fermentation

MDPI

Article

The Optimization of the Nutrient Medium Composition for the Submerged Cultivation of the *Mycolicibacterium neoaurum* Strain VKM Ac-3067D in a 100 L Bioreactor Under Controlled Conditions by Mathematical Planning

Vera V. Yaderets, Nataliya V. Karpova, Elena V. Glagoleva, Alexandra S. Shibaeva and Vakhtang V. Dzhavakhiya *

Laboratory of Biotechnology of Industrial Microorganisms, Department of Biotechnology and Technology of Bioorganic Synthesis Products, Russian Biotechnological University (ROSBIOTECH), Moscow 125080, Russia; verayaderetz@yandex.ru (V.V.Y.)

* Correspondence: vahoru@mail.ru

Abstract: The biotechnological production of carotenoids offers a promising alternative to their chemical synthesis or extraction from plants. *Mycolicibacterium* species have shown potential as pigment-producing microorganisms. However, bacterial strains typically exhibit lower productivity compared to fungal and yeast strains. Earlier, we enhanced the β-carotene biosynthesis in *M. neoaurum* strain VKM Ac-3067D by modifying the cultivation medium. Key changes included replacing glucose with glycerol and soybean meal with skimmed milk powder (SMP) and increasing the urea content from 0.5 to 1.0 g/L. To further optimize β-carotene yield, a steepest ascent method was applied combining factorial design with a gradient-based optimization (Wilson–Box method). The resulting regression model showed that the most influential factors were the glycerol concentration and SPM use. The in-flask fermentation of the Ac-3067D strain in a medium containing 25.5 g/L of glycerol (carbon source) and 12.80 g/L of SMP (nitrogen source) increased β-carotene yield to 318.4 ± 8.3 mg/kg. In a 15 L bioreactor, β-carotene yield increased to 432.3 ± 10.4 mg/kg, while the biomass concentration reached 23.2 ± 1.2 g/L. The further scaling up to a 100 L bioreactor increased both β-carotene yield (450.4 ± 8.2 mg/kg) and biomass concentration (25.2 ± 1.1 g/L). Thus, β-carotene production technology using the *M. neoaurum* strain AC-3067D was successfully scaled up from 750 mL flasks to a 100 L bioreactor, confirming its potential for industrial-scale application.

Keywords: mathematical planning; β-carotene; *Mycolicibacterium neoaurum*; nutrient medium; fermentation; bioreactor; scaling up

check for updates

Academic Editor: Ricardo Aguilar-López

Received: 31 December 2024
Revised: 24 January 2025
Accepted: 28 January 2025
Published: 7 February 2025

Citation: Yaderets, V.V.; Karpova, N.V.; Glagoleva, E.V.; Shibaeva, A.S.; Dzhavakhiya, V.V. The Optimization of the Nutrient Medium Composition for the Submerged Cultivation of the *Mycolicibacterium neoaurum* Strain VKM Ac-3067D in a 100 L Bioreactor Under Controlled Conditions by Mathematical Planning. *Fermentation* **2025**, *11*, 82. https://doi.org/10.3390/fermentation11020082

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

A high biological activity of carotenoids and their wide range of applications determine the ongoing interest of researchers in finding effective methods for their production [1–5]. According to the available data, there are three main methods of the production of these compounds, chemical synthesis [6,7], extraction [3,6,8–10], and biosynthesis, which is based on the use of various microorganisms including fungi, yeast, bacteria, and microalgae [2–5].

To date, the majority of commercial carotenoids on the global market are of a chemical origin [11,12] that is explained by low production costs, relatively high yield of target products, and the lack of the productivity dependence on a season [12]. However, chemical synthesis has several drawbacks. First, not all carotenoids can be produced via chemical

synthesis. Second, toxic by-products are generated during their production, thus contributing to environmental pollution. Third, chemically synthesized compounds can differ from their natural analogues in their isomeric composition and effectiveness, and may also cause allergic and other undesirable reactions. For example, natural astaxanthin consists mainly of esterified forms (95%), while synthetic astaxanthin is non-esterified; as a result, natural astaxanthin is 50-fold more effective in relation to the singlet oxygen neutralization and 20-fold more effective in scavenging of free radicals [13]. In addition, a global trend to prefer natural carotenoids has been observed in recent years among consumers. All these facts suggest that in the nearest future, the focus of carotenoid production may shift towards the use of natural sources.

Technologies related to the carotenoid extraction from natural plant sources are certainly positively assessed by consumers. However, the stability of the manufacturing processes is complicated by a seasonality of carotenoid sources, relatively low yield, and the need for the purification of the target product from other metabolites that does not completely meet the requirements of industrial production, and it is often labor-consuming, and requires significant land areas for the cultivation of target plants [14].

Bacteria represent another potential type of producers suitable for the microbiological production of carotenoids, because they are widely used in the large-scale production of various biological compounds and have a short life cycle [3,15–17]. The suitable producers may include either naturally pigmented bacteria, or genetically modified bacteria, which initially were not able to synthesize pigments.

The ability of a producer strain to synthesize compounds with specific properties depends not only on individual characteristics of the strain, but also on the conditions, under which the target product is obtained. Determining the composition of a cultivation medium is an effective method for increasing biosynthetic activity of microorganisms producing biologically active compounds [18,19]. The medium composition can be determined by two ways: empirical selection, or the use of mathematical design of experiments. The traditional empirical selection method is widely used for determining the optimal cultivation conditions for microorganisms [19]. However, experimental design makes it possible to simultaneously vary all factors and to provide a quantitative evaluation of the influence of key factors and interactions on the yield of the target parameter with smaller errors compared to the traditional one-factor methods [20–24]. The ultimate goal of the mathematical modeling is the controlled cultivation of producer strains to achieve the maximum yield of the biomass or target metabolites.

For example, modeling of the nutrient medium composition for *Candida melibiosica* strain 2491 enabled its optimization, so its use for the yeast cultivation increased the phytase biosynthesis by 22.5% [23]. This approach was also used in the planning of β-carotene production by *Rhodotorula glutinis* ATCC 4054. Rice bran, molasses, and sugarcane bagasse were tested as substrates. As a result, rice bran was found to be the best inexpensive substrate for the biomass growth and β-carotene production. Using mathematical planning, the optimal contents of medium components were determined, including sucrose (18.6 g/L), NaCl (0.66 g/L), and KH_2PO_4 (1.01 g/L). In addition, the optimal amount of the inoculum, pH (5.4), and stirring rate were established, which provided a several-fold increase in the biosynthesis of the target product [24].

Fast-growing, non-pathogenic bacteria from the genus *Mycolicibacterium*, which have been shown to synthesize β-carotene and lycopene as well as several other pigments [17,24–26], can be considered as promising producers of carotenoids. In our earlier studies, we developed the *M. neoaurum* strain AC-501/22 via chemical mutagenesis and optimized its cultivation conditions in a 3 L bioreactor to achieve a β-carotene yield of 262.4 mg/kg of dry biomass [27]. The strain was deposited in the All-Russian Collection of Microorganisms (accession num-

ber *M. neoaurum* VKM AC-3067D). The purpose of this study was the optimization of the nutrient medium composition using mathematical planning and its approbation under conditions of a submerged cultivation of the AC-3067D strain during the scaling up of the process from flasks to a 100 L bioreactor.

2. Materials and Methods

2.1. Reagents and Media Components

Skimmed milk powder was manufactured by HiMedia Laboratories (Mumbai, India). Urea was manufactured by NeoFroxx GmbH (Einhausen, Germany). Skimmed deodorated soybean flour was purchased from Soyanta 200 (Irkutsk, Russia). Citric acid was manufactured by Component-reactiv Ltd. (Moscow, Russia). Agar was manufactured by Difco (Detroit, MI, USA). Inorganic salts, glycerol, glucose, and organic solvents (acetone, hexane, benzene) were purchased from Acros Organics (Geel, Belgium).

2.2. Producer Strain

M. neoaurum strain AC-3067D was stored in the working collection of the Laboratory of Biotechnology of Industrial Microorganisms of the Russian Biotechnological University (Moscow, Russia).

2.3. Nutrient Media for M. neoaurum Cultivation and Maintenance

M. neoaurum VKM Ac-3067D was cultivated on the A1 medium of the following composition (g/L): agar, 17.0; glucose, 10.0; soybean meal, 10.0; citric acid, 2.2; urea, 0.5; NH_4Cl, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; and $CaCO_3$, 1.5 (pH 6.8–7.2). After being stored on an agarized medium at 4 °C for one month, the cultures were transferred to a fresh medium. For long-term preservation, the cultures were freeze-dried with a powdered milk as a carrier and stored at 4 °C.

2.4. Cultivation of M. neoaurum VKM Ac-3067D on a Liquid Nutrient Medium

The control liquid medium (AM medium) used for the further modification had the following composition (g/L): glycerol, 20.0; skimmed milk powder (SMP), 10.0; citric acid, 2.2; urea, 1.0; NH_4Cl, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; and $CaCO_3$, 1.5 (pH 6.8–7.2).

The inoculum was prepared as follows. A tube containing the bacterial culture was supplemented with 10 mL of a sterile physiological solution. The agar surface was gently scraped using an inoculation loop, and the obtained cell suspension was transferred into 750 mL flasks containing 100 mL of the AM medium followed by the 48-h incubation of flasks at 220 rpm and 35 °C in an Innova 44 shaker (New Brunswick, Germany). The obtained inoculate (10 vol. %) was then transferred to fresh flasks containing 100 mL of the same medium and cultivated for 72 h under identical conditions. The resulting culture broth was either used to inoculate the bioreactor, or dried and then analyzed for the carotenoid content.

2.5. Carotenoid Content Determination

The carotenoid content in the cell biomass of *M. neoaurum* VKM Ac-3067D was measured spectrophotometrically [27]. The procedure was conducted under low illumination with the room temperature maintained below 20 °C. A 30-mL aliquot of culture broth was centrifuged for 5 min at 7500 rpm. The supernatant was discarded, and the remaining biomass underwent a 3-fold extraction using 10 mL of acetone. The resulting acetone extracts were pooled, transferred into a separating funnel, and mixed with petroleum ether (10 mL). After vigorous shaking, the resulted emulsion was broken by a adding a saturated

NaCl solution dropwise. Once the acetone layer separated, the solution was re-extracted with petroleum ether. The petroleum ether extracts were pooled and filtered through a glass filter. The absorption spectrum of the carotenoid extract was recorded at 450 nm using a Thermo Spectronic spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with petroleum ether used as a reference. The total carotenoid content was determined using the following formula:

$$Carotenoid\ content\ (\mu g/g\ d.w.) = \frac{V(mL) \times A_{450} \times 10^6}{2592 \times 100 \times m(g)}, \quad (1)$$

where A_{450} is the experimentally determined adsorption of the measured solution; 2592 is the value of a 1% extinction; and m is the weight of dried cells (g).

2.6. Nutrient Medium Optimization Using the Mathematical Planning Method (Complete Factorial Experiment, CFE 2^3)

2.6.1. Nutrient Medium Optimization by a Complete Factorial Experiment (CFE)

To perform the nutrient medium optimization by CFE [28], three factors were chosen: glycerol concentration (X1), SPM concentration (X2), and urea concentration (X3). Variations of these factors were arranged on two levels, bottom (–1) and upper (+1). The plan center (Z) was chosen based on the research executed on the influence of these components on the β-carotene biosynthesis and the biomass yield [28]. The sizes of variation steps (δ) for these factors are shown in Table 1.

Table 1. The plan center and the variation step of the studied factors.

Factors	**X1**	**X2**	**X3**
Variables	Glycerol	SMP	Urea
Concentration, g/L	20.0	10.0	1.0
δ, g/L	2.5	1.5	0.15
Low level (–1), g/L	17.5	7.5	0.85
High level (+1), g/L	22.5	11.5	1.15

The number of experiments N was determined by the following formula:

$$N = n \times k, \quad (2)$$

where n is the number of the levels of variation and k is the number of factors.

The regression coefficients were determined using Formulas (3) and (4):

$$b_0 = \frac{\sum \overline{y}}{N}, \quad (3)$$

$$b_i = \frac{\sum [(x)]_{iu} \overline{y}_u}{N}, \quad (4)$$

where N is the number of experiments calculated by Formula (2), $\bar{y}$ is the experimentally obtained value of the parameter, x_{iu} is the value of the variable in the corresponding column of the experiment plan, u is the number of the experimental variant, and i is the number of a factor.

The coefficients of interaction (b_{12}, b_{13}, b_{23}, b_{123}) were determined using Formulas (5) and (6):

$$b_{ij} = \frac{\sum_{u=l}^{N} x_{iu} x_{ji} \overline{y}_u}{N} \quad (5)$$

$$b_{ijk} = \frac{\sum_{u=l}^{N} x_{iu} x_{ji} x_{ku} \overline{y}_u}{N} \quad (6)$$

where x_{iu}, x_{ju}, and x_{ku} are variable values in the corresponding column of the planning matrix and $\bar{y}_u$ is the average value of the optimization parameter.

The line-by-line dispersion was determined by Formula (7):

$$S^2_{y_{ul}} = \frac{\sum (y_{ul})^2 - \frac{(\sum y_{ul})^2}{m}}{m-1}, \quad (7)$$

where u is the row number in the planning matrix, l is the number in each row, and m is the number of parallel experiments.

A single-value dispersion is calculated using Formulas (8) and (9):

$$S^2_{b_i} = \frac{\sum S^2_{y_{ul}}}{mN^2}, \quad (8)$$

$$S_{b_i} = \sqrt{S^2_{b_i}}. \quad (9)$$

The error determination is calculated by Formula (10):

$$|\,b_i\,| = t \times S_{bi}, \quad (10)$$

where t is Student's coefficient at $t_{0.9S}$ determined as a tabulated value for the given number of the degrees of freedom (f).

Degrees of freedom were calculated using Formulas (11) and (12):

$$f = (m-1) \cdot N, \quad (11)$$

where m is the number of parallel experiments and N is the total number of experiments.

$$f_{ad} = N - N', \quad (12)$$

where N is the number of experiments and N' is the number of significant regression coefficients.

The dispersion of adequacy was calculated using Formula (13):

$$S^2_{ad} = \frac{\sum (\overline{y}_u - y_u)^2}{N - N'}, \quad (13)$$

where N is the total number of experiments, N' is the number of significant regression coefficients including b_0, $\bar{y}$ is the experimentally obtained factor value, and y is the factor value calculated by the obtained regression formula.

Then, using Formula (14), the dispersion of reproduction was calculated:

$$S^2_{\overline{y}} = \frac{\sum S^2_{y_{ul}}}{mM}. \quad (14)$$

The Fisher criterion was calculated using Formula (15):

$$F_p = \frac{S^2_{ad}}{S^2_{\overline{y}}}. \quad (15)$$

The uniformity and reproducibility of the experiment were evaluated by the Cochran test (Gp) using table data.

2.6.2. Nutrient Medium Optimization Using the Steepest Ascent Method

The nutrient medium optimization by the steepest ascent method was performed according to [29]. After the assessment of the adequacy of the regression equation and the fulfillment of the $F_m > F_p$ condition, the next optimization stage is carried out using the above-mentioned method. First, the L_i coefficient is found according to Formula (16) to calculate the steepest ascent program:

$$L_i = \delta \times b_i, \tag{16}$$

where δ is the variation step and b_i is the regression coefficient.

The L_i value can be either positive or negative. Therefore, the factor can either increase or decrease. The factor with the maximum $|L_i|$ value is considered as the basic one. Then, the new γ_i coefficients are calculated using Formula (17):

$$\gamma_i = \frac{L_i}{|L_{\max}|}. \tag{17}$$

In the case of the base factor (hbase, Formula (18)), the step for the movement along the gradient is calculated according to Formula (19). First, the "reserve" for the movement along each factor from the basic level up to a practical worthwhile value in the decreasing or increasing direction is calculated by Formula (19).

$$h_{base} = \frac{\Delta i}{n}, \tag{18}$$

$$\Delta i = Xi_{\max} - Xi_0 \ (\Delta i = Xi_0 - Xi_{\min}), \tag{19}$$

were n is the number of steps towards the maximum or minimum value of the Xi_{max} factor. The n number is chosen arbitrarily; as a rule, it varies within 5–8 steps.

The movement step for other factors is calculated using Formula (20):

$$h = h_{base} \times \gamma_i \tag{20}$$

If the regression coefficient of a planning factor is insignificant, then during a calculation of the steepest ascent program, it can be used at the basic level. A new matrix is constructed on the basis of the performed calculations, and then new experiments are performed.

All experiments on the mathematical planning were performed three times, each in three replications. The data shown in the Section 3 represent the means of these experiments.

2.7. M. neoaurum Fermentation in a 3 L Bioreactor

2.7.1. Inoculum Preparation

The procedure of inoculum obtaining was similar to that described in Section 2.4; the following medium composition was used (g/L): glycerol, 25.5; powdered milk, 12.8; citric acid, 2.2; urea, 1.0; NH_4Cl, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; and $CaCO_3$, 1.5 (pre-sterilization pH 6.8–7.2). Prior to the inoculation of the bioreactor, the inoculum was microscoped using a Carl Zeiss Primo Star microscope (Carl Zeiss, Jena, Germany) for the quality control. The inoculum volume further used for the bioreactor seeding was 10% of the fermentation medium volume (150 mL). The inoculum was added in the bioreactor via a sterile inoculum feeding line.

2.7.2. Bioreactor Preparation and Fermentation

M. neoaurum fermentation in a 3 L bioreactor was performed as described earlier [27]. After the completion of the process, the biomass was inactivated by a 30-min heating at 80–85 °C with continuous stirring, then transferred out into a collection vessel, centrifuged, and dried in a Martin Christ ALPHA 2-4LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

2.8. M. neoaurum Fermentation in a 15 L Bioreactor

2.8.1. Inoculum Preparation

An inoculum was produced in accordance with the procedure described in Section 2.4; the medium composition was the same as described in Section 2.7.1. The inoculum obtained in flasks was combined under sterile conditions into one 2 L flask with the bottom outlet. The total volume of the inoculum was 1.0 L. Prior to the inoculation of the bioreactor, the quality of inoculum was examined using a Carl Zeiss Primo Star microscope (Carl Zeiss, Jena, Germany). The inoculum was transferred into the bioreactor via a sterile inoculum feeding line.

2.8.2. Bioreactor Preparation

The fermentation system used in the study consisted of a computer-based automatic control unit and three 15 L bioreactors (Prointech-Bio, Pushchino, Russia) connected via the coupling elements with the feed lines for the direct steam, compressed air, and water. The effective volume of bioreactors was 10 L. The units for monitoring culture broth parameters included a Buk-3 thermal sensor connected to a controller (Keklab Group, Moscow, Russia); an InPro 6800/12/220 oxygen sensor (Mettler Toledo, Greifensee, Switzerland) connected to a controller regulating the work of the mechanical stirring device; and an InPro3300/225/PT1000 pH sensor (Mettler Toledo, Greifensee, Switzerland) connected via a controller to a peristaltic pump delivering a titrating solution.

Bioreactors were equipped with 0.2 µm fine filters for compressed air. The preparation and sterilization of the fermentation medium (see Section 2.8.1 for the medium composition) were carried out directly within the bioreactor. Preliminarily, bioreactors with the air filters were sterilized for 1 h at 121 ± 2 °C. Then, all components of the fermentation medium were sequentially loaded into each bioreactor through a receiver, and the volume of the medium was adjusted to 10.0 L with purified water. If necessary, the pH level was adjusted to 6.8–7.2. The medium was sterilized for 1 h at 125 ± 1 °C by introducing direct steam into the bioreactor through a bubbler, sample collector, and bottom outlet.

2.8.3. *M. neoaurum* Fermentation

The pre-inoculation fermentation regime is shown in Table 2. The level of pO_2 was controlled by changing the air supply volume to culture broth volume ratio (manual mode) via changes in the stirrer's rpm number (automatic mode). For both regimes, the fermentation process duration was 82 h. The basic fermentation parameters are shown in Table 3.

Table 2. Pre-inoculation fermentation regime (15 L bioreactor).

Parameter	Value
Medium volume	10.0 L
Temperature	35 ± 1 °C
Aeration	0.1 L/min

Table 2. *Cont.*

Parameter	Value
Stirring rate	250 rpm
pO_2 level	100% of saturation
Pressure within the bioreactor	0.03–0.05 MPa
Medium pH	6.8–7.2

Table 3. Basic parameters for the *M. neoaurum* strain AC-3067D fermentation in a 15 L bioreactor.

Parameter	Value
Temperature	35 ± 1 °C
Aeration	5–10 L/min
pH	6.8–7.2
Stirring rate	400–450 rpm to maintain the required level of dissolved oxygen
Feeding addition	Glycerol (2.5 g/L) after 30 h of fermentation
Fermentation time	72–76 h
Process control	The first sampling to control the culture purity and crude biomass weight is performed after 18–24 h of fermentation. The further sampling is performed as required, but at least one time a day.

Upon the completion of fermentation, the biomass of *M. neoaurum* was either inactivated (see Section 2.7.2) or transferred via a seed train to seed a 100 L bioreactor.

2.9. M. neoaurum Fermentation in a 100 L Bioreactor

2.9.1. Inoculum Preparation

The inoculum for the bioreactor was obtained as described in Section 2.8.

2.9.2. Bioreactor Preparation

A 100 L bioreactor was connected via the control unit with the feed lines for the steam, compressed air, and water as well as with an automatic control unit. The effective volume of the bioreactor ranged from 60 to 70 L. The measurement devices required to control fermentation medium parameters included a Buk-3 thermal sensor connected to a controller (Keklab Group, Moscow, Russia), an InPro 6800/12/220 sensor for dissolved oxygen (Mettler Toledo, Greifensee, Switzerland), connected via a controller to the mechanical stirring device, and an InPro3300/225/PT1000 pH sensor (Mettler Toledo, Greifensee, Switzerland) intended to manage the supply of a titrant solution by a peristaltic pump via the controller.

The fermentation medium was mixed by a mechanical stirring device characterized by the motor located on the top and the end seal; the stirring rate ranged from 50 to 600 rpm. The pO_2 level was regulated by manual adjusting the air flow volume compared to the fermentation medium volume and by automatic change in the stirring rate. The medium was sterilized directly in the bioreactor. After sterilization, the air flow rate and the pressure within the bioreactor were set to be 35 L/min and 0.03 MPa, respectively; the dissolved oxygen concentration in the medium was set to 100%. After the cooling of the bioreactor to 29–30 °C, a sterile medium sample was taken for a microbiological analysis.

2.9.3. *M. neoaurum* Fermentation

The pre-inoculation fermentation regime is shown in Table 4. The pO_2 level was regulated by manual adjusting the air flow rate relative to the volume of the culture medium with the automatic changes in the rpm number of the stirrer. For both regimes,

the process was conducted for 72–74 h. The basic fermentation parameters are shown in Table 5.

Table 4. Pre-inoculation fermentation regime (100 L bioreactor).

Parameter	Value
Medium volume	70.0 L
Temperature	35 ± 1 °C
Aeration	3.5 L/min
Stirring rate	250 rpm
pO_2 level	100% of saturation
Pressure within the bioreactor	0.03–0.05 MPa
Medium pH	6.8–7.2

Table 5. Basic parameters for a *M. neoaurum* strain AC-3067D fermentation in a 100 L bioreactor.

Parameter	Value
Temperature	35 °C
Aeration	35–70 L/min
pH	Maintained at 6.8–7.2 using a sterile 10% HCl solution
Stirring rate	400–450 rpm to maintain the required level of dissolved oxygen
Feeding addition	50% glycerol solution (2.5 g/L of medium) after 30 h of fermentation
Fermentation time	72–80 h

Upon the completion of fermentation, the biomass of *M. neoaurum* was inactivated (see Section 2.7.2) and collected into a collector. After centrifugation, it was dried in a Martin Christ ALPHA 2-4LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

3. Results

3.1. Optimization of Nutrient Medium Composition by CFE 2^3

In this study, the composition of the nutrient medium was optimized by the steepest ascent method, which combines CFE with the Wilson–Box method. The planning factors included the concentrations of glycerol, a surfactant (SMP), and urea (see Table 1). The output parameter of optimization (y) was determined as the content of β-carotene (mg/g) in the culture broth (CB). The number of experiments was determined using Formula (2) (N = 8 for this study); based on this, the experimental planning matrix for CFE 2^3 was constructed (Table 6). Using this matrix, a series of experiments was conducted, each in three replications. The results are shown in Table 3.

The mathematical model equation should be the following:

$$\overline{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3,$$

where $\bar{y}$ is the process optimization parameter, b_i is a regression coefficient indicating the influence of factors on the optimization parameters, and x is the value of a factor level in encoded units. The homogeneity and reproducibility of the experiment were evaluated using Cochran's criterion (Gp). For the obtained data, Gp = 0.2594, which is less than the tabulated value for the used degrees of freedom (0.2594 < 0.5157). Thus, we concluded that the obtained data are homogenous and reproducible.

Table 6. The experimental matrix in natural and encoded units of factor values and the results of the experiment (output parameter values).

Experiment No.	Encoded Factors			Natural-Scale Factors, g/L			β-Carotene Yield, mg/kg			
	x_1	x_2	x_3	x_1	x_2	x_3	y_1 *	y_2 *	y_3 *	$\bar{y}_u$ **
1	−1	−1	−1	17.5	8.5	0.85	182.5	170.4	186.2	179.70
2	+1	−1	−1	22.5	8.5	0.85	206.2	196.5	188.4	197.03
3	−1	+1	−1	17.5	11.5	0.85	190.6	198.5	188.6	192.57
4	+1	+1	−1	22.5	11.5	0.85	248.3	220.4	229.5	232.73
5	−1	−1	+1	17.5	8.5	1.15	180.8	188.5	183.8	184.37
6	+1	−1	+1	22.5	8.5	1.15	204.3	227.4	214.5	215.40
7	−1	+1	+1	17.5	11.5	1.15	196.6	202.6	220.7	206.63
8	+1	+1	+1	22.5	11.5	1.15	216.7	200.8	218.7	212.07

* y_1, y_2, and y_3 represent the numerical values of the response function for each of the replications obtained after the completion of the experiment, mg/kg; ** $\bar{y}_u$ is the mean arithmetical value of the response function, mg/kg.

To determine the direction of action for each factor, regression coefficients were calculated using Formulas (3) and (4): b_0 = 202.56, b_1 = 11.75, b_2 = 8.44, and b_3 = 2.05. Then, using the data presented in Table 6, the significance of the calculated regression coefficients was determined for m = 3, where m is the number of parallel experiments. The line-by-line dispersion was calculated using Formula (7), where m = 3 represents the number of parallel experiments and N = 8 represents the total number of experiments. The results are shown in Table 7.

Table 7. Determination of line-by-line dispersions.

u	y_{ul}			$\sum(y_{ul})^2$	$(\sum y_{ul})^2$	$\frac{(\sum y_{ul})^2}{m}$	$S^2_{y_{ul}}$
1	182.5	170.4	186.2	97,012.85	290,628.81	96,876.27	68.29
2	206.2	196.5	188.4	116,625.25	349,399.21	116,466.40	79.42
3	190.6	198.5	188.6	111,300.57	333,737.29	111,245.76	27.40
4	248.3	220.4	229.5	162,899.3	487,483.24	162,494.41	202.44
5	180.8	188.5	183.8	102,003.33	305,919.61	101,973.20	15.06
6	204.3	227.4	214.5	139,459.5	417,574.44	139,191.48	134.01
7	196.6	202.6	220.7	128,406.81	384,276.01	128,092.00	157.40
8	216.7	200.8	218.7	135,109.22	404,750.44	134,916.81	96.20
							$\sum$ = 780.24

Using Formulas (8) and (9), a single-value dispersion (S_{bi}) was calculated to be equal to 2.02. Then, using Formula (10) with t = 2.09, the error determination was calculated (tS_{b_i} = 4.21). Therefore, regression coefficients are significant in the case when $|bi| > 4.21$, and the regression equation takes the following form:

$$y = 202.56 + 11.75x_1 + 8.44x_2 \quad (21)$$

To evaluate the adequacy of the obtained linear approximation equation, Equation (21), the β-carotene yield (y) was calculated for each variant. The x_1 and x_2 values were taken from Table 6. The results of the performed calculations are shown in Table 8.

Using Formula (12), the dispersion of adequacy S^2_{ad} was determined at $N - N' = 5$. According to the performed calculations, S^2_{ad} = 164.33. Using Formula (13), the dispersion of reproducibility S^2_y = 32.51 was calculated. The Fisher criterion Fp = 5.05 was calculated by Formula (14).

Table 8. The determination of the deviation square for the average values obtained experimentally and those calculated by the linear approximation equation.

u	y_{ul}	$\bar{y}_u$	$\bar{y}_u - y_u$	$(\bar{y}_u - y_u)^2$
1	182.38	179.70	−2.68	7.18
2	205.87	197.03	−8.84	78.10
3	199.25	192.57	−6.69	44.72
4	222.75	232.73	9.99	99.75
5	182.38	184.37	1.99	3.95
6	205.87	215.40	9.53	90.81
7	199.25	206.63	7.38	54.45
8	222.75	212.07	−10.68	114.04
				$\sum = 493.00$

The tabulated value of the Fisher criterion (F_t) is located at the intersection of the values of the degree of freedom of the dispersion of adequacy (f_{ad} = 5) and the number of degrees of freedom of the dispersion of reproducibility (f = 16).

Since $F_p > F_t$ (5.05 > 2.85), then, first, the equation is inadequate (i.e., the process cannot be described by the linear approximation equation). Second, the obtained results evidence that the process is close to the near-optimum area, when the influence of the interaction between factors is enhanced.

If the process cannot be described by the linear approximation equation, it is possible to use a complete factorial experiment with allowance for all interactions between factors. A new planning matrix and the results obtained during the experiment implementation are shown in Table 9.

Table 9. Experimental plan and obtained results.

Experiment No.	Factors at a Natural Scale, g/L			Encoded Factors			Auxiliary Graphs in a CFE 23 Matrix				B-Carotene Yield, mg/kg			
	x_1	x_2	x_3	x_1	x_2	x_3	x_1x_2	x_2x_3	x_1x_3	$x_1x_2x_3$	y_1 *	y_2 *	y_3 *	$\bar{y}_u$ **
1	17.5	8.5	0.85	−1	−1	−1	+1	+1	+1	−1	182.5	170.4	186.2	179.70
2	22.5	8.5	0.85	+1	−1	−1	−1	+1	−1	+	206.2	196.5	188.4	197.03
3	17.5	11.5	0.85	−1	+1	−1	−1	−1	+1	+1	190.6	198.5	188.6	192.57
4	22.5	11.5	0.85	+1	+1	−1	+1	−1	−1	−1	248.3	220.4	229.5	232.73
5	17.5	8.5	1.15	−1	−1	+1	+1	−1	−1	+	180.8	188.5	183.8	184.37
6	22.5	8.5	1.15	+1	−1	+1	−1	−1	+1	−1	204.3	227.4	214.5	215.40
7	17.5	11.5	1.15	−1	+1	+1	−1	+1	−1	−1	196.6	202.6	220.7	206.63
8	22.5	11.5	1.15	+1	+1	+1	+1	+1	+1	+1	216.7	200.8	218.7	212.07

* y_1, y_2, and y_3 represent the numerical values of the response function for each of the replications obtained after the completion of the experiment, mg/kg; ** $\bar{y}_u$ is the mean arithmetical value of the response function, mg/kg.

The regression equation obtained by the use of CFE 2^3 is the following:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + + b_{13}x_1x_3 + + b_{23}x_2x_3 + b_{123}x_1x_2x_3.$$

Using Formulas (3)–(6), coefficients of regression were calculated. The regression equation has the following form:

$$y = 202.56 + 11.75x_1 + 8.44x_2 - 6.05x_2x_3 - 6.5x_1x_2x_3 \quad (22)$$

Then, the adequacy of the obtained linear approximation equation, Equation (22), was determined. To achieve this, a dry biomass yield (y) was calculated for each variant using the regression equation. The results of this calculation are shown in Table 10.

Table 10. The deviation square for the means determined experimentally and by the linear approximation equation.

u	y_{ul}	$\bar{y}_u$	$\bar{y}_u - y_u$	$(\bar{y}_u - y_u)^2$
1	194.49	179.70	−14.79	218.67
2	193.76	197.03	3.27	10.70
3	187.15	192.57	5.42	29.39
4	234.85	232.73	−2.12	4.50
5	170.27	184.37	14.10	198.69
6	217.98	215.40	−2.58	6.65
7	211.36	206.63	−4.73	22.37
8	210.64	212.07	1.43	2.04
				$\sum = 493.00$

The dispersion of adequacy was calculated using Formula (13). According to the performed calculations, the dispersion of adequacy was equal to 98.60. The dispersion of reproduction calculated earlier by Formula (14) was 32.51. The Fisher criterion F_p calculated by Formula (15) was equal to 3.03. The tabulated value of the Fisher criterion (F_t) was determined at $f_{ad} = 3$ and $f = 16$. Since $F_p < F_t$ (3.03 < 3.24), then, the obtained regression equation (Formula (21)) is adequate to the studied process. Therefore, the biomass yield within the studied concentration range is positively influenced by an increase in the glycerol and SMP concentrations. At the same time, the effect of these factors is enhanced in the case of a combined action of x_1 and x_2 or x_1, x_2, and x_3 factors (if these factors are at the highest coordination level).

3.2. The Optimization of the Nutrient Medium Composition by the Steepest Ascent Method

Based on the results of the performed CFE, one can suppose the efficiency of the steepest ascent method for the further medium optimization. Since the coefficient of regression b_3 was determined as insignificant, then the urea concentration was further considered as a constant variable equal to 1.0 g/L.

The initial data required for the construction of a new planning matrix are shown in Table 11.

Table 11. The calculation of the steepest ascent for the determination of the quantitative ratio of medium components.

Factor and Experiment Characteristics	X_1 **Glycerol Concentration**	X_2 **SPM Concentration**
Base level, g/L	22.5	11.5
Maximum level, g/L	30.0	15.0
Stock, Δ_i	7.5	4.5
Variation interval (δ_i)	2.5	1.5
Coefficient of regression (b_i)	11.75	8.44
Production $L_i = b_i\delta_i$	29.34	12.66
Coefficient (γ_i)	0.26	0.59
Steepest ascent step (h_i), g/L	1.5	0.65

Since the value of the γ_i coefficient for glycerol is less than that for SMP, this factor was considered as the base one. The steepest ascent steps for glycerol and SMP are presented in Table 11. In all variants, the urea concentration was 1.0 g/L. A new experimental design matrix was created, and the β-carotene yield was determined. The results are shown in Table 12.

Table 12. The planning matrix for the experiment based on the steepest ascent method.

Experiment No.	X_1 (Glycerol Concentration, g/L)	X_2 (SMP Concentration, g/L)	β-Carotene Yield, mg/kg
1 (initial medium)	22.5	11.5	248.3 ± 5.7
2	24.0	12.15	284.5 ± 8.2
3	25.5	12.80	318.4 ± 8.3
4	27.0	13.45	292.4 ± 6.4
5	28.5	14.10	252.1 ± 5.5
6	30.0	14.75	224.6 ± 8.2

The maximum β-carotene yield (318.4 ± 8.3 mg/kg) was obtained for variant 3, where the nutrient medium contained 25.5 g/L of glycerol and 12.8 g/L SMP. The further increase in the glycerol and SMP concentrations up to 30.0 and 14.75 g/L, respectively, was accompanied with the inhibition of a pigment accumulation process.

Thus, a new composition of the fermentation medium was proposed. The next stage of work included the examination of this medium composition for the cultivation of *M. neoaurum* in a 3 L bioreactor.

3.3. M. neoaurum Fermentation in a 3 L Bioreactor

Earlier, we studied the effect of the temperature, dissolved oxygen level, and acidity of a culture medium on the biomass accumulation and β-carotene production by *M. neoaurum* [27]. The optimal pH values were found to fall within the range of 6.8–7.0, and the optimal O_2 concentration was 50%. Therefore, these parameters were maintained at a constant level during *M. neoaurum* fermentation in the bioreactor as described in the Section 2.

After 30 h of fermentation, a sterile 50% glycerol solution (2.5 g/L of fermentation medium) was added under controlled optimal pH and O_2 concentration (6.8–7.2 and 50%, respectively). Glycerol addition started automatically, when pH exceeded 7.0, and the dissolved oxygen concentration decreased. The results are shown in Figure 1. The productivity of *M. neoaurum* in terms of the β-carotene production and the maximum biomass increase was recorded after 72 h of fermentation and reached 370.5 mg/kg and 25.4 g/L, respectively.

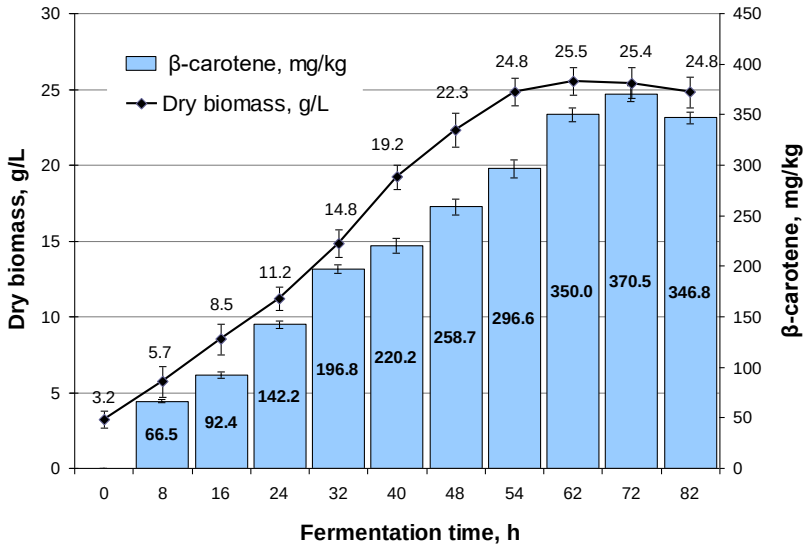


Figure 1. Biomass yield and β-carotene accumulation during fermentation of *M. neoaurum* in 3 L fermenter with culture feeding by 2.5 g/L glycerol after 30 h of cultivation.

3.4. M. neoaurum Fermentation in a 15 L Bioreactor

The fermentation of *M. neoaurum* in a 15 L bioreactor was performed under the same conditions as described in Section 3.3. The results are shown in Figure 2. The β-carotene productivity of *M. neoaurum* and the maximum biomass yield observed after 72 h of fermentation reached 432.3 ± 10.4 mg/kg and 23.2 ± 1.2 g/L, respectively.

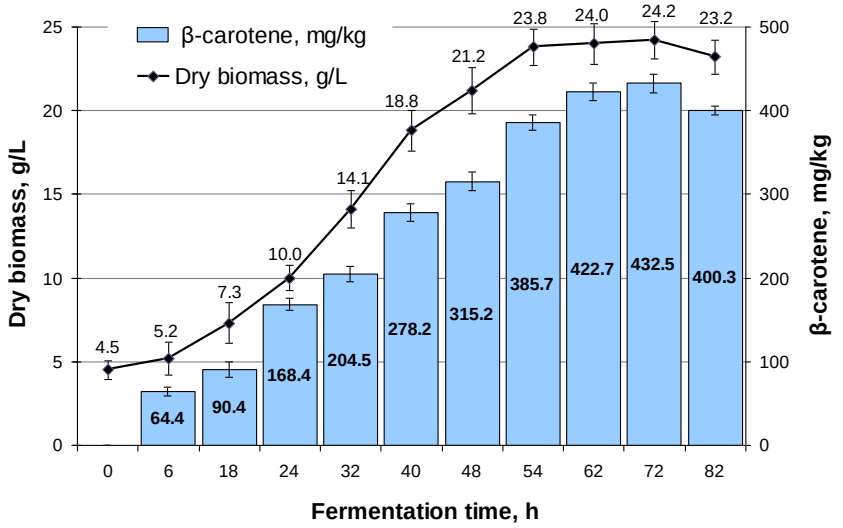


Figure 2. Biomass yield and β-carotene accumulation during fermentation of *M. neoaurum* in 15 L fermenter with culture feeding (50% glycerol, 2.5 g/L) after 30 h of cultivation.

3.5. M. neoaurum Fermentation in a 100 L Bioreactor

The fermentation of *M. neoaurum* in a 100 L bioreactor was performed under the constant pH (6.8–7.2), and the pO_2 value of 50% or more. After 30 h of fermentation, a sterile 50% glycerol solution (2.5 g/L of fermentation medium) was added in an automatic mode. The results are shown in Figure 3. The β-carotene productivity and the maximum biomass yield of the Ac-3067D strain observed after 72 h of fermentation reached 450.4 ± 8.2 mg/kg and 25.2 ± 1.1 g/L, respectively.

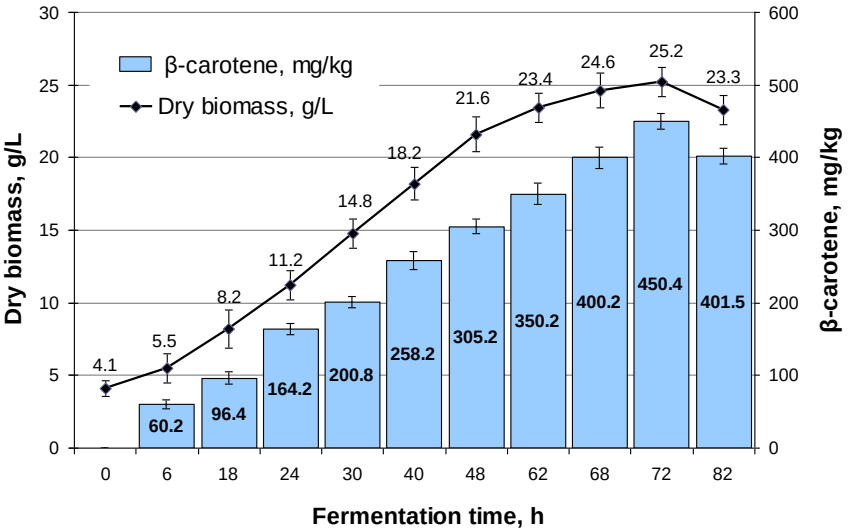


Figure 3. Biomass yield and β-carotene accumulation during fermentation of *M. neoaurum* in 100 L fermenter with culture feeding (50% glycerol, 2.5 g/L) after 30 h of cultivation.

4. Discussion

One of the most challenging tasks for biotechnological processes based on the use of overproducing strains is the selection and optimization of fermentation conditions. The analysis of numerous data shows that the growth of a culture and the biosynthesis of secondary metabolites are influenced by such factors as the viability of the planting material, the composition of the cultivation medium, and physicochemical conditions of fermentation. However, one should note that optimal conditions for the cell growth and the target product biosynthesis may not coincide [29–31].

Traditional approaches for the optimization of the β-carotene production are based on the change in one parameter at a time. However, this approach often does not allow a researcher to determine variables responsible for the optimal result, since such approaches do not take into account possible interaction between different factors [24]. One of the effective methods for optimizing cultivation conditions is a combination of the experimental and mathematical modeling with the conduction of a computational experiment, which includes an important step, namely, the determination of a mathematical model, i.e., a regression equation, which characterizes the relationship between the optimization parameter and the main factors. An approach based on the design of experiments (DOE) makes it possible to not only efficiently evaluate the main effects and interactions using a minimal number of experiments, but also relatively rapidly conclude on the significance of various components of the nutrient medium as well as to determine its qualitative and quantitative composition [20–24]. Mathematical modeling is widely used to determine optimal cultivation conditions for highly active strains. Numerous publications describe different examples of the DOE use for the development of processes occurring in biotechnological plants. However, one should note that this study represents the first attempt to use mathematical modeling for the optimization of the *M. neoaurum* cultivation conditions.

This study investigated the influence of three key factors (glycerol, SMP, and urea) on the β-carotene productivity of *M. neoaurum*. We found that glycerol and SMP provided the greatest impact on the pigment yield. The stimulation of the carotenoid biosynthesis can be explained by the fact that glycerol is involved in the biosynthesis of isoprenoids representing precursors of carotenoids (Figure 4). Use of glycerol as a carbon source for the β-carotene biosynthesis was also demonstrated in some other studies. For example, Bindea et al. [32] studied the effect of pH and glycerol concentration on a pigment accumulation. In addition, a positive effect of a glycerol addition into the nutrient medium on the carotenoid biosynthesis was reported by Suwaleerat et al. [33] for *Rhodococcus opacus* PD630. According to the obtained regression equation, the factors that have the greatest influence on the biosynthesis of β-carotene by *M. neoaurum* cells include glycerol and SMP concentrations. Using the mathematical planning method, the optimal values of these concentrations were determined as 25.5 and 12.80 g/L, respectively.

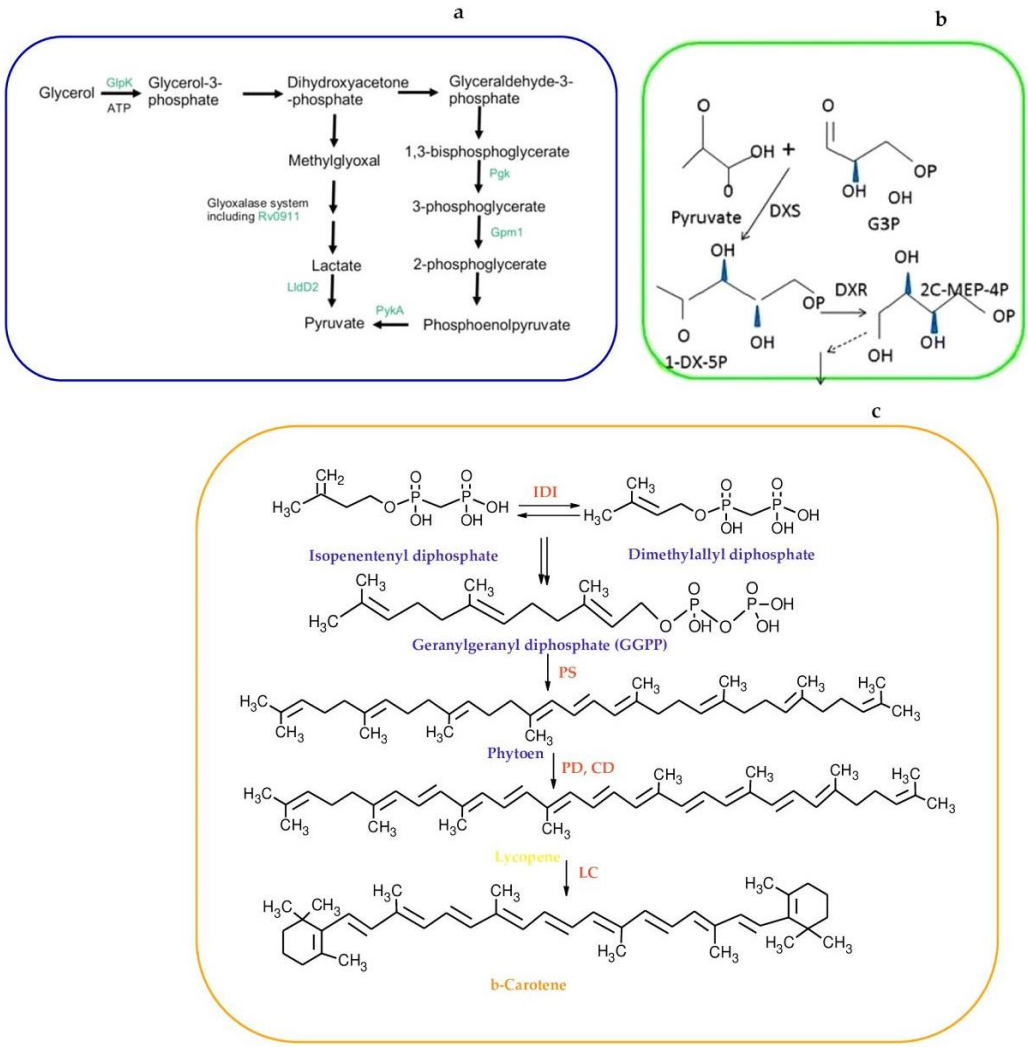


Figure 4. Proposed pathway for carotenoid biosynthesis in *M. neoaurum* based on published data: (**a**) pyruvate production from glycerol [34]; (**b**) biosynthetic pathways of isoprenoids [35]; (**c**) general scheme of carotenoid biosynthesis [36]. 1-DX-5P, deoxyxylulose 5-phosphate; 2C-MEP-4P, 2-C-methyl-D-erythritol 4-phosphate; CD, carotene desaturase; DXR, 1-deoxy-D-xylose 5-phosphate reducto-isomerase; DXS, 1-deoxy-D-xylose 5-phosphate synthase; G3P, glycerolaldehyde-3-phosphate; Glpk, glycerol kinase; Gpm1, phosphoglycerate mutase 1; IDI, diphosphate isomerase; LC, lycopene β-cyclase; LldD2, lactate dehydrogenase; PD, phytoene desaturase; Pgk, phosphoglycerate kinase; PS, phytoene synthase; PykA, pyruvate kinase; Rv0911, putative glyoxalase.

The influence of the nitrogen sources on the development and morphology of microorganisms and also on the synthesis of secondary metabolites is determined by the importance of this element as a constituent of nucleotides and amino acids representing building blocks of enzymes providing the biosynthetic processes. However, according to some data, the greatest effect on the carotenoid biosynthesis is provided by carbon rather than nitrogen [37], which was confirmed in the current study.

As a rule, microbial biosynthesis technologies are initially developed under laboratory conditions and then scaled up to the pilot and then industrial scales. The main task of the scale-up process is to increase the volume of production while maintaining or even increasing the productivity of the used strain [38,39]. High cell density in fermenters significantly differs from their natural growth conditions and results in some stresses related to various environmental factors such as changes in temperature, pH, osmotic concentrations, etc. [40].

Published data related to the industrial technologies of β-carotene production by *M. neoaurum* are rather poor. The majority of publications are focused on optimizing fermentation conditions for such producers as *Blakeslea trispora* and *Rhodotorula* spp. There is also a study describing the optimization of fermentation conditions of *Yarrowia lipolytica*. A successful modification of the original strain and optimization of batch fermentation conditions for a 5 L bioreactor provided a β-carotene yield of 2.7 g/L [41]. Another study reports about the fermentation of a genetically modified *Yarrowia lipolytica* strain, YL-C0, in a 5 L bioreactor with the constant glucose concentration of 10.0 g/L, pO_2 level maintained at 20–30% by adjusting the stirring rate, and pH level maintained at 5.5 using a 15% ammonia solution; under such conditions, the maximum β-carotene yield reached 1.7 g/L [42].

It is known that the reduction in the concentration of carbon and/or nitrogen sources causes some disorders in the biosynthesis of secondary metabolites including β-carotene. In the case of periodic processes, this problem can be solved by additional feeding of the limiting substrate, thus avoiding a suppressive effect of initially high substrate concentrations and increasing the yield of a target metabolite. Therefore, after 30 h of fermentation, a sterile 50% glycerol solution (2.5 g/L of a fermentation medium) was added under controlled optimal pH and pO_2 conditions (6.8–7.2 and 50%, respectively). A continuous supply of a 50% glycerol solution during fermentation also helps to stabilize the pH level due to the formation of organic acids during its metabolization [43]. Such pH stabilization promotes more active biosynthesis of β-carotene.

In this study, *M. neoaurum* cultivation in flasks under selected conditions resulted in a β-carotene yield of 318.4 ± 8.3 mg/kg. When the optimized medium was tested in a 3 L bioreactor, β-carotene and dry biomass yields were 370.5 ± 8.0 mg/kg and 25.4 ± 1.0 g/L, respectively. These results allowed us to perform a step-by-step scaling up of the process in 15 L and 100 L bioreactors. For the 15 L bioreactor, β-carotene and dry biomass yields reached 432.3 ± 10.4 mg/kg and 22.2 ± 1.2 g/L, respectively. In the case of the 100 L bioreactor, the strain productivity for β-carotene was 450.4 ± 11.0 mg/kg, while the dry biomass yield was 25.2 ± 1.1 g/L. Note that the scaling up from the 3 L to 100 L bioreactor provided an increase in the β-carotene yield by 21.7% that can be associated with the improved aeration conditions.

Aeration is a key parameter, which should be considered for the production of this class of pigments, since the biosynthesis of carotenoids is an aerobic process. The air flow rate during microbial fermentation is an important factor providing the nutrient absorption, growth rate, cell mass accumulation, and carotenoid biosynthesis. In addition, according to some published data, a decrease in the oxygen content in the culture medium affects the production of carotenoids and xanthophylls [31]. The effect of aeration can depend on the microorganism species.

Moreover, the development of a biotechnological process should take into account such factors as the design of the bioreactor, used raw materials, and fermentation type (batch, fed-batch, or continuous). These factors play a very important role in achieving the desired yield of target metabolites, including β-carotene [31].

5. Conclusions

The performed study showed that the biosynthesis of β-carotene by *M. neoaurum* cells is influenced mainly by the glycerol and SMP concentration. Using mathematical planning, we optimized a nutrient medium composition for *M. neoaurum* fermentation and β-carotene biosynthesis and determined the optimal glycerol and SMP concentrations (25.5 and 12.8 g/L, respectively). Under such cultivation conditions, the β-carotene production by the *M. neoaurum* strain AC-3067D in 750 mL flasks was 318.4 ± 8.3 mg/kg. In the case of a 15 L bioreactor, the β-carotene and biomass yields were 432.3 ± 10.4 mg/kg and

23.2 ± 1.2 g/L, respectively. The further scaling up to a 100 L bioreactor increased the yield of β-carotene and the biomass up to 450.4 ± 8.2 mg/kg and 25.2 ± 1.1 g/L, respectively.

The obtained results demonstrate the potential of the application of the *M. neoaurum* strain AC-3067D for industrial β-carotene production. The further work planned with this strain includes determining cultivation conditions and optimal fermentation parameters for pilot-scale and industrial conditions with the process scaling up to a 1000 L bioreactor.

Author Contributions: Conceptualization, V.V.Y. and E.V.G.; methodology, N.V.K., E.V.G. and A.S.S.; software, V.V.Y.; validation, E.V.G., N.V.K. and V.V.Y.; formal analysis, V.V.D.; investigation, N.V.K., V.V.Y. and V.V.D.; data curation, V.V.Y.; writing—original draft preparation, N.V.K. and A.S.S.; writing—review and editing, V.V.Y. and V.V.D.; visualization, V.V.Y. and A.S.S.; supervision, V.V.D. and E.V.G.; project administration, V.V.D.; funding acquisition, V.V.D. All authors have read and agreed to the published version of the manuscript.

Funding: The study was carried out within the State Assignment of the Ministry of Science and Higher Education of the Russian Federation (theme no. 123012000071-1).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author(s).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Maoka, T. Carotenoids: Distribution, function in nature, and analysis using LC-photodiode array detector (DAD)-MS and MS/MS system. *Mass Spectrom* **2023**, *12*, A0133. [CrossRef]
2. Maoka, T. Carotenoids as natural functional pigments. *J. Nat. Med.* **2020**, *74*, 1–16. [CrossRef]
3. Yaderets, V.V.; Karpova, N.V.; Glagoleva, E.V.; Petrova, K.S.; Shibaeva, A.S.; Dzhavakhiya, V.V. Carotenoids: Overview of the main methods and conditions of their preparation. *Proc. Univ. Appl. Chem. Biotechnol.* **2024**, *14*, 41–54. (In Russian) [CrossRef]
4. Ashokkumar, V.; Flora, G.; Sevanan, M.; Sripriya, R.; Chen, W.H.; Park, J.H.; Rajesh Banu, J.; Kumar, G. Technological advances in the production of carotenoids and their applications—A critical review. *Bioresour. Technol.* **2023**, *367*, 128215. [CrossRef] [PubMed]
5. Foong, L.C.; Loh, C.W.L.; Ng, H.S.; Lan, J.C. Recent development in the production strategies of microbial carotenoids. *World J. Microbiol. Biotechnol.* **2021**, *37*, 12. [CrossRef] [PubMed]
6. Ernst, H. Recent advances in industrial carotenoid synthesis. *Pure Appl. Chem.* **2002**, *74*, 2213–2226. [CrossRef]
7. Lim, K.C.; Yusoff, F.M.; Shariff, M.; Kamarudin, M.S. Astaxanthin as feed supplement in aquatic animals. *Rev. Aquac.* **2017**, *10*, 738–773. [CrossRef]
8. Botella-Pavía, P.; Rodríguez-Concepción, M. Carotenoid biotechnology in plants for nutritionally improved foods. *Physiol. Plant.* **2006**, *126*, 369–381. [CrossRef]
9. Gong, M.; Bassi, A. Carotenoids from microalgae: A review of recent developments. *Biotechnol. Adv.* **2016**, *34*, 1396–1412. [CrossRef] [PubMed]
10. Rodriguez-Amaya, D.B. Update on natural food pigments – A mini-review on carotenoids, anthocyanins, and betalains. *Food Res. Int.* **2019**, *124*, 200–205. [CrossRef] [PubMed]
11. Choi, S.S.; Kim, G.D. Production of carotenoids by bacteria: Carotenoid productivity and availability. *J. Life Sci.* **2022**, *32*, 411–419. [CrossRef]
12. El Baky, H.H.A.; El Baroty, G.S.; Mostafa, E.M. Optimization growth of *Spirulina (Arthrospira) platensis* in photobioreactor under varied nitrogen concentration for maximized biomass, carotenoids and lipid contents. *Recent Pat. Food. Nutr. Agric.* **2018**, *11*, 40–48. [CrossRef] [PubMed]
13. Aneesh, P.A.; Ajeeshkumar, K.K.R.G.; Kumar Lekshmi, R.; Anandan, C.N.; Ravishankar, S.M. Bioactivities of astaxanthin from natural sources, augmenting its biomedical potential: A review. *Trends Food Sci. Technol.* **2022**, *125*, 81–90. [CrossRef]
14. Frusciante, S.; Diretto, G.; Bruno, M.; Ferrante, P.; Pietrella, M.; Prado-Cabrero, A.; Rubio-Moraga, A.; Beyer, P.; Gomez-Gomez, L.; Al-Babili, S.; et al. Novel carotenoid cleavage dioxygenase catalyzes the first dedicated step in saffron crocin biosynthesis. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 12246–12251. [CrossRef]
15. Asker, D. Isolation and characterization of a novel, highly selective astaxanthin-producing marine bacterium. *Agric. Food Chem.* **2017**, *65*, 101–9109. [CrossRef] [PubMed]

16. Silva, T.P.; Paixão, S.M.; Alves, L. Ability of *Gordonia alkanivorans* strain 1B for high added value carotenoids production. *RSC Adv.* **2018**, *5*, 58055. [CrossRef]
17. Tran, T.; Dawrs, S.N.; Norton, G.J.; Virdi, R.; Honda, J.R. Brought to you courtesy of the red, white, and blue pigments of nontuberculous mycobacteria. *AIMS Microbiol.* **2020**, *6*, 434–450. [CrossRef] [PubMed]
18. Igreja, W.S.; Maia, F.A.; Lopes, A.S.; Chisté, R.C. Biotechnological production of carotenoids using low-cost substrates is influenced by cultivation parameters: A review. *Int. J. Mol. Sci.* **2021**, *22*, 8819. [CrossRef] [PubMed]
19. Pagels, F.; Vasconcelos, V.; Guedes, A.C. Carotenoids from Cyanobacteria: Biotechnological potential and optimization strategies. *Biomolecules* **2021**, *11*, 735. [CrossRef]
20. Dyaa, A.; Soliman, H.; Abdelrazak, A.; Samra, B.N.; Khojah, E.; Ahmed, A.F.; El-Esawi, M.A.; Elsayed, A. Optimization of carotenoids production from *Rhodotorula* sp. strain ATL72 for enhancing its biotechnological applications. *J. Fungi* **2022**, *8*, 160. [CrossRef]
21. Xu, X.; Liu, W.; Niu, H.; Hua, M.; Su, Y.; Miao, X.; Chi, Y.; Xu, H.; Wang, J.; Sun, M.; et al. Study on the fermentation effect of *Rhodotorula glutinis* utilizing tofu whey wastewater and the influence of *Rhodotorula glutinis* on lying hens. *Front. Nutr.* **2023**, *10*, 1125720. [CrossRef]
22. Siziya, I.N.; Yoon, D.J.; Kim, M.; Seo, M.J. Enhanced production of C30 carotenoid 4,4′-diaponeurosporene by optimizing culture conditions of *Lactiplantibacillus plantarum* subsp. *plantarum* KCCP11226T. *J. Microbiol. Biotechnol.* **2022**, *32*, 892–901. [CrossRef] [PubMed]
23. Stanchev, V.; Georfiev, D.; Gargova, S. Mathematical modeling of the nutrient medium composition for the production of yeast phytase. *Bulg. J. Agric. Sci.* **2010**, *16*, 628–634.
24. Husseiny, S.M.; Abdelhafez, A.A.; Ali, A.A.; Sand, H.M. Optimization of b-carotene production from *Rhodotorula glutinis* ATCC 4054 growing on agro-industrial substrate using Plackett–Burman design. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* **2017**, *88*, 1637–1646. [CrossRef]
25. da Costa Cardoso, L.A.; Kanno, K.Y.F.; Karp, S.G. Microbial production of carotenoids—A review. *Afr. J. Biotechnol.* **2017**, *16*, 139–146. [CrossRef]
26. Kerr, S.; Calé, C.; Cabral, J.M.; van Keulen, F. Factors enhancing lycopene production by a new *Mycobacterium aurum* mutant. *Biotechnol. Lett.* **2004**, *26*, 103–108. [CrossRef]
27. Yaderets, V.; Karpova, N.; Glagoleva, E.; Shibaeva, A.; Dzhavakhiya, V. Enhanced β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 by combining mutagenesis, strain selection, and subsequent fermentation optimization. *Fermentation* **2023**, *9*, 1007. [CrossRef]
28. Rozhnov, E.D. *Modeling of Biotechnological Processes: Methodological Recommendations for Performing Laboratory Work, Conducting Practical Classes and Organizing Independent Work of Students for the Speciality 04/19/2011—Biotechnology*; Altai State Technical University: Biysk, Russia, 2019; pp. 40–96.
29. Zhernosekova, I.V.; Chernogor, N.P.; Tymchuk, A.A.; Vinnikov, A.I. Methods of experiments planning by optimization of the nutrient medium for Streptomycete. *Visnyk Dnipropetrovsk Univ. Biol. Ecol.* **2001**, *18*, 20–28. [CrossRef]
30. Zeni, J.; Colet, R.; Cence, K.; Tiggeman, L.; Toniazzo, G.; Cansian, R.L.; Di Luccio, M.; Oliveira, D.; Valduga, E. Screening of microorganisms for production of carotenoids. *CyTA J. Food* **2011**, *9*, 160–166. [CrossRef]
31. Mata-Gómez, L.C.; Montañez, J.C.; Méndez-Zavala, A.; Aguilar, C. Biotechnological production of carotenoids by yeasts: An overview. *Microb. Cell Fact.* **2014**, *13*, 12. [CrossRef] [PubMed]
32. Bindea, M.; Rusu, B.; Rusu, A.; Trif, M.; Leopold, L.F.; Dulf, F.; Vodnar, D.C. Valorification of crude glycerol for pure fractions of docosahexaenoic acid and β-carotene production by using *Schizochytrium limacinum* and *Blakeslea trispora*. *Microb. Cell Fact.* **2018**, *17*, 97. [CrossRef] [PubMed]
33. Suwaleerat, T.; Thanapimmetha, A.; Srisaiyoot, M.; Chisti, Y.; Srinophakun, P. Enhanced production of carotenoids and lipids by *Rhodococcus opacus* PD630. *J. Chem. Technol. Biotechnol.* **2018**, *93*, 2160–2169. [CrossRef]
34. Baughn, A.D.; Rhee, K.Y. Metabolomics of central carbon metabolism in *Mycobacterium tuberculosis*. *Microbiol. Spectr.* **2014**, *2*. [CrossRef] [PubMed]
35. Paniagua-Michel, J.; Olmos-Soto, J.; Ruiz, M.A. Pathways of carotenoid biosynthesis in bacteria and microalgae. *Methods Mol. Biol.* **2012**, *892*, 1–12. [CrossRef]
36. Janisch, N.; Levendosky, K.; Budell, W.C.; Quadri, L.E.N. Genetic underpinnings of carotenogenesis and light-induced transcriptome remodeling in the opportunistic pathogen *Mycobacterium kansasii*. *Pathogens* **2023**, *12*, 86. [CrossRef]
37. Zheng, X.; Hu, R.; Chen, D.; Chen, J.; He, W.; Huang, L.; Lin, C.; Chen, H.; Chen, Y.; Zhu, J.; et al. Lipid and carotenoid production by the *Rhodosporidium toruloides* mutant in cane molasses. *Biores. Technol.* **2021**, *326*, 124816. [CrossRef] [PubMed]
38. Dzhavakhiya, V.; Savushkin, V.; Ovchinnikov, A.; Glagolev, V.; Savelyeva, V.; Popova, E.; Novak, N.; Glagoleva, E. Scaling up a virginiamycin production by a high-yield *Streptomyces virginiae* VKM Ac-2738D strain using adsorbing resin addition and fed-batch fermentation under controlled conditions. *3 Biotech* **2016**, *6*, 240. [CrossRef]

39. Schmidt, F.R. Optimization and scale up of industrial fermentation processes. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 425–435. [CrossRef]
40. Liu, W.C.; Gong, T.; Wang, Q.H.; Liang, X.; Chen, J.J.; Zhu, P. Scaling-up fermentation of *Pichia pastoris* to demonstration-scale using new methanol-feeding strategy and increased air pressure instead of pure oxygen supplement. *Sci. Rep.* **2016**, *6*, 18439. [CrossRef]
41. Jing, Y.; Wang, J.; Gao, H.; Jiang, Y.; Jiang, W.; Jiang, M.; Xin, F.; Zhang, W. Enhanced β-carotene production in *Yarrowia lipolytica* through the metabolic and fermentation engineering. *J. Ind. Microbiol. Biotechnol.* **2023**, *50*, kuad009. [CrossRef]
42. Liu, L.; Qu, Y.L.; Dong, G.R.; Wang, J.; Hu, C.Y.; Meng, Y.H. Elevated β-carotene production using codon-adapted CarRA&B and metabolic balance in engineered *Yarrowia lipolytica*. *Front. Microbiol.* **2021**, *12*, 627150. [CrossRef]
43. Hunter, G.J. The oxidation of glycerol by Mycobacteria. *Biochem. J.* **1953**, *55*, 320–328. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.