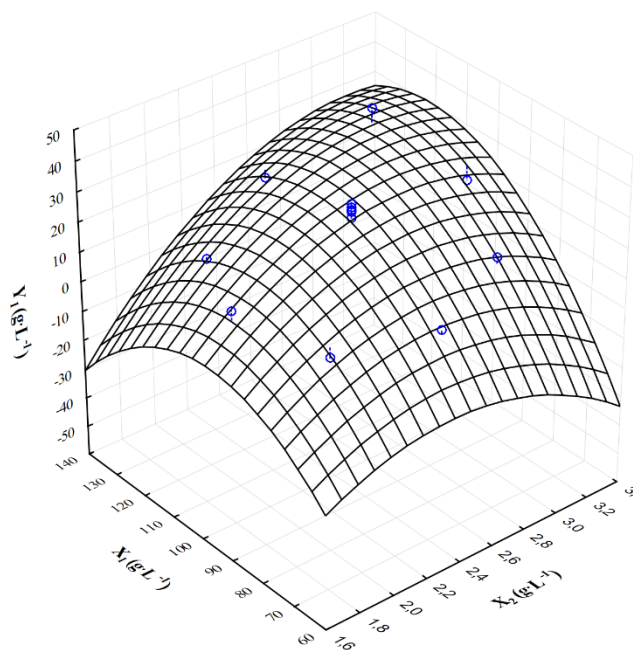
Results included statistical analysis to verify the homogeneity of Brown–Forsyth’s variance test. The test showed homogeneity of variance ( $p > 0.01$ ).

The coefficients of determination ( $R^2$ ) of the predicted models of  $Y_1$ ,  $Y_2$  and  $Y_3$  were 0.9265, 0.8882 and 0.9266, respectively. The coefficient of determination informs about the variability part of the dependent variable that was explained by the model. The result set indicated that 80% of the variability was included in these mathematical models. The value of the determination coefficient above 0.75 indicates a high coherence between the predicted and experimental values [40]. The significance of each coefficient was determined using the Fisher (*Snedecor*) test and  $p$ -value. The corresponding variable is usually considered as

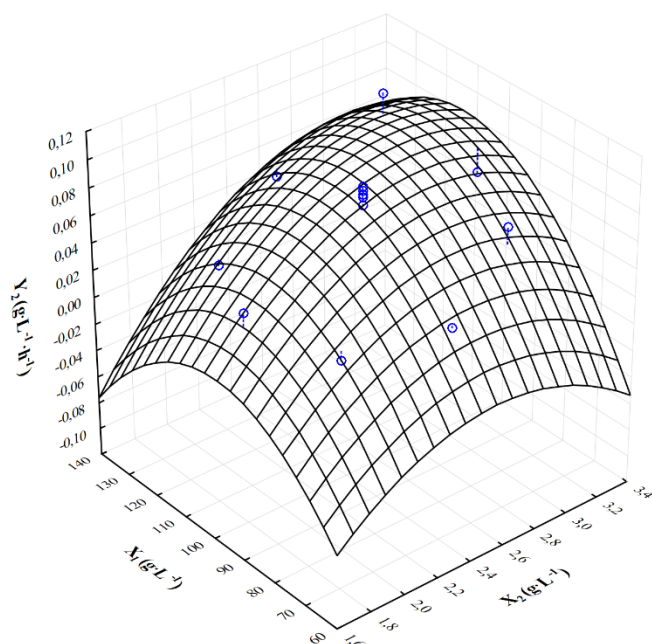
significant when the absolute F-value becomes larger and the  $p$ -value becomes smaller [41]. The F-values of  $Y_1$ ,  $Y_2$  and  $Y_3$  were 1120.76, 0.0078 and 869.91, respectively, while the  $p$ -values of  $Y_1$ ,  $Y_2$  and  $Y_3$  were less than 0.01, which indicated that the models were significant.

The ANOVA analysis showed that the input variables  $X_1$ ,  $X_1^2$ ,  $X_2$  and  $X_2^2$  in both the linear and quadratic terms of variability were significant for the  $Y_1$ ,  $Y_2$ , and  $Y_3$  models. It can be readily observed that  $X_1X_2$  was significant for the model  $Y_1$ . The respective  $p$ -values ( $p$ -values > 0.01) of the models  $Y_1$ ,  $Y_2$  and  $Y_3$  in *Lack of Fit* test were 0.0312, 0.0202 and 0.0510, which demonstrated that *Lack of Fit* test for the three models was not significant. These results indicated that the models were applicable to accurately predicting the variation. Thus, the mathematical models were satisfactory to perform statistical analyses [42].

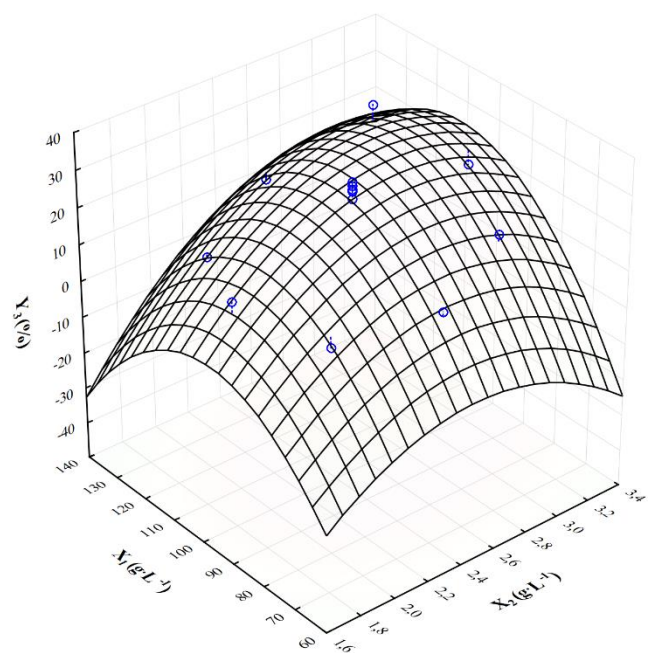
Figures 1–3 show the response surface plots of changes in the output variables as a function of the crude glycerol concentration ( $X_1$ ) and the ammonium nitrate concentration ( $X_2$ ). The response surface analysis was conducted with the following constant concentrations:  $\text{KH}_2\text{PO}_4 = 0.2 \text{ g}\cdot\text{L}^{-1}$  and  $\text{MgSO}_4\cdot 7\text{H}_2\text{O} = 0.2 \text{ g}\cdot\text{L}^{-1}$ . The highest concentration of citric acid ( $Y_1 = 38.15 \text{ g}\cdot\text{L}^{-1}$ ) was achieved when the initial concentration of waste glycerol and ammonium nitrate was  $120.0 \text{ g}\cdot\text{L}^{-1}$  and  $3.0 \text{ g}\cdot\text{L}^{-1}$ , respectively. In the case of decrease in ammonium nitrate concentration of  $2.0 \text{ g}\cdot\text{L}^{-1}$ , a significant decrease in the final concentration of citric acid ( $Y_1 = 11.32 \text{ g}\cdot\text{L}^{-1}$ ) was observed. The lower concentration of crude glycerol ( $80.0 \text{ g}\cdot\text{L}^{-1}$ ) and ammonium nitrate ( $1.8 \text{ g}\cdot\text{L}^{-1}$ ) in the culture medium resulted in noticeable reduction in final citric acid concentration ( $Y_1 = 5.60 \text{ g}\cdot\text{L}^{-1}$ ) (Figure 1). In addition, the highest volumetric rate of citric acid biosynthesis ( $Y_2 = 0.106 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ) was obtained at the initial concentration of crude glycerol of  $X_1 = 120 \text{ g}\cdot\text{L}^{-1}$  and ammonium nitrate  $X_2 = 3.0 \text{ g}\cdot\text{L}^{-1}$  in the culture medium. Similarly, reduction in the nitrogen source to  $X_2 = 2.0 \text{ g}\cdot\text{L}^{-1}$  resulted in a significant reduction in the volume rate of citric acid biosynthesis (Figure 2). Interaction analysis for the yield of citric acid biosynthesis showed that range of input variables values were  $X_1 = 100.0\text{--}120.0 \text{ g}\cdot\text{L}^{-1}$  and  $X_2 = 2.5\text{--}3.0 \text{ g}\cdot\text{L}^{-1}$ . If concentrations of crude glycerol and ammonium nitrate were reduced below  $80 \text{ g}\cdot\text{L}^{-1}$  and  $2.5 \text{ g}\cdot\text{L}^{-1}$ , respectively, significant reduction in the yield of citric acid biosynthesis was observed (Figure 3).



**Figure 1.** Response surface plot (3D) of the influence of the crude glycerol concentration ( $X_1$ ) and ammonium nitrate concentration ( $X_2$ ) on the final citric acid concentration ( $Y_1$ ); blue points—points where the measurement was made.



**Figure 2.** Response surface plot (3D) of the influence of the crude glycerol concentration ( $X_1$ ) and ammonium nitrate concentration ( $X_2$ ) on the volumetric rate yield of citric acid biosynthesis ( $Y_2$ ); blue points—points where the measurement was made.



**Figure 3.** Response surface plot (3D) of the influence of the crude glycerol concentration ( $X_1$ ) and ammonium nitrate concentration ( $X_2$ ) on the yield of citric acid biosynthesis ( $Y_3$ ); blue points—points where the measurement was made.

## 2.2. Determination of Optimum Medium Constituents

In order to determine the optimal values of the input variables ( $X_1$ ,  $X_2$  and  $X_3$ ) on the grounds of three selected final responses, the final concentration of citric acid ( $Y_1$ ), the volumetric rate ( $Y_2$ ), and biosynthesis yield ( $Y_3$ ), the method of utility function was used [43–46]. The utility function was defined as the following Equation (1):

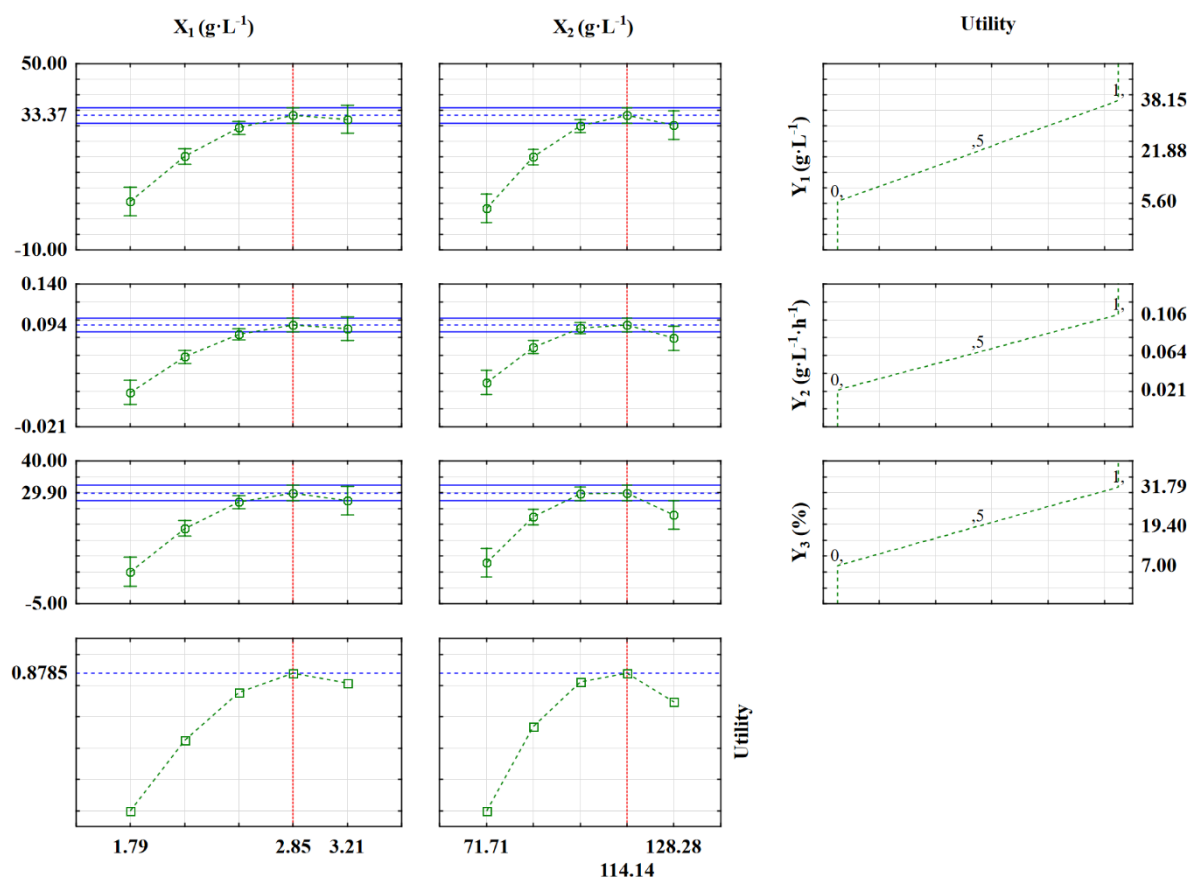
$$U = f(u_1, u_2, \dots, u_n), \quad (1)$$

and a function that satisfies the following conditions:

$$U'(x) > 0, U''(x) < 0. \quad (2)$$

The value of each response was evaluated using a dimensionless linear function with values in the interval [0.1]. The detailed  $u_i$  was assigned as follows:  $u_i = 0$  if response was of low value,  $u_i = 0.5$  if response was of minimal value, and  $u_i = 1$  if response was of high value. After determining the utility function for each output variable, a model of total utility was built using the spline function method for fitting the response surface to the utility value. To obtain the optimal values of the input variables, the optimum method of grid node was used.

Figure 4 shows the profiles of approximate values and their utility determined using the optimum method of grid node. The highest utility value of 0.84 was obtained for the following optimal values of the analysed culture medium composition parameters: waste glycerol concentration—114.14  $\text{g}\cdot\text{L}^{-1}$  and ammonium nitrate—2.85  $\text{g}\cdot\text{L}^{-1}$ . On the grounds of basic research, constant concentrations of  $\text{KH}_2\text{PO}_4 = 0.2 \text{ g}\cdot\text{L}^{-1}$  and  $\text{MgSO}_4\cdot 7\text{H}_2\text{O} = 0.2 \text{ g}\cdot\text{L}^{-1}$  were assumed. For such composition of the culture medium, a triplicated verification experiment was performed. The model was verified and validated by comparing experimental and predicted response values as shown in Table 1. The experimental and predicted response values were consistent with each other, indicating the success of the mathematical model in obtaining optimum experimental conditions.



**Figure 4.** Optimal values utility of the analysed culture medium parameters for the highest final concentration of citric acid ( $Y_1$ ), volumetric rate of citric acid biosynthesis ( $Y_2$ ), and yield of citric acid biosynthesis ( $Y_3$ ). The blue line represents usability. The red line determines the optimal concentrations of the components of the culture medium, waste glycerol and ammonium nitrate.

**Table 1.** Comparison of predicted and experimental response values of verification model.

Response	Optimal Variable Value (g·L <sup>-1</sup> )		Predicted	Experimental
	Crude Glycerol	Ammonium Nitrate		
Final concentration of citric acid (Y <sub>1</sub> ) (g·L <sup>-1</sup> )			36.42	33.69
Volumetric rate of citric acid biosynthesis (Y <sub>2</sub> ) (g·L <sup>-1</sup> ·h <sup>-1</sup> )	114.14	2.85	0.101	0.095
Yield of citric acid biosynthesis (Y <sub>3</sub> ) (%)			32.18	30.26

### 2.3. Biosynthesis of the Citric Acid

The biosynthesis of citric acid was performed on an enlarged scale in the Biomer 10 bioreactor. The final concentration of citric acid (Y<sub>1</sub>), the volumetric rate (Y<sub>2</sub>), the yield of citric acid biosynthesis (Y<sub>3</sub>), and other parameters characterizing the biosynthesis process in submerged cultures in the bioreactor are presented in Table 2. The final concentration of citric acid in the culture medium was significantly higher than that in the verification experiment and amounted to Y<sub>1</sub> = 69.70 g·L<sup>-1</sup>. In addition, the bioprocess was characterized by a high yield (61.00%) and the volumetric rate of citric acid biosynthesis (0.183 g·L<sup>-1</sup>·h<sup>-1</sup>). Qualitative and quantitative analysis of organic acids showed the presence of only citric acid in the culture medium, which proved the high chemical purity of the obtained product.

**Table 2.** Kinetic parameters of citric acid biosynthesis by *Aspergillus niger* PD-66 in optimized medium containing crude glycerol as a carbon source.

Symbol	Unit	Parameters	Results
t	h	Culture time	157
G <sub>K</sub>	g·L <sup>-1</sup>	Final concentration of glycerol in the medium	0.50
Y <sub>1</sub>	g·L <sup>-1</sup>	Monohydrate citric acid concentration in culture medium	69.70
Y <sub>2</sub>	g·L <sup>-1</sup> ·h <sup>-1</sup>	Volumetric rate of monohydrate citric acid biosynthesis	0.183
Y <sub>3</sub>	% (m/m)	Yield of citric acid biosynthesis with respect to introduced substrate	61.00
K <sub>EF</sub>	% g·L <sup>-1</sup> ·h <sup>-1</sup>	Efficiency coefficient of monohydrate citric acid biosynthesis	27.08
X <sub>K</sub>	g·L <sup>-1</sup>	Biomass concentration in culture medium	19.40
Y <sub>X/S</sub>	% (m/m)	Yield of biomass biosynthesis	17.00

The literature lacks studies on the assessment of the impact of waste glycerol on the biosynthesis of citric acid. There are also few comparative studies on the biosynthesis of metabolites by microorganisms in media containing waste or anhydrous glycerol as the main carbon source. This is probably due to the belief that glycerol slows down the growth rate of filamentous fungi and does not favor the production of citric acid by *Aspergillus niger*.

According to research conducted by Wittwen et al. [47], glycerol in *Aspergillus niger* cells is phosphorylated to 3-phosphoglycerol, similar to *Aspergillus nidulans* and *Saccharomyces cerevisiae*. This reaction is catalyzed by glycerol kinase, which in *Saccharomyces cerevisiae* is a product of the GUT1 gene. Then, 3-phosphoglycerol is oxidized to phosphodihydroxyacetone by the FAD<sup>+</sup>-dependent 3-phosphoglycerol dehydrogenase. Finally, phosphodihydroxyacetone is incorporated into the glycolytic pathway [34,37,48].

Various carbon sources were used for the production of citric acid by *Aspergillus niger*. The highest yields are obtained when monosaccharides (glucose) and disaccharides (sucrose) are used as substrates in submerged cultures. Unfortunately, they increase production costs due to their high price [22]. In order to reduce the cost of citric acid production, cheap carbon sources are sought that can be used by *Aspergillus niger* (e.g., apple pomace, sweet potato hydrolysates, sugar cane cake, pineapple pomace) [49–51].

Comparing the results obtained in this study with those of other authors, Zohu et al., in a medium with maize waste, obtained an accumulation of citric acid at the level of 100.4 g·L<sup>-1</sup> and a yield of 94.11% in submerged cultures of *Aspergillus niger* SIIM M288 [52]. On the other hand, Hu et al., in a study with the same strain, obtained a yield of 74.9% [53].

In submerged cultures of *Aspergillus niger* using two types of dates, GHARS and MECH DE-GALA, as a carbon source, the concentration of citric acid was  $42.25 \text{ g}\cdot\text{L}^{-1}$  and  $36.60 \text{ g}\cdot\text{L}^{-1}$ , respectively [54]. In a study with beet molasses as a substrate, citric acid concentrations of  $19.13 \text{ g}\cdot\text{L}^{-1}$  and  $34.62 \text{ g}\cdot\text{L}^{-1}$  were obtained with a sugar concentration of  $200 \text{ g}\cdot\text{L}^{-1}$  and  $150 \text{ g}\cdot\text{L}^{-1}$ , respectively [55]. In the process of optimizing the biosynthesis process, Aboyeji et al. obtained a yield of  $4.36 \text{ mg}\cdot\text{mL}^{-1}$  from sweet potato starch hydrolysates [56].

### 3. Materials and Methods

#### 3.1. Major Substrate and Microorganisms

The major substrate, waste glycerol, was collected from Wratysławia Biodiesel S.A. Wrocław, Poland and stored in room at  $20 \text{ }^\circ\text{C}$ . The chemical characteristics of the substrate are presented in Table 3.

**Table 3.** The chemical composition of waste glycerol.

Property	Unit	Value
Glycerin	% (m/m)	88.30
Sodium chloride	% (m/m)	3.92
Methanol	% (m/m)	0.04
Water	% (m/m)	6.67
Matter Organic Non-Glycerol	% (m/m)	1.20
pH	-	7.40

The fungal strain of *Aspergillus niger* PD-66 used in this work was obtained from Pure Cultures Collection maintained at the Department of Food Biotechnology and Analysis at the Wrocław University of Economics and Business. The culture was regularly sub-cultured and maintained at  $4 \text{ }^\circ\text{C}$ . *Aspergillus niger* spores were produced on potato dextrose agar (PDA) plates for 10 days at  $30 \text{ }^\circ\text{C}$  and washed with 25 mL sterilized distilled water to prepare the inoculum. Spore suspension was collected in a 500 mL Erlenmeyer flask.

#### 3.2. Experimental Procedure

The culture medium used in the study contained waste glycerol,  $\text{NH}_4\text{NO}_3$  (p.a., Chempur, Piekary Śląskie, Poland)  $\text{KH}_2\text{PO}_4$  (p.a., Chempur, Piekary Śląskie, Poland),  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  (p.a., Chempur, Piekary Śląskie, Poland) and tap water. The initial pH of medium was adjusted to 3.0 by adding 5M HCl. The medium with nutrients was autoclaved at  $121 \text{ }^\circ\text{C}$  for 30 min. According to the created experiment plan, variants of the composition of culture media were obtained, which are presented in Table 4.

**Table 4.** Composition of culture media used to optimize the concentration of waste glycerol and ammonium nitrate.

Run	Crude Glycerol	$\text{NH}_4\text{NO}_3$	$\text{KH}_2\text{PO}_4$	$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$
	[ $\text{g}\cdot\text{L}^{-1}$ ]	[ $\text{g}\cdot\text{L}^{-1}$ ]	[ $\text{g}\cdot\text{L}^{-1}$ ]	[ $\text{g}\cdot\text{L}^{-1}$ ]
1	80.00	2.00	0.20	0.20
2	80.00	3.00	0.20	0.20
3	120.00	2.00	0.20	0.20
4	120.00	3.00	0.20	0.20
5	71.71	2.50	0.20	0.20
6	128.28	2.50	0.20	0.20
7	100.00	1.79	0.20	0.20
8	100.00	3.20	0.20	0.20
9	100.00	2.50	0.20	0.20
10	100.00	2.50	0.20	0.20
11	100.00	2.50	0.20	0.20
12	100.00	2.50	0.20	0.20
13	100.00	2.50	0.20	0.20

For the optimisation procedure, small-scale batch fermentation experiment was conducted in 500 mL Erlenmeyer flasks holding 100 mL of the medium. After autoclaving, the medium was inoculated with a spore suspension of  $1 \times 10^5 \text{ mL}^{-1}$ . The Erlenmeyer flasks with their contents were incubated in a shaker GFL 3033 (Lauda Dr. R. Wobser GMBH & CO. KG, Lauda-Königshofen Germany) at  $30 \text{ }^\circ\text{C}$  on  $200 \text{ rpm}\cdot\text{min}^{-1}$  for 15 days. All of above experiments were conducted three times.

The batch cultivations on large scale were performed in BIOMER 10 (model, manufacturer, Wrocław, Poland) bioreactor with total volume of 7 L and working volume of 5 L. Temperature was maintained at  $30 \text{ }^\circ\text{C}$ . To prevent foam formation, rapeseed oil was added automatically using level sensor control. The aeration rate was set  $1.0 \text{ L}\cdot\text{min}^{-1}$  and the dissolved oxygen tension was kept at 80% saturation. In the subsequent days of the bioprocess, the rotational speed of the agitator shaft was gradually increased from 300 to  $800 \text{ rpm}\cdot\text{min}^{-1}$ .

### 3.3. Determination of Citric Acid and Glycerol

For HPLC analysis, centrifuged samples ( $8000 \times g$ , 15 min) were filtered through nylon filters (diameter  $0.22 \text{ }\mu\text{m}$ ) prior to diluting 1:1 with distilled water. For analysis of glycerol and organic acid, Perkin Elmer HPLC (Series 200, Waltham, 940 Winter St, United States model, manufacturer, city, state abbreviation,) was used with the Eurokat H65 (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) column at  $60 \text{ }^\circ\text{C}$ , with constant flow rate  $0.6 \text{ mL}\cdot\text{min}^{-1}$  HPLC water eluent. For detection, RI Perkin Elmer Series 2000 detector and a variable wavelength UV/VIS CE detector (Series 200, Waltham, 940 Winter St, United States) (model, manufacturer, city, (state abbreviation if USA and Canada), country) at 210 nm were used.

### 3.4. Experimental Design

In the present study, Statistica 13.3 software (Statsoft Inc., Tulsa, OK, USA) was employed to perform the experiment design and development results. Analysis of variance (ANOVA) and RSM (Response Surface Methodology) were employed to determine the regression coefficients, statistical significance of the model terms and fit of the experimental data to mathematical models, which aims at optimizing the overall region for three response variables.

A quadratic model used to predict the response variables is shown as the following Equation (3).

$$Y = b_0 + b_1X_1 + b_2X_1^2 + b_3X_2 + b_4X_2^2 + b_5X_1X_2, \quad (3)$$

where Y is the predicted dependent variable,  $X_1$  and  $X_2$  are the independent variables and  $\beta_i$  are the regression coefficients.

In the present study, parameters of culture medium for citric acid biosynthesis were optimized using response surface methodology. RSM is a statistical technique used to design experiments, build models, evaluate the effects of the factor and search for the optimal conditions of factors required for desired responses. Moreover, the central composite rotatable design is the most widely used method in RSM. In this study, the central composite rotatable design was used to identify relationship between the response function and bioprocess variables. It was also used to optimize the culture medium parameters of citric acid concentration, rate, and yield of biosynthesis.

Our preliminary experiment demonstrated that the concentration of crude glycerol and ammonium nitrate had significant influence on the final concentration of citric acid, volumetric rate, and yield of its biosynthesis. The values of other bioprocess variables were determined based on previous experiments:  $\text{KH}_2\text{PO}_4$ — $0.2 \text{ g}\cdot\text{L}^{-1}$ ,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ — $0.2 \text{ g}\cdot\text{L}^{-1}$ , temperature  $30 \text{ }^\circ\text{C}$ , mixing speed  $200 \text{ rpm}\cdot\text{min}^{-1}$ , and pH 3.0. Therefore, the crude glycerol ( $X_1$ ) and ammonium nitrate ( $X_2$ ) were chosen as independent variables, while response variables were the final concentration of citric acid ( $Y_1$ ), volumetric rate ( $Y_2$ ) and yield ( $Y_3$ ) of citric acid biosynthesis. Then, the natural values of the central point (plan nucleus)



and variable step ( $\Delta X_i$ ) were determined. The natural and coded levels of variables of the central point and variable step are provided in Tables 5 and 6.

**Table 5.** Actual value of independent variable at the center point and the change in variable step.

Independent Variables, $X_i$	Unit	Center Point $X_i^0 = \frac{X_i^{\max} + X_i^{\min}}{2}$	Variable Step $\Delta X = \frac{X_i^{\max} - X_i^{\min}}{2}$
$X_1$	$g \cdot L^{-1}$	120.0	20.0
$X_2$	$g \cdot L^{-1}$	2.5	0.5

$X$ —encoded value,  $X_i$ —actual value,  $X_i^0$ —actual value in the center of the domain,  $\Delta X$ —variable step.

**Table 6.** Experimental range and levels of variables.

Independent Variables, $X_i$	Unit	Levels and Ranges				
		(−1.414)	(−1)	(0)	(+1)	(+1.414)
$X_1$	$g \cdot L^{-1}$	71.71	80.00	100.00	120.00	128.28
$X_2$	$g \cdot L^{-1}$	1.79	2.00	2.50	3.20	3.00

Levels of each variable are axial ( $\pm 1$ ), central (0) and corner ( $\pm 1.414$ ).

From the variables selected for testing, the rotatable plan was created. The experimental design consists of four factorial points, four axial points at distance 1.414 from the centre and five replicates of the central point. The number of experiments at the central point is 5, which results from the requirement that the variance of the Y value has to be the same at the central point of the plan as well as at the points lying on the sphere within the radius of 1. The experiments were carried out in three independent repetitions in random order. The distance of 1.414 between axial point and central point was calculated from the following Equation (4):

$$\alpha = \sqrt[4]{2^n}, \tag{4}$$

where  $\alpha$  is the distance,  $n$  is a number of independent variables. In this study,  $n = 2$  and  $\alpha = 1.414$ .

The conformity of the model was determined by regression analysis and ANOVA analysis ( $p < 0.01$ ). The significance of the regression coefficient was analysed by F-test. The relationship between independent variables ( $X_1, X_2$ ) and response variables ( $Y_1, Y_2$  and  $Y_3$ ) was illustrated by response surface plots.

Table 7 summarizes the experimental and predicted values of the response variables using the central composite rotatable design. ANOVA analysis of response surface quadratic model was used to estimate the relationship between response variables and independent variables of regression models. The results are presented in Tables 8–10.

The quadratic models of  $Y_1, Y_2$  and  $Y_3$  in actual and coded variables were demonstrated as the following Equations (5)–(10).

The equation in terms of actual variables:

$$Y_1 (g \cdot L^{-1}) = -210.91 + 70.22X_1 - 21.02X_1^2 + 2.42X_2 - 0.02X_2^2 + 0.47X_1X_2, \tag{5}$$

$$Y_2 (g \cdot L^{-1} \cdot h^{-1}) = -0.69 + 0.25X_1 - 0.06X_1^2 + 0.008X_2 - 0.00005X_2^2 + 0.0008X_1X_2, \tag{6}$$

$$Y_3 (\%) = -250.78 + 2.94X_1 - 0.02X_1^2 + 88.05X_2 - 21.47X_2^2 + 0.31X_1X_2. \tag{7}$$

The equation in terms of coded variables:

$$Y_1 (g \cdot L^{-1}) = 28.30 + 6.17X_1 - 6.57 X_1^2 + 6.03X_2 - 5.26X_2^2 + 4.70X_1X_2, \tag{8}$$

$$Y_2 (g \cdot L^{-1} \cdot h^{-1}) = 0.08 + 0.01 X_1 - 0.02X_1^2 + 0.02 X_2 - 0.01 X_2^2 + 0.01X_1X_2, \tag{9}$$

$$Y_3 (\%) = 28.30 + 3.17X_1 - 7.09X_1^2 + 5.72 X_2 - 5.37X_2^2 + 3.07X_1X_2. \tag{10}$$

**Table 7.** Actual and coded variables with the experimental and predicted values.

Run	Actual and Coded Variables				Results					
					Y <sub>1</sub> (g·L <sup>-1</sup> )		Y <sub>2</sub> (g·L <sup>-1</sup> ·h <sup>-1</sup> )		Y <sub>3</sub> (%)	
	Crude Glycerol X <sub>1</sub>		Ammonium Nitrate X <sub>2</sub>		Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	80.00	-1	2.00	-1	5.60	8.97	0.021	0.028	7.00	10.02
2	80.00	-1	3.00	1	13.65	11.64	0.063	0.051	17.06	15.32
3	120.00	1	2.00	-1	11.32	11.92	0.031	0.036	9.43	10.21
4	120.00	1	3.00	1	38.15	33.38	0.106	0.091	31.79	27.82
5	71.72	-1.414	2.50	0	7.70	6.44	0.029	0.031	10.74	9.63
6	128.28	1.414	2.50	0	21.23	23.89	0.059	0.065	16.55	18.61
7	100.00	0	1.79	-1.414	12.37	9.26	0.037	0.027	12.37	9.472
8	100.00	0	3.20	1.414	21.82	26.32	0.065	0.082	21.82	25.66
9	100.00	0	2.50	0	28.35	28.30	0.084	0.084	28.35	28.30
10	100.00	0	2.50	0	25.78	28.30	0.077	0.084	25.78	28.30
11	100.00	0	2.50	0	29.28	28.30	0.087	0.084	29.28	28.30
12	100.00	0	2.50	0	30.33	28.30	0.090	0.084	30.33	28.30
13	100.00	0	2.50	0	27.77	28.30	0.083	0.084	27.77	28.30

**Table 8.** Variance analysis of regression equation Y<sub>1</sub>.

Source	Sum of Square	Degree of Freedom	Mean Squares	F-Value	p-Values
Model	1120.76	5	224.15	17.66	0.0008
X <sub>1</sub>	304.450	1	304.50	103.91	0.0005
X <sub>1</sub> <sup>2</sup>	300.13	1	300.13	102.42	0.0005
X <sub>2</sub>	290.98	1	290.98	99.29	0.0006
X <sub>2</sub> <sup>2</sup>	192.17	1	192.17	65.58	0.0013
X <sub>1</sub> X <sub>2</sub>	88.20	1	88.20	30.10	0.0054
Lack of fit	77.14	3	25.71	8.77	0.0312
Pure error	11.72	4	2.93		
Cor total	1209.62	12			

R<sup>2</sup> = 92.65%; Adjusted R<sup>2</sup> = 87.41%; *p* < 0.01 is significant; L—linear term; Q—quadratic term.

**Table 9.** Variance analysis of regression equation Y<sub>2</sub>.

Source	Sum of Square	Degree of Freedom	Mean Squares	F-Value	p-Values
Model	0.0078	5	0.0016	11.12	0.0032
X <sub>1</sub>	0.0011	1	0.0011	43.61	0.0027
X <sub>1</sub> <sup>2</sup>	0.0023	1	0.0023	87.66	0.0007
X <sub>2</sub>	0.0031	1	0.0031	117.63	0.0004
X <sub>2</sub> <sup>2</sup>	0.0015	1	0.0015	57.82	0.0016
X <sub>1</sub> X <sub>2</sub>	0.0003	1	0.0003	10.21	0.0331
Lack of fit	0.0009	3	0.00029	11.28	0.0202
Pure error	0.0001	4	0.00003		
Cor total	0.0088	12			

R<sup>2</sup> = 88.82%; Adjusted R<sup>2</sup> = 80.83%; *p* < 0.01 is significant; L—linear term; Q—quadratic term.

**Table 10.** Variance analysis of regression equation  $Y_3$ .

Source	Sum of Square	Degree of Freedom	Mean Squares	F-Value	p-Values
Model	869.91	5	9.85	17.67	0.0008
$X_1$	80.5393	1	80.5393	27.48	0.0063
$X_1^2$	349.8102	1	349.8102	119.37	0.0004
$X_2$	262.0668	1	262.0668	89.43	0.0007
$X_2^2$	200.4206	1	200.4206	68.39	0.0012
$X_1X_2$	37.8140	1	37.8140	12.90	0.0230
Lack of fit	57.2059	3	19.0686	6.51	0.0510
Pure error	11.7219	4	2.9305		
Cor total	938.8329	12			

$R^2 = 92.66\%$ ; Adjusted  $R^2 = 87.41\%$ ;  $p < 0.01$  is significant; L—linear term; Q—quadratic term.

#### 4. Conclusions

The test results confirmed the usefulness of the method of optimizing the culture medium composition for the citric acid biosynthesis using the utility function. Optimization studies included: ANOVA analysis, determination of optimal values of input variables and their interactions as well as mathematical models in the form of quadratic functions describing the dependence of the final concentration of citric acid ( $Y_1$ ), volumetric rate ( $Y_2$ ), and yield of biosynthesis ( $Y_3$ ) on the method parameters. The correlation coefficients of  $R^2$  for the three models  $Y_1$ ,  $Y_2$ ,  $Y_3$  amounting to 92.65%, 88.82%, and 92.65%, respectively, showed good agreement between the experimental and predicted parameters of citric acid biosynthesis. The optimal values of input variables were determined:  $X_1 = 114.14 \text{ g}\cdot\text{L}^{-1}$  and  $X_2 = 2.85 \text{ g}\cdot\text{L}^{-1}$ . The bioreactor experiment showed that the average final concentration of citric acid was  $69.70 \text{ g}\cdot\text{L}^{-1}$  and the efficiency coefficient of citric acid biosynthesis was  $27.08\% \cdot \text{g}\cdot\text{L}^{-1} \cdot \text{h}^{-1}$ .

The obtained results confirmed the usefulness of the utility function method to optimize the parameters of biological processes. The presented approach to optimizing the composition of the culture medium using the utility function allows to increase the knowledge about the studied process. Analysis of the results showed that the designed experimental systems allowed identification of relationships describing the interaction of independent variables with response variables. In the studied area of variability, quadratic functions were a satisfactory reflection of the actual bioprocess function. Utility function has enabled poly-optimization by considering three criteria for maximizing the citric acid biosynthesis. The utility function also reflects the purpose of the designed study. The applied utility function determined the feasibility area in which the productivity of the process is maximum. The identified condition allowed to increase the efficiency of the process by 55%.

It should be emphasized that the proposed method makes it possible to determine the optimum based on a number of criteria, the list of which can be freely extended, which is important from a practical point of view.

In addition, this present study specified that crude glycerol by-product from biodiesel production could be used for producing high amount of citric acid without any pretreatment. It is forecast that the production of biodiesel in the years 2023–2025 will amount to 46 billion L, and for every 100 kg of biodiesel obtained, there is approximately 11 kg of glycerin phase [57]. On the other hand, the citric acid market is growing year by year and will reach 3.29 million t by 2028 [58]. This arrangement will enable the use of crude glycerol without pretreatment as a cheap energy source for the biosynthesis of citric acid. This would solve the ecological problems related to the disposal of the glycerine phase generated in the production of biodiesel. The bioconversion of crude glycerol by microorganisms allows to avoid the inconveniences associated with its chemical conversion, involving the use of high temperatures or pressures and consequently high costs [59]. The production of citric acid at a lower cost is in high demand. Therefore, innovations are needed to solve

the problems associated with increasing the scale of production in fermentation processes. Hence, cheap substrates, such as by-products from the agro-industry, have the potential to reduce costs and environmental problems.

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