fermentation

MDPI

Article

Enhanced β-Carotene Production in *Mycolicibacterium neoaurum* Ac-501/22 by Combining Mutagenesis, Strain Selection, and Subsequent Fermentation Optimization

Vera Yaderets, Nataliya Karpova, Elena Glagoleva, Alexandra Shibaeva and Vakhtang 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.Y.); ashatanr@mail.ru (N.K.); glagolevaev@mail.ru (E.G.); aleksandrashibaeva@mail.ru (A.S.)

* Correspondence: vahoru@mail.ru

Abstract: A continuing interest of scientists regarding the development of new β-carotene production technologies is due to the high biological activity of this compound and its wide application range. Bacteria are considered among the possible β-carotene producers convenient for industrial use. The purpose of this study was to develop a *Mycolicibacterium neoaurum* strain with an enhanced ability for β-carotene production and to optimize the fermentation conditions to improve the final yield of the target compound. Using chemical mutagenesis with N-nitroso-N-methylurea along with further strain selection, a *M. neoaurum* strain Ac-501/22, whose productivity was 2.7-fold higher than that of the parental strain Ac-501, was developed. The effect of nitrogen and carbon sources as well as cultivation conditions on the growth of *M. neoaurum* Ac-501/22 and β-carotene production were studied to select the optimal fermentation regime. Due to an increase in the temperature of cultivation from 30 to 35 °C, replacement of glucose with glycerin (20.0 g/L) and degreased soybean flour with powdered milk (10.0 g/L), and increase in the urea content from 0.5 to 1.0 g/L, the level of β-carotene production was improved to 183.0 mg/kg that was 35% higher than in the control. Further strain fermentation in a 3 L bioreactor using an optimized medium with the pH level maintained at 7.0–7.2 and 50% pO_2 provided the maximum output of the target compound (262.4 mg/kg of dry biomass) that confirmed the prospects of the developed strain as an industrial β-carotene producer.

Keywords: carotenoids; *Mycolicibacterium neoaurum*; cultivation; mutagenesis; biofermenter

Citation: 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. https://doi.org/10.3390/fermentation9121007

Academic Editor: Mohamed Koubaa

Received: 28 October 2023
Revised: 23 November 2023
Accepted: 27 November 2023
Published: 6 December 2023

1. Introduction

Carotenoids ("*carota*" (Lat.)—carrot, "*eidos*" (Gr.)—form) represent the group of the largest number of organic pigments synthesized by higher plants, algae, phototrophic bacteria, and some filamentous fungi and yeasts [1–3]. To date, scientists have revealed the structure of 1158 carotenoids synthesized by 691 different species [4]. According to their chemical structure, carotenoids belong to terpenoids, whose carbon backbone consists of eight C5-isoprene units of isopentyl diphosphate. Due to this fact, carotenoids are also called C40-isoprenoids [2,5]. In microbial or plant cells, these compounds may be present in a free form or form glycosides, protein–carotene complexes, or ethers with long-chain fatty acids.

The high biological activity of this group of compounds and a wide range of their use [1–6] explain the constant interest of scientists toward the development of efficient technologies for their production. To date, industrial production of carotenoids can be realized in several possible ways. First, they can be produced by chemical synthesis; this method is used for astaxanthin, canthaxanthin, and β-carotene [7,8]. The second method is based on carotenoid extraction from natural sources (plants and algae) [9–11]. Finally, the

third way is the biosynthesis of carotenoids using various microbial cultures, such as fungi, yeasts, bacteria, and microalgae [2,3].

A common producer of a wide range of biologically active compounds including carotenoids is a cyanobacterium *Spirulina platensis* [12,13]. Though spirulina provides a high yield of pigments (carotenoids, phycocyanin, and chlorophyll) and is characterized by a high content of proteins (55–70% of the total biomass) and essential fatty acids (5–8%) [13], a large-scale cultivation of this cyanobacterium is confronted with some problems. First, a large-scale cultivation of *S. platensis* requires the Zarukk medium and a large volume of freshwater. For example, production of 1 kg of dry spirulina biomass (powder) requires ~1000 L of water [12]. The system of open ponds, which is most commonly used for the microalgae biomass production, requires ~11–13 mln. liters of fresh water per ha per year [14]. Second, the process of carotene biosynthesis in cyanobacteria is significantly influenced by the illumination level and the temperature of the cultivation medium [13,14]. Thus, the dependence of spirulina growth and development on a large volume of freshwater and hardly controlled factors makes the production of biologically active compounds by microalgae to be more expensive compared to the technologies based on the use of bioreactors.

The main carotenoids produced by microbial biosynthesis in bioreactors are β-carotene, astaxanthin, and lycopene. Their producers are the heterothallic fungus *Blakeslea trispora* and yeasts *Rhodotorula* sp., *Rhodosporidium* sp., and *Xanthophyllomyces* sp. The advantages of the use of filamentous fungi and yeasts include the possibility of their cultivation on agroindustrial wastes, such as corn steep, wheat and rice bran, silage, whey, sugar-cane cake, glycerin, etc. [2,3]. At the same time, bacteria are also interesting as carotenoid producers, since they are widely used in the industrial production of various biologically active substances. Bacteria are also able to grow on a number of substrates and may synthesize a wide range of carotenoids; in addition, they are characterized by shorter fermentation cycles that increase the profitability of the production. Moreover, pigment extraction from bacterial cells is easier than from microalgae, fungi, and yeasts, which are characterized by thick cell walls [15].

Among bacterial species studied as possible carotenoid producers, one should mention *Arthrospira platensis*, *Cyanobium* sp., *Synechococcus* sp., and *Cyanobacterium aponinum*. These and other carotenoid-producing cyanobacteria are reviewed in detail in [1]. However, the expediency of their use on an industrial scale is still under question, since their productivity is significantly lower than that of microalgae [1,16].

Soil bacteria demonstrate better productivity; for example, *Gordonia alkanivorans* cultivated on a sulfate-containing medium under light was able to synthesize lutein, canthaxanthin, and astaxanthin at a level of >2 mg/g of dry weight in total [17]. Rapid-growing nonpathogenic *Mycolicibacterium* strains able to synthesize β-carotene and some other pigments also can be considered as promising carotenoid producers [18]. However, the productivity of bacterial strains is inferior to that of fungal and yeast cultures.

Mycolicibacteria represent mycelium-free actinomycetes. The *M. neoaurum* strain Ac-501 used in our study is characterized by immobile cells, which do not form spores. A young culture forms curved rods (1.5–4.0 × 0.4–0.8 μm) with an uneven contour; they can also be starry, sometimes quite long and with side outgrowths. An old culture represents single and grouped cocci (0.8–1.0 μm). Being cultivated on corn meal glucose agar or agar supplemented with a soybean meal, the strain forms rounded and slightly wrinkled colonies with uneven edges. Some *Mycolicibacterium* strains are used in industrial microbiology to transform plant sterols into the main precursors of steroid drugs.

The purpose of this study was the development of a *M. neoaurum* strain, characterized by an enhanced carotenoid production, and optimization of its cultivation conditions to maximize the accumulation of a carotenoid-containing biomass.

2. Materials and Methods

2.1. Reagents and Nutrient Media Components

Nutrient medium components used in the study included: fructose, sucrose, arabinose, meat peptone, yeast extract, and skimmed milk powder (all from HiMedia Laboratories Ltd., Mumbai, India); lactose and soy peptone (Conda Pronadisa, Madrid, Spain); yeast peptone (Angel Yeasters Rus., Ltd., Lipetsk, Russia; urea (NeoFroxx GmbH, Einhausen, Germany); citric acid (Component-Reactiv, Ltd., Moscow, Russia); inorganic salts, glycerin, glucose, mannitol, sorbitol, and organic solvents (hexane, benzene, acetone) (all from Acros Organics, Geel, Belgium); agar (Difco, Detroit, MI, USA); skimmed deodorized soybean meal (Yanta Ltd., Irkutsk, Russia); pea meal (Garnets Ltd., Vladimir, Russia).

N-nitroso-N-methylurea, β-carotene, and lycopene were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Microorganisms

M. neoaurum strain Ac-501 was obtained from the work collection of the Laboratory of Biotechnology of Physiologically Active Compounds of the Russian Biotechnological University (Moscow, Russia). *M. neoaurum* strain Ac-501/22 was obtained from Ac-501 by chemical mutagenesis.

2.3. Composition of Media for Cultivation and Maintenance of M. neoaurum Ac-501 and Ac-501/22

M. neoaurum Ac-501 and *M. neoaurum* Ac-501/22 were maintained on the A1 medium with 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; $CaCO_3$, 1.5 (pH 6.8–7.2). After one month of storage on the agarized medium at 4 °C, cultures were reinoculated onto fresh medium. For long-term storage, cultures were freeze-dried using powdered milk as a carrier and stored at 4 °C.

2.4. Cultivation of M. neoaurum Ac-501 and M. neoaurum Ac-501/22 on a Liquid Nutrient Medium

The inoculate was obtained by the following way. Ten milliliters of a sterile physiological solution were added into a tube with bacterial culture, then the surface layer of cells was accurately scraped with an inoculation loop and the resulting cell suspension was transferred into 750 mL flasks with 100 mL of the agar-free A1 medium (A medium). This medium (without glucose or soybean meal) was also used as the basic medium for selection of optimal cultivation conditions with a sequential addition (according to the experiment scheme) of the following components: fructose, sucrose, lactose, arabinose, mannitol, sorbitol, glycerin, soybean peptone, powdered milk, pea meal, yeast peptone, yeast extract, and meat peptone. Each component was added in the amount of 10.0 g. The urea amount varied depending on the task within 0.25–2.0 g/L.

Flasks with the inoculated culture were placed in an Innova 44 shaker (New Brunswick, Germany) and incubated for 48 h at 220 rpm and a temperature of 25–40 °C (depending on experimental conditions). The resulting inoculate (10 vol. %) was transferred into fresh flasks with 100 mL of the same medium and cultivated for 48–72 h (depending on the experiment) under the same conditions. The resulting culture broth was used for the inoculation of a bioreactor or was dried and used to determine the content of carotenoids.

2.5. Mutagenesis

After a 48 h growth of *M. neoaurum* Ac-501, 100 mL of culture broth was centrifuged for 5 min at 7500 rpm. Concentrated bacterial biomass was resuspended in 10 mL of phosphate buffer (pH 6.2). Five milliliters of the obtained cell suspension was mixed with 5 mL of 2% N-nitroso-N-methylurea (NMU) and incubated for 0–160 min at 30 °C. During incubation, 2 mL aliquots of the cell suspension were sampled at certain time intervals, added to 10 mL of a phosphate buffer (pH 7.0), then centrifuged for 5 min at 7500 rpm. After removal of

the supernatant, the procedure was repeated. After the removal of the second portion of supernatant, the residue was resuspended in 10 mL of the same phosphate buffer (the first dilution), and a series of sequential tenfold dilutions of treated cells was prepared. To obtain monocolonies, 0.1 mL aliquot of a cell suspension was taken from each tube for inoculation in a Petri plate containing agarized medium. After inoculation, the plates were turned upside down, placed into a thermostat, and incubated for 5 days at 30 °C. At the end of incubation, the number of grown colonies was calculated.

2.6. Carotenoid Content Determination

The content of carotenoids in the cell biomass of *M. neoaurum* Ac-501 and *M. neoaurum* Ac-501/22 was determined spectrophotometrically [19]. The analysis was carried out under conditions of low illumination; the temperature in the room did not exceed 20 °C. The culture broth (30 mL) was centrifuged for 5 min at 7500 rpm. The obtained supernatant was decanted, and the remaining biomass was thrice extracted with 10 mL of acetone. Acetone extracts were combined, transferred into a separating funnel, and supplemented with 10 mL of petroleum ether. After a thorough shaking, the obtained emulsion was destroyed by a drop-by-drop addition of a saturated NaCl solution. After the separation of the acetone layer, it was extracted again by petroleum ether. The resulting petroleum extracts were combined and filtered through a glass filter. The absorption spectrum of the carotenoid extract was measured at 450 nm using a Thermo Spectronic spectrophotometer and petroleum ether as the reference sample. The content of total carotenoids was calculated using the following formula:

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

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).

2.7. Carotenoid Determination by Thin-Layer Chromatography

A qualitative determination of carotenoids was carried out according to [20–22] with some modifications. The analysis and all preliminary procedures were performed under low illumination. Dry biomass of *M. neoaurum* Ac-501 or *M. neoaurum* Ac-501/22 (0.5 g) was thrice extracted with 20 mL of the acetone:methanol mix (7:2). The extracts were combined and centrifuged for 5 min at 5000 rpm. A transparent supernatant was collected and 4-fold concentrated using a rotary evaporator. A concentrate (100 µL) and reference solutions of β-carotene and lycopene were applied onto a Sorbfil chromatographic plate (Imid Ltd., Krasnodar, Russia). The diameter of the resulting spot was 3–4 mm. The plate was placed into a chromatographic camera with the following mix of organic solvents: hexane:benzene (5:1), hexane:benzene (9:1), hexane:acetone (98:2), and benzene:acetone (25:1). When the solvent front reached the plate edge, the plate was taken out of the camera.

The qualitative composition of carotenoids contained in the sample was determined visually in a comparison with the witness samples. The *Rf* value was calculated using the following formula:

$$Rf = \frac{l}{L}$$

where l is the distance between the sample application point and the center of a spot characterized by the adsorption zone (mm) and L is the distance between the start point and the edge of the eluent front.

2.8. Dry Biomass Obtaining

To obtain a dry biomass, aliquots of the culture broth were sampled at the given time intervals, transferred to special trays, and dried for 4–6 h at 80 °C until a constant weight in a drying chamber (Binder, Tuttlingen, Germany).

2.9. Fermentation of M. neoaurum Ac-501/22 in a 3 L Bioreactor

2.9.1. Obtaining of the Inoculate

An inoculum was obtained according to the procedure described in Section 2.4 using a modified medium (AM, see Section 3.7) of the following composition (g/L): glycerin, 20.0; powdered milk, 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; $CaCO_3$, 1.5 (pre-sterilization pH 6.8–7.2). Prior to inoculation, the inoculate was microscopically examined using a Carl Zeiss Primo Star microscope (Carl Zeiss, Jena, Germany) to control its quality, and a sample of fermentation medium was taken from a bioreactor for the microbiological and biochemical control. The volume of inoculate was 10% of the fermentation medium volume (150 mL). The inoculum was introduced into the bioreactor using a sterile inoculum feeding line.

2.9.2. Bioreactor Preparation

A submerged fermentation of the strain was carried out using a 3 L bioreactor manufactured by Prointech-Bio (Pushchino, Russia) equipped with an integrated computer control providing regulation of the basic fermentation parameters (pH, stirring rate, temperature, and pO_2). Devices intended to measure culture broth characteristics during fermentation included a Buk-3 thermal sensor connected with the controller (Keklab Group, Moscow, Russia); an InPro3300/225/PT1000 pH sensor (Mettler Toledo, Greifensee, Switzerland) connected via controller with a peristaltic pump providing a titrating solution; and an InPro 6800/12/220 oxygen sensor (Mettler Toledo, Greifensee, Switzerland) connected via controller with the mechanical stirring device.

After loading of an AM nutrient medium (1.5 L, pre-sterilization pH 6.8–7.2), the bioreactor was placed into an autoclave for sterilization (60–90 min at 121 °C). Additional liquid components (glycerin, acid and alkali solutions, distilled water) were sterilized separately in flasks with the bottom hose barbs equipped with connecting hoses and needles. After sterilization, the bioreactor was mounted onto a fermentation stand and connected with sensors, air input units, water cooling system, and electric motor. The initial pO_2 value in the medium was set to be equal to 100%.

2.9.3. *M. neoaurum* Ac-501/22 Fermentation

The fermentation regime prior to inoculation is described in Table 1. Fermentation was carried out in two regimes: (1) stirring and registration of basic technological parameters (pH, pO_2); (2) fed-butch fermentation at controlled levels of pH (6.8–7.2) and pO_2. The pO_2 level was regulated by changes in the volume of air supply per volume of culture broth (manual mode) and changes in the number of revolutions of a stirrer (automatic mode). In both regimes, fermentation lasted for 72 h. Then, the biomass was inactivated for 30 min at 80–85 °C under stirring, poured out into a collector, centrifuged, and dried using a Martin Christ ALPHA 2-4LD plus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

Table 1. Pre-inoculation fermentation regime.

Parameter	Value
Medium volume (with allowance for a steam condensate)	1.5 L
Temperature	35 ± 1 °C
Aeration	0.1 L/L/min
Stirring rate	250 rpm
pO_2 level	100% of saturation
Pressure within a fermenter	0.03–0.05 MPa
Medium pH	6.8–7.2

2.10. M. neoaurum Ac-501/22 Identification

DNA isolation from a sample was carried out as described in [23]; the resulting concentration of the obtained DNA preparations was 30–50 μg/mL, and RNA presented only in trace amount (<1%). Universal primer systems for 16S rRNA providing detection of both eubacteria (11f-1492r) and archaea (8fa-A915R) were used. The amplification was performed in 50 μL of the following reaction mix: 1× buffer for BioTaq DNA polymerase (17 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$); 12.5 nmoles of each dNTP; 50 ng of template DNA; 5 pmoles of each primer; and 3U BioTaq DNA polymerase enzyme (Dialat Ltd., Moscow, Russia).

Amplification conditions included initial denaturation for 9 min at 94 °C, annealing for 1 min at 55 °C, and elongation for 2 min at 72 °C followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, elongation for 2 min at 72 °C, and a final extension for 7 min at 72 °C.

PCR products were electrophoretically analyzed in 1% agarose at the electric field intensity of 6 V/cm. Isolation and purification of PCR products from agarose gel was carried out using a WizardPCRPreps kit (Promega, Madison, WI, USA) according to the manufacture's recommendations.

The sequencing of the obtained PCR fragments was performed as described by Sanger et al. [24] using an Applied Biosystems 3730 genetic analyzer (ThermoFisher Scientific, Waltham, MA, USA) and a BigDyeTerminator v.3.1 cycle sequencing kit (ThermoFisher Scientific, USA). The procedure was carried out at the "Bioengineering" Resource Sharing Center of the Federal Research Center "Fundamentals of Biotechnology". The sequencing was performed in both forward and reverse directions using amplifying and internal primers.

The alignment and editing of the obtained sequences were carried out using a Bioedit program package (https://bioedit.software.informer.com/, accessed on 28 September 2023). A phylogenetic analysis of the obtained nucleotide sequences was carried out based on a BLAST algorithm of the NCBI GenBank (http://www.ncbi.nlm.nih.gov, accessed on 28 September 2023). To exclude chimeres, the sequences were examined using an online CHECK_CHIMERA system from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu, accessed on 28 September 2023).

2.11. Data Treatment

Each experiment on the effect of different factors on the strain growth and carotenoid accumulation was carried out three times, each in three replications, and the calculated mean values were used for the following calculations. The statistical treatment of the obtained results was carried out using a Statistica 10 software package. The significance of difference between the control and treated variants was determined using a *t*-test for independent variables.

3. Results

3.1. Breeding of M. neoaurum Ac-501/22 Characterized by an Enhanced Carotenoid Biosynthesis

The chosen parental strain (*M. neoaurum* Ac-501) cultivated on the agarized A1 medium formed rounded and slightly rugous yellow colonies with uneven edges. The productivity of this strain was 44.4 ± 1.2 mg/kg of β-carotene. The synthesized pigment was identified by a TLC analysis using lycopene and β-carotene as reference samples as well as by a spectrophotometric analysis with the wavelengths corresponding to lycopene (474 nm) and β-carotene (450 nm) [22,25]. The TLC chromatogram showed that the biomass contained only one pigment, whose *Rf* coincided with that of the β-carotene reference sample (Table 2). Therefore, we confirmed that the basic pigment synthesized by Ac-501 was β-carotene. Indeed, the TLC analysis used in this study cannot be considered as a reliable analytical method providing an unambiguous conclusion about the structure of pigments in *M. neoaurum* cells. At the next stage of our study, we plan to perform a more

detailed characterization of the composition and structure of carotenoids present in the produced cell biomass.

Table 2. *Rf* values of reference lycopene and β-carotene samples and products present in the extract from the *Mycolicibacterium neoaurum* Ac-501 biomass.

Eluent (Mobile Phase)	Biomass Extract		Reference Samples	
	β-Carotene	Lycopene	β-Carotene	Lycopene
Hexane:benzene (5:1)	0.98 ± 0.01	–	0.98 ± 0.01	0.80 ± 0.01
Hexane:benzene (9:1)	0.74 ± 0.01	–	0.74 ± 0.01	0.64 ± 0.01
Benzene:acetone (25:1)	0.98 ± 0.01	–	0.98 ± 0.01	0.81 ± 0.01
Hexane:acetone (98:2)	0.54 ± 0.01	–	0.54 ± 0.01	0.45 ± 0.01

Prior to a multi-step mutagenesis of the parental strain followed by a selection of single colonies with altered morphological traits, a preliminary experiment was arranged to study the effect of the mutagenic factor (NMU) on the cell survival rate (Figure 1).

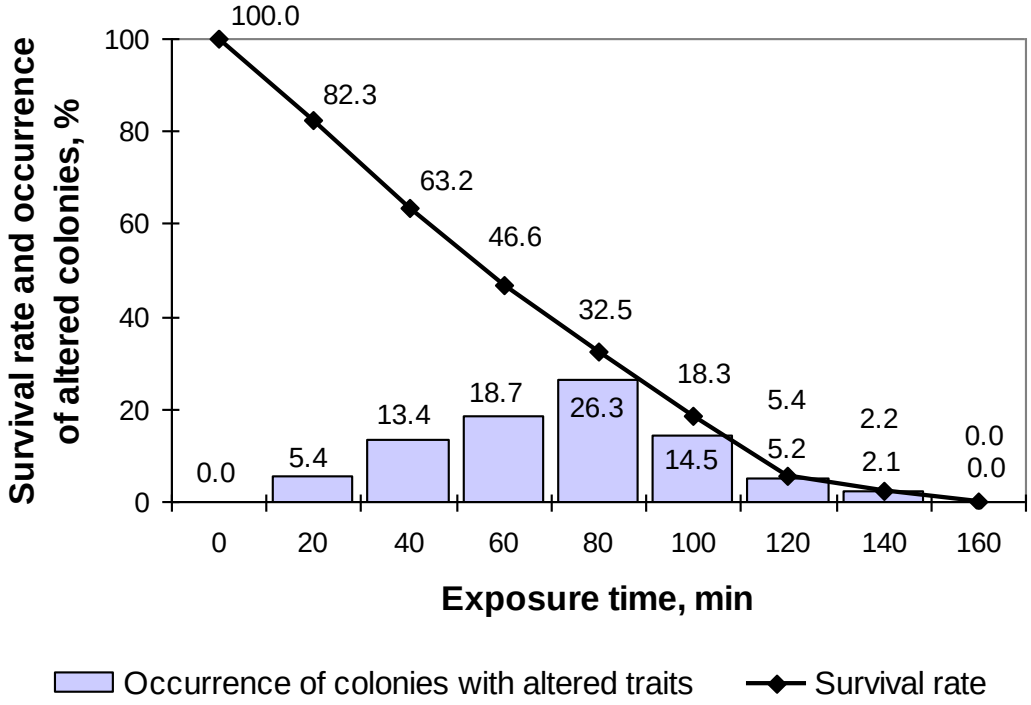

Figure 1. Effect of different times of exposure to NMU on the survival rate and the occurrence of *Mycolicibacterium neoaurum* Ac-501 colonies with altered morphological traits.

The main selection criterion was the change in the colony color from yellow to orange or carrot. According to Figure 1, the optimum exposure of Ac-501 to NMU was 80 min, since, in this case, the maximum number of colonies with the target trait (26.3%) was registered. The selected colonies were re-inoculated on liquid nutrient media to determine the β-carotene content in their biomass. The most productive strain was treated with NMU again and so on. In the course of this multi-step process, several basic types of colonies with the altered morphology have been obtained at different stages of the process: (1) beige rugous colonies with an even edge, (2) yellow-orange rugous colonies with uneven edge, (3) bright orange rugous colonies with uneven edge, and (4) beige microcolonies. The most productive colonies corresponded to the types 2 and 3. After five mutagenesis cycles, a strain able to synthesize 122.3 mg/kg of β-carotene was obtained. This strain was called *M. neoaurum* Ac-501/22 (Figure 2).

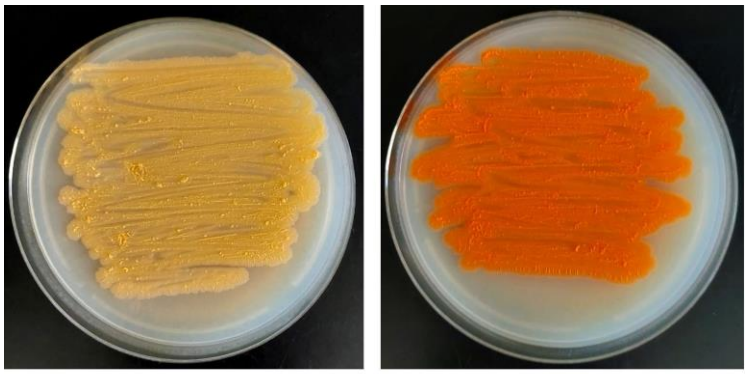

Figure 2. Parental and mutant strains of *Mycolicibacterium neoaurum:* (**a**) Ac-501, (**b**) Ac-501/22.

3.2. Mutant Strain Identification

To confirm that the mutant *M. neoaurum* strain Ac-501/22 belongs to the corresponding genus, the nucleotide sequence of 16S rRNA was analyzed and compared with that of the parental strain. The results of the performed BLAST analysis are shown in Figure S1. The obtained sequence completely (100%) coincided with that of a reference *M. neoaurum* strain NRRL B-3805 on the whole length available for reliable interpretation, so the induced mutations did not involve the 16S rRNA gene.

3.3. Effect of Temperature on the Growth of M. neoaurum Ac-501/22 and Carotenoid Accumulation

The effect of temperature on the level of β-carotene biosynthesis in the mutant strain Ac-501/22 was studied by strain cultivation at four different temperatures (25, 30, 35, and 40 °C). The initial inoculate was grown at 30 °C, since this cultivation temperature was considered as the control [26,27].

According to the obtained results, the optimum cultivation temperature was equal to 35 °C. In this case, the culture grew more intensively and reached the stationary growth phase after 50 h of fermentation; at the same time, in the case of cultivation at 30 (control) and 25 °C, this phase was reached only after 65 h of cultivation (Figure 3). The content of β-carotene in the bacterial biomass produced after 50 h of cultivation at 35 °C was 136.2 μg/kg that was 12.4% higher than in the control (Table 3). In the case of cultivation at 40 °C, the growth rate also increased, and the culture reached the stationary growth phase more rapidly than the control. However, the weight of the resulting biomass was 18.4% lower than that obtained for the cultivation temperature of 35 °C. At the same time, no negative influence of the heightened temperature on the biosynthesis of β-carotene was observed, since the amount of β-carotene was insignificantly lower than that produced under optimal growth conditions (Table 3). Thus, in all further experiments, the temperature of cultivation of Ac-501/22 was chosen to be 35 °C.

Table 3. Effect of cultivation temperature on the growth, biomass accumulation, and β-carotene production in *Mycolicibacterium neoaurum* Ac-501.

Cultivation Temperature, °C	Growth Period, h	Biomass Accumulation, g/L	Growth Rate, g/L/h	β-Carotene Content, mg/kg
25	65	8.5 ± 0.5	0.13	113.8 ± 1.5
30	65	12.2 ± 1.2	0.17	122.3 ± 0.75
35	50	15.2 ± 0.7	0.28	136.2 ± 1.5
40	50	12.4 ± 1.1	0.23	128.5 ± 0.5

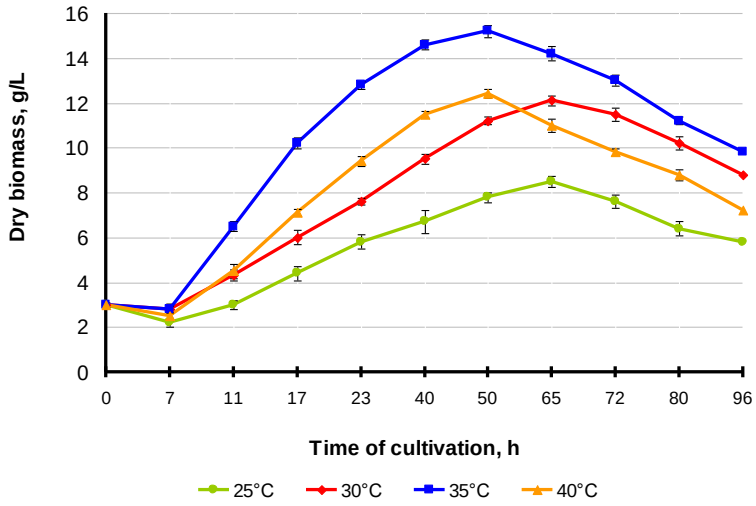

Figure 3. Dynamics of biomass accumulation of *Mycolibacterium neoaurum* Ac-501/22 at different temperature regimes.

3.4. Effect of Carbon Source on Carotenoid Accumulation in the Mycolicibacterium neoaurum Ac-501/22 Biomass

The ability of the mutant strain Ac-501/22 to utilize carbon sources such as sucrose, fructose, lactose, arabinose, mannitol, sorbitol, and glycerin was evaluated compared to the glucose-containing medium (Figure 4); the duration of the culture growth was 50 h. According to the obtained results, the strain was able to utilize all sugars and organic alcohols included in the study. The maximum productivity in terms of the amount of carotenoids per 1 g of dry biomass was registered for glucose replacement with glycerin (156.7 mg/kg); it exceeded the control value by 15%. In addition, the dry biomass weight increased by 15.1% compared to the control. Glucose replacement with lactose or sucrose resulted in a biomass increase by 30.3 and 19.7%, respectively; however, in the case of sucrose, the content of β-carotene in the biomass was 13.4% lower than in the control, while in the case of lactose, it was almost the same as in the control. Polyatomic organic alcohols used in this experiment did not provide any influence on biomass accumulation. In relation to the β-carotene content, glucose replacement with mannitol did not result in any changes, while in the case of sorbitol use, the content of the target compound decreased by 12.2%. Based on these data, glycerin was chosen as the carbon source for the further experiments.

3.5. Effect of Nitrogen Source on Carotenoid Accumulation in the M. neoaurum Ac-501/22 Biomass

Nitrogen sources evaluated in the experiment included soybean flour (control), powdered milk, pea meal, yeast extract, meat peptone, yeast peptone, and soybean peptone. The contents of the total protein and total nitrogen in the above-mentioned nutrient medium components are shown in Supplementary Materials (Table S1). According to the obtained results, Ac-501/22 was able to utilize all tested substrates (Figure 5). The maximum β-carotene production (170.8 mg/kg) was observed for the powdered milk and exceeded that in the control medium by 23.4%; the biomass yield was 19.7% higher than in the control. High biomass and β-carotene yields were also obtained for the media containing meat peptone (17.6 g/L and 162.3 mg/kg, respectively) and pea meal (17.8 g/L and 160.5 mg/kg, respectively).

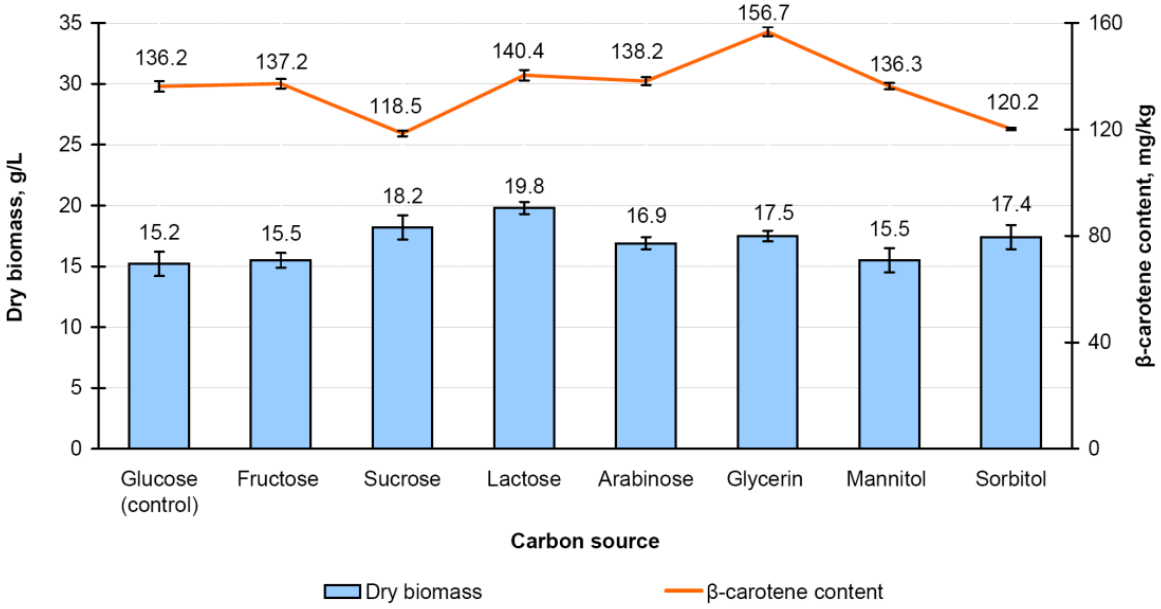

Figure 4. Effect of carbon source on biomass accumulation and β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 after 50 h of cultivation.

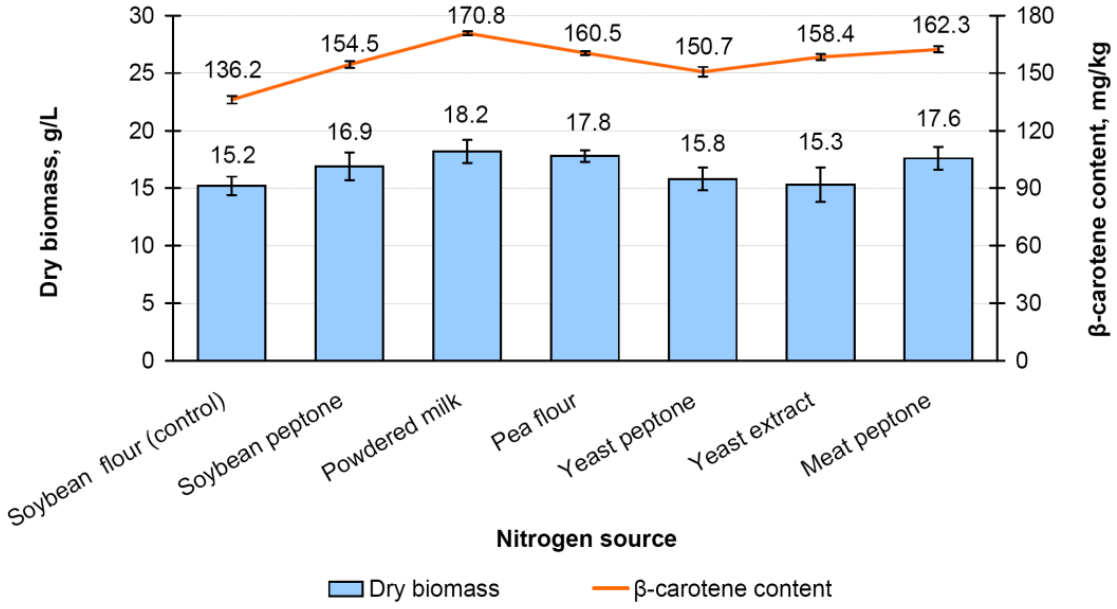

Figure 5. Effect of nitrogen source on biomass accumulation and β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 after 50 h of cultivation.

3.6. Effect of the Urea Content on Biomass Accumulation and Carotenoid Content in M. neoaurum Ac-501/22

Since urea represents an additional nitrogen source, which can be effectively used by microorganisms, an additional experiment was arranged to determine the concentration of this medium component that provided optimal growth of *M. neoaurum* and β-carotene production. The studied urea concentration range was 0.25–1.5 g/L; the nutrient medium also contained soybean flour as the main nitrogen source. Urea concentration equal to 0.5 g/L was considered as the control. The results of the experiment are shown in Figure 6.

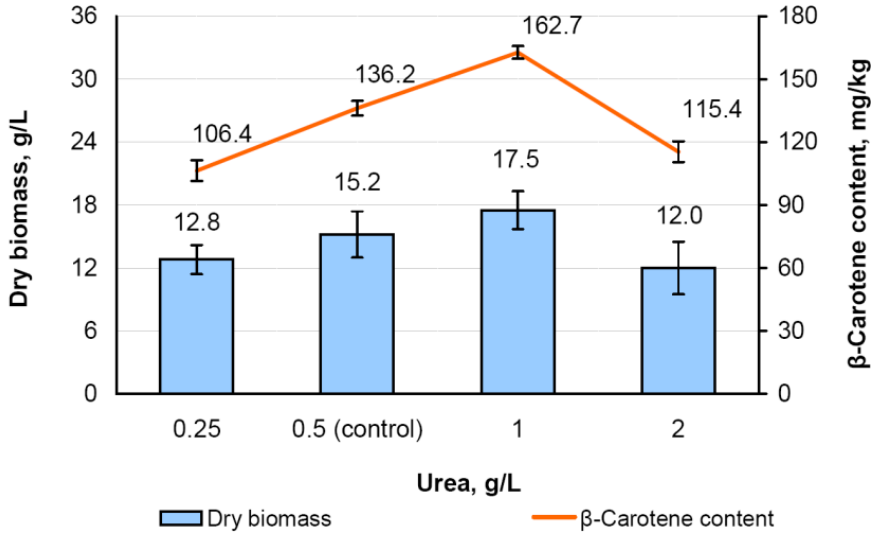

Figure 6. Effect of the urea concentration on biomass accumulation and β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 after 50 h of cultivation.

The maximum strain productivity was obtained at the urea concentration of 1.0 g/L: the biomass and β-carotene yields were 17.5 g/L and 162.7 mg/kg that exceeded the control values by 15.2 and 19.5%, respectively. A further increase in the urea content in the nutrient medium to 2.0 g/L inhibited both strain growth and β-carotene production by 21 and 15.2%, respectively, compared to that in the control. A decrease in the urea content below that in the control to 0.25 g/L also negatively influenced the strain productivity: compared to the control, the biomass β-carotene yields decreased by 15.7 and 21.8%, respectively.

3.7. M. neoaurum Ac-501/22 Cultivation in a Modified Medium

Based on the results of the performed experiments, a modified medium (AM medium) was developed to provide the maximum productivity of the *M. neoaurum* Ac-501/22 strain. The medium contained glycerin (20.0 g/L) as the carbon source and a combination of powdered milk (10.0 g/L) and urea (1.0 g/L) as the nitrogen source. The medium A (see Section 2.4) was used as the control. The results of strain cultivation in the modified medium compared to the control medium are shown in Figure 7. The use of optimized AM medium provided a reliable and significant increase in the strain productivity in relation to the biomass yield (+26.3%) and β-carotene production (+35%) compared to the control medium.

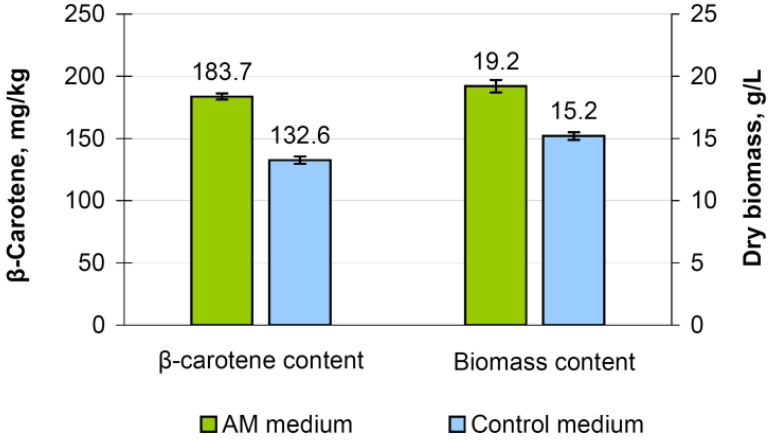

Figure 7. Effect of the optimized AM medium on biomass accumulation and β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 after 50 h of cultivation.

3.8. M. neoaurum Ac-501/22 Fermentation in a 3 L Bioreactor under Laboratory Conditions

The strain was cultivated in three parallel replications using a fermentation system consisting of three 3 L bioreactors with the optimized AM medium. Fermentation was carried out in two different regimes. The first regime included registration of basic technological parameters (pH, pO_2) under constant stirring, and the second regime included fed-batch fermentation under controlled pH and pO_2 levels.

3.8.1. *M. neoaurum* Ac-501/22 Fermentation in the Registration Regime

The regime of registration of the basic technological parameters maintained aeration at the level of 2 L/min, stirring at 450 rpm, and temperature at 35 °C. During culture growth, an increase in the medium pH from 6.9 to 8.5 was observed (Figure 8). The biomass content after 48 h fermentation reached 17.8 g/L (Figure 7); during further fermentation, the biomass began to decrease, since the culture reached the next life cycle stage (lysis). The level of β-carotene production after 48 h fermentation reached 176.8 mg/kg. A significant pH increase at 72 h of fermentation confirmed the occurring cell lysis.

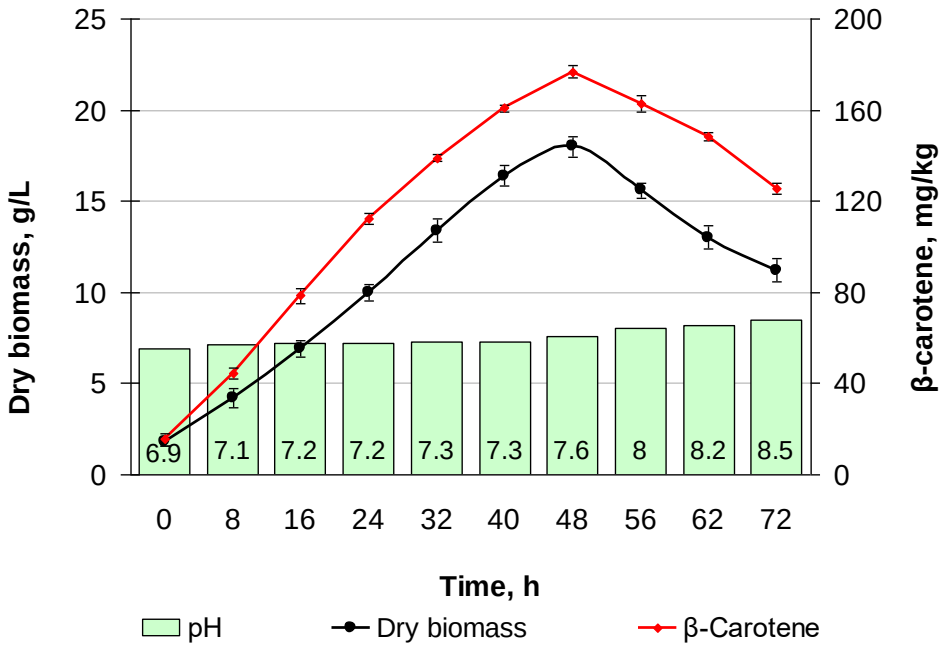

Figure 8. Cultivation of *M. neoaurum* Ac-501/22 in the regime of registration of basic technological parameters.

3.8.2. Fermentation of *M. neoaurum* Ac-501/22 under Controlled pH Regime

To study the effect of pH level on the productivity of the Ac-501/22 strain revealing the optimal pH value, a series of fermentation was carried out at three different pH values (6.0, 7.0, and 8.0) maintained stable by feeding the fermentation medium with 5% HCl via a peristaltic pump in an automated mode. Fermentation in the regime of registration of basic technological parameters without any regulation of the active medium acidity was considered as the control. The results of the experiment are shown in Figure 9. The maximum productivity indices were obtained after 48 h of fermentation at pH 7.0–7.2: the biomass and β-carotene yields reached 19.6 g/L and 205.6 mg/kg, respectively, that exceeded the control values by 10 and 17.6%, respectively.

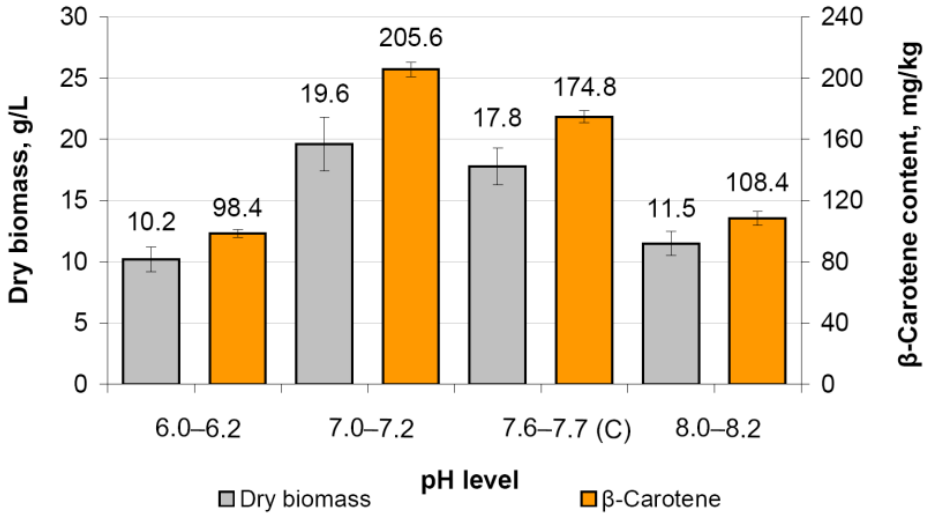

Figure 9. Effect of different pH levels on biomass accumulation and β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 after 48 h of fermentation.

3.8.3. Determination of the Optimal pO_2 Level for Fermentation of *M. neoaurum* Ac-501/22

The content of dissolved oxygen in a fermentation medium is one of the important parameters influencing the productivity of microbial biosynthesis of biologically active compounds. The evaluation of the effect of different concentrations of dissolved oxygen on biomass growth and β-carotene productivity of *M. neoaurum* Ac-501/22 was carried out under an automatically controlled medium pH (7.0–7.2). The content of dissolved oxygen in the medium was varied by regulating the rotational speed of a stirring device and changing the amount of the consumed air. The maximum productivity (in terms of β-carotene) and the maximum biomass yield were observed at the dissolved oxygen content equal to 50% (215.8 mg/kg and 22.6 g/L, respectively; see Figure 10).

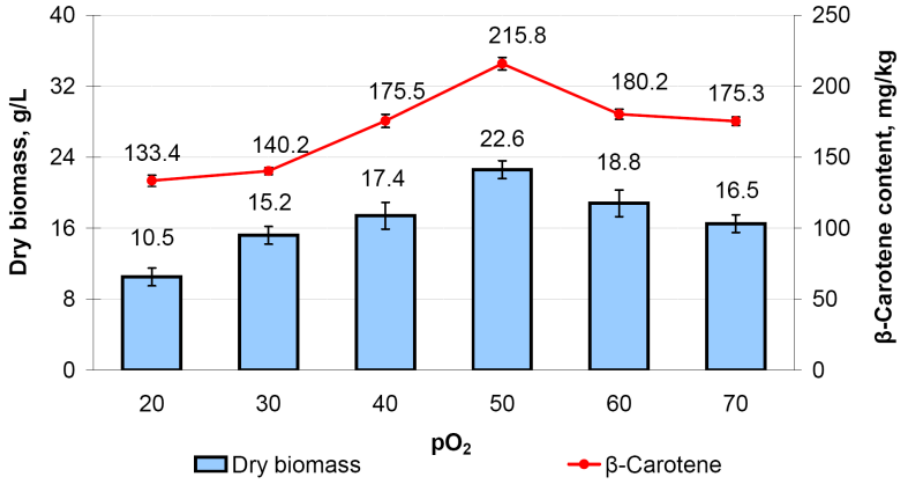

Figure 10. Effect of different concentrations of dissolved oxygen (pO_2) on biomass accumulation and β-carotene production in *Mycolicibacterium neoaurum* Ac-501/22 after 48 h of fermentation.

3.8.4. Productivity of *M. neoaurum* Ac-501/22 Cultivated in a 3 L Bioreactor with Controlled pH and pO_2 Levels and Additional Carbon Source Supply

A sterile glycerin solution (2.5 g/L) was fed into the bioreactor in an automated mode after 24 h of fermentation at the maintained optimal pH and pO_2 levels (7.0–7.2 and 50%, respectively). The results of the experiment are shown in Figure 11. Glycerin addition after 24 h of fermentation positively influenced both studied parameters. After 52 h of fermentation, the biomass and β-carotene yields reached 24.8 g/L and 262.4 mg/kg, respectively.

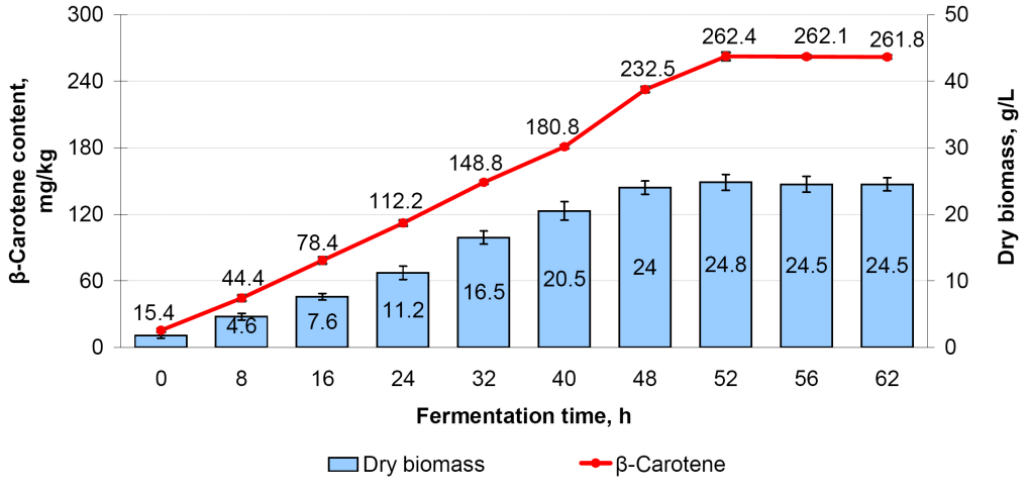

Figure 11. Accumulation of the biomass and β-carotene in a culture broth of *Mycolicibacterium neoaurum* Ac-501/22 under conditions of a fed-batch cultivation with addition of glycerin (5 g/L) at the 24th h of fermentation.

4. Discussion

The research activity related to the large-scale microbiological synthesis of carotenoids is focused mainly on the search for highly active strains and optimization of nutrient media and fermentation conditions. Biotechnological production of carotenoids is based mainly on yeast cultures [28–30]. Nevertheless, bacteria, which are widely used in the large-scale production of biologically active compounds [15,31,32], can also be considered as potential carotenoid producers, especially pigmented bacteria naturally producing this type of compounds or genetically modified non-pigmented bacteria [33].

The main pigments synthesized by bacteria from the genus *Mycolicibacterium* (synonym *Mycobacterium*) are α- and β-carotene, lycopene, zeaxanthin, and astaxanthin [34–37]. Therefore, bacteria from this genus can be used as producers of these compounds. Note that the biosynthetic activity of *Mycolicibacterium* does not depend on the illumination intensity and environmental salinity as in the case of cyanobacteria, halophiles, or microalgae [38]. For example, stimulation of carotenoid biosynthesis in microalgae *Dunaliella salina* requires the maintenance of the NaCl concentration in the cultivation medium at the level of 18–27% and a provision of a 12 h light stress within 15 days [39].

To date, the data on the use of *Mycolicibacterium* (including *M. neoaurum*) strains as carotene producers are very limited. For example, *M. rubrum* strain no. 44 was reported to accumulate carotenoids up to 6.5–7.0 mg/g of dry biomass after 140–150 h fermentation in the ANKUM fermentation system [40]. Authors noted that the carotene biosynthesis occurred only after 50 h of culture growth. By this time, the intensive growth stage has already finished, the basic nutrients have been consumed, and metabolites, which negatively influenced the further strain growth but stimulated the production of new enzymes involved in the carotene biosynthesis with further oxidation to xanthophylls, have been accumulated. In other representatives of the genus *Mycolicibacterium*, such as *M. lacticolum*, *M. phlei*, and *M. neoaurum*, the biosynthesis of carotenoids occurs in parallel with the biomass growth [40].

The majority of the current studies on *Mycolicibacterium* are focused on the ability of the strains to transform sterols of animal and plant origin to basic intermediates of steroid drugs [41]. Nevertheless, the ability of the studied *M. neoaurum* strain to produce β-carotene as the only pigment as well as a rapid growth of this species on available substrates indicate interesting prospects of this strain for a large-scale production of this pigment.

Today, the main way to develop overproducing strains, including carotenoid producers, is genetic engineering [42,43]. In the case of the genus *Mycobacterium*, Elamin et al. [44] reported the development of genetically modified *M. smegmatis* MC2-155 strain, whose lycopene productivity was 1.41 mg/g of dry biomass. However, the design of such overproducers still remains to be very complex, and the stability of the obtained strains in a long-term scale is always questionable because of a probable loss of the newly acquired biological activity. Moreover, in the case of microbial strains intended for use as a carotene-enriched feed additive, genetic engineering methods are unacceptable, because of the GMO-prohibiting legislation in many countries. Thus, microorganisms with the improved ability to synthesize biologically active compounds, which are intended for use as food or feed additives, are developed via chemical or physical induction of the corresponding mutations followed by selection for the desired traits. Note that selection is a very important stage in the process of obtaining highly productive strains. Of course, this method is not devoid of some subjectivism, but the fact that changes in the productivity of a target product correlate with changes in the morphological traits of a mutant strain is confirmed by a number of publications [28,45]. In our study, strain selection was based on the change of a colony color from yellow to bright orange, since this trait correlates with the strain ability to produce carotenoids [28]. After five mutagenesis cycles with the use of NMU as a mutagen, we obtained the *M. neoaurum* Ac-501/22 strain characterized by a 2.7-fold increase in β-carotene productivity compared to the parental strain.

Along with the development and maintenance of an overproducing strain, a lot of attention should be paid to the optimization of strain cultivation conditions, since the effect of temperature and nutrient medium composition on the strain growth and synthesis of secondary metabolites is well-known [31,46–52]. For example, LB medium supplementation with glucose, sucrose, and xylose resulted in a 1.8–3.1-fold increase in the carotenoid yield from the biomass of *Microbacterium paraoxydans* [49]. In our study, glucose replacement with sucrose or lactose resulted in an increased biomass yield of *M. neoaurum* Ac-501/22 (by 19.7 and 30.2%, respectively). However, in the case of glucose replacement with sucrose, the β-carotene content decreased by 13%. In the case of lactose, the content of β-carotene almost coincided with that in the control. Among all the carbon sources tested, the maximum effect on the carotenoid biosynthesis in *M. neoaurum* cells was observed for glycerin (+15%). The positive effect of glycerin on the biosynthesis of carotenoids was also revealed for the *Rhodococcus opacus* strain PD630 [50]. This effect can be explained by the involvement of glycerin in the biosynthesis of isoprenoids representing carotenoid precursors [50]. The putative scheme of glycerin involvement in carotenoid biosynthesis is shown in Figure 12.

Like carbon, nitrogen is also an important component of the nutrient medium influencing the cell growth and biosynthesis of secondary metabolites. According to some data, the biosynthesis of β-carotene is influenced by both nature and concentration of the nitrogen source [31,51]. For example, the maximum biomass yield in *Rhodotorula glutinis* var. *glutinis* was reported for the medium containing casein hydrolysate as the nitrogen source [52]. Moreover, authors demonstrated the effect of the nitrogen source on the ratio of carotenoids in a dry biomass of this fungus. For example, cultivation of the fungus on a peptone-containing medium promoted torulene production, whereas a medium containing NH4NO3 promoted the biosynthesis of β-carotene [52]. According to the results obtained in our study, the maximum effect on the *M. neoaurum* biomass yield and the target pigment production was observed for soybean flour replacement with dried milk, meat peptone, and pea meal. None of the studied nitrogen sources influenced the direction of the carotenoid biosynthesis.

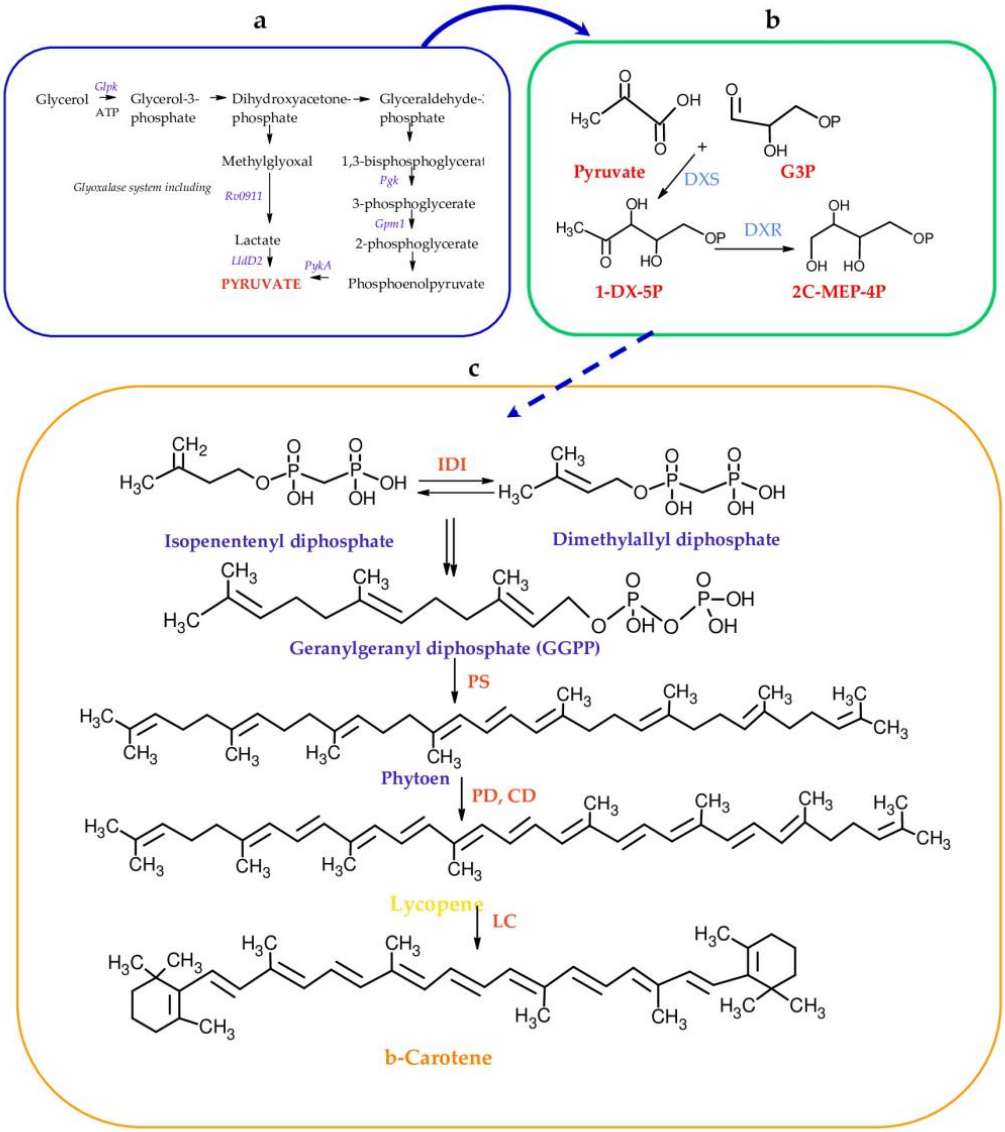

Figure 12. Scheme of glycerol involvement in the carotenoid biosynthesis pathway of *M. neoaurum* proposed on the basis of published data. (**a**) Pyruvate production from glycerol (adapted from [53]); (**b**) isoprenoid biosynthetic pathway (adapted from [54]); (**c**) general scheme of the carotenoid biosynthesis according to [55]. Glpk, glycerol kinase; Pgk, phosphoglycerate kinase; Gpm1, phosphoglycerate mutase 1; PykA, pyruvate kinase; Rv0911, putative glyoxalase; LldD2, lactate dehydrogenase; DXS, 1-deoxy-D-xylose-5-phosphate synthase; DXR, 1-deoxy-D-xylose 5-phosphate reducto-isomerase; 2C-MEP-4P, 2-C-methyl-D-erythritol 4-phosphate; G3P, glycerinaldehyde-3-phosphate; 1-DX-5P, deoxyxylulose 5-phosphate; IDI, diphosphate isomerase; PS, phytoene synthase; PD, phytoene desaturase; CD, carotene desaturase; LC, lycopene β-cyclasa.

Urea is an additional nitrogen source for microorganisms. We found that an increase in the content of this component in a nutrient medium from 0.5 to 1.0 g/L resulted in a 15.2% increase in the biomass yield and 19.5% increase in the β-carotene content. A further increase in the urea content to 2.0 g/L inhibited the growth of *M. neoaurum*. The positive effect of urea on the microbial growth and biosynthesis of secondary metabolites is well-known. For example, Brabender et al. [46] showed that ammonium sulfate replacement with an equivalent (by nitrogen) amount of urea caused an increase in the growth rate of *Yarrowia lipolitica*, a reduction in the time required for reaching a stationary growth phase from 72 to 48 h, and an increase in the OD_{600} from 25 to 35. Therefore, based on the obtained results, we can consider that urea, along with the optimization of the main nitrogen source

(dried milk), also significantly contributes to the growth of *M. neoaurum* and β-carotene biosynthesis. The effect of nitrogen on the growth and morphology of the microorganism as well as on the biosynthesis of secondary metabolites is probably explained by the fact that nitrogen is an important element of amino acids, which form enzymes involved in the biosynthesis of various metabolites. At the same time, according to some data, carbon provides a more significant influence on the carotenoid biosynthesis than nitrogen [47].

Temperature is an important factor in carotenoid accumulation, since it influences the growth and development of microorganisms, functioning of enzymes, and biosynthetic pathways. However, different strains have different responses to temperatures, since they are characterized by specific optimal growth conditions and stress tolerance [31,56]. In some cases, high temperatures promote an increased yield of one carotenoid, but suppress production of another one [56,57]. For example, cultivation of the *Lactiplantibacillus plantarum* subsp. *plantarum* strain KCCP11226T at 30 °C provided the maximum biomass density, but the maximum carotenoid yield was observed at 25 °C [48]. In our study, an increase in the *M. neoaurum* cultivation temperature from 30 to 35 °C caused a reduction in the time required to achieve the stationary growth phase, an increase in the biomass and fermentation broth yields, and an increase in the content of β-carotene in the fermentation broth. A similar simultaneous increase in the biomass and carotenoid yields was demonstrated for two *Muricauda* sp. isolates, YUAB-SO-11 and YUAB-SO-45 [56]. An improved growth rate and β-carotene biosynthesis in *M. neoaurum* Ac–501/22 is explained mainly by the fact that 35 °C is the optimal temperature for the growth of this strain and biosynthesis of secondary metabolites. However, we cannot completely exclude the hypothesis that such a temperature can be considered as a stress factor stimulating the production of carotenes, since the cultivation temperature of the parental *M. neoaurum* Ac–501 strain, as well as a number of other *Mycolicibacterium* strains used in biotechnological production, did not exceed 30 °C [26,58]. This hypothesis is confirmed by the reduction in the strain growth rate and biomass accumulation at 40 °C, while β-carotene production remained at the level comparable to that in the optimal growth conditions.

One of the key aspects for increasing the yield of the target compounds under lab-scale or large-scale fermentation is the maintenance of optimal fermentation conditions at a constant level to reduce microbial stress and improve the direction of the metabolism. For each industrial strain, the optimal cultivation conditions are selected individually. For example, *R. opacus* cultivation with a periodic addition of nutrient medium components (ammonium acetate or acetic acid) resulted in the doubled yields of the biomass and carotenoids; note also that the intensification of the carotene biosynthesis did not require pH maintenance at a stable level [59]. Our study revealed a dependence of the biomass yield of *M. neoaurum* Ac–501/22 and β-carotene accumulation on the level of pH and pO_2. The maintenance of the pH level within 6.9–7.2 and the pO_2 level at 50% provided an increase in the biomass and β-carotene yields by 39 and 48.4%, respectively (Figure 8).

Thus, the performed multi-step induced mutagenesis of the *M. neoaurum* strain Ac–501 with further selection resulted in a new *M. neoaurum* strain Ac–501/22, whose β-carotene production was three-times higher than that of the parental strain (44.4 and 136.2 mg/kg, respectively). The further optimization of the submerged cultivation conditions of the new strain (at the flask level) provided an increase in the biomass and β-carotene yields by 26.3 and 35.0%, respectively. The further optimization of the fermentation conditions in a 3 L bioreactor allowed us to further improve these two parameters (biomass yield was 24.8 g/L, β-carotene content 262.4 mg/kg). At the next stage of this study, we plan to select the optimal fermentation parameters for the cultivation of this strain under pilot and large-scale conditions.

5. Conclusions

The performed study resulted in a sixfold increase in the β-carotene productivity of *M. neoaurum* Ac–501/22 compared to the initial value. These results confirm good prospects for the use of *M. neoaurum* Ac–501/22 as a β-carotene producer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9121007/s1, Figure S1: Nucleotide sequence of the 16S rRNA gene of *M. neoaurum* strain Ac 501/22. Table S1: Total nitrogen/protein content in each of the tested nitrogen sources.

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

Funding: The research 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 authors declare that the data supporting the findings of this study are available within the main text of the manuscript and Supplementary Materials. Raw data are available from the corresponding author upon reasonable request.

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

References

1. Pagels, F.; Vasconcelos, V.; Guedes, A.C. Carotenoids from cyanobacteria: Biotechnological potential and optimization strategies. *Biomolecules* **2021**, *11*, 735. [CrossRef] [PubMed]
2. Maoka, T. Carotenoids as natural functional pigments. *J. Nat. Med.* **2020**, *74*, 1–16. [CrossRef] [PubMed]
3. 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]
4. 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]
5. Novoveská, L.; Ross, M.E.; Stanley, M.S.; Pradelles, R.; Wasiolek, V.; Sassi, J.F. Microalgal carotenoids: A review of production, current markets, regulations, and future direction. *Mar. Drugs* **2019**, *17*, 40. [CrossRef] [PubMed]
6. Meléndez-Martínez, A.J. An overview of carotenoids, apocarotenoids, and vitamin A in agro-food, nutrition, health, and disease. *Mol. Nutr. Food Res.* **2019**, *63*, e1801045. [CrossRef] [PubMed]
7. Ernst, H. Recent advances in industrial carotenoid synthesis. *Pure Appl. Chem.* **2002**, *74*, 2213–2226. [CrossRef]
8. Lim, K.C.; Yusoff, F.M.; Shariff, M.; Kamarudin, M.S. Astaxanthin as feed supplement in aquatic animals. *Rev. Aquacult.* **2017**, *10*, 738–773. [CrossRef]
9. Botella-Pavía, P.; Rodríguez-Concepción, M. Carotenoid biotechnology in plants for nutritionally improved foods. *Physiol. Plant* **2006**, *126*, 369–381. [CrossRef]
10. Gong, M.; Bassi, A. Carotenoids from microalgae: A review of recent developments. *Biotechnol. Adv.* **2016**, *34*, 1396–1412. [CrossRef]
11. 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]
12. Liu, C.; Li, L.J.; Wu, C.Y.; Guo, K.N.; Li, J.H. Growth and antioxidant production of *Spirulina* in different NaCl concentrations. *Biotechnol. Lett.* **2016**, *38*, 1089–1096. [CrossRef] [PubMed]
13. Maddiboyina, B.; Vanamamalai, H.K.; Roy, H.; Ramaiah; Gandhi, S.; Kavisri, M.; Moovendhan, M. Food and drug industry applications of microalgae *Spirulina platensis*: A review. *J. Basic Microbiol.* **2023**, *63*, 573–583. [CrossRef]
14. Chinnasamy, S.; Bhatnagar, A.; Hunt, R.W.; Das, K. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresour. Technol.* **2010**, *101*, 3097–3105. [CrossRef]
15. Choi, S.S.; Kim, G.D. Production of carotenoids by bacteria: Carotenoid productivity and availability. *J. Life Sci.* **2022**, *32*, 411–419.
16. 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]

17. Silva, T.P.; Paixão, S.M.; Alves, L. Ability of *Gordonia alkanivorans* strain 1B for high added value carotenoids production. *RSC Adv.* **2016**, *5*, 58055. [CrossRef]
18. 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]
19. Allahkarami, S.; Sepahi, A.A.; Hosseini, H.; Razavi, M.R. Isolation and identification of carotenoid-producing *Rhodotorula* sp. from Pinaceae forest ecosystems and optimization of in vitro carotenoid production. *Biotechnol. Rep.* **2021**, *32*, e00687. [CrossRef]
20. Barreiro, C.; Barredo, J.L. *Microbial Carotenoids: Methods and Protocols*; Humana New York: New York, NY, USA, 2018; p. 344.
21. Zeb, A.; Murkovic, M. Thin-layer chromatographic analysis of carotenoids in plant and animal samples. *J. Planar Chromatogr.* **2010**, *23*, 94–103. [CrossRef]
22. Minyuk, G.S.; Solovchenko, A.E. Express analysis of microalgal secondary carotenoids by TLC and UV-Vis spectroscopy. In *Microbial Carotenoids*; Methods in Molecular Biology; Springer: Amsterdam, The Netherlands, 2018; p. 7395.
23. Bulygina, E.S.; Kuznetsov, B.B.; Marusina, A.I.; Turova, T.P.; Kravchenko, I.K.; Bykova, S.A.; Kolganova, T.V.; Gal'chenko, V.F. A study of nucleotide sequences of nifH genes of some methanotrophic bacteria. *Mikrobiology* **2002**, *71*, 425–432.
24. Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **1977**, *84*, 5463–5467. [CrossRef] [PubMed]
25. Checheta, O.V.; Safonova, E.F.; Slivkin, A.I. Structure of chemical compounds, methods and process control: Thin-layer chromatography characteristics of β-carotene. *Pharm. Chem. J.* **2012**, *46*, 321–323. [CrossRef]
26. Tekucheva, D.N.; Nikolayeva, V.M.; Karpov, M.V.; Timakova, T.A.; Shutov, A.V.; Donova, M.V. Bioproduction of testosterone from phytosterol by *Mycolicibacterium neoaurum* strains: "one-pot", two modes. *Bioresour. Bioproces* **2022**, *9*, 11. [CrossRef]
27. Andriushina, V.A.; Rodina, N.V.; Stytsenko, T.C.; Hue, L.D.; Druzhinina, A.V.; Iaderets, V.V.; Voishvillo, N.E. Conversion of soybean sterols into 3,17-diketosteroids using actinobacteria *Mycobacterium neoaurum*, *Pimelobacter simplex*, and *Rhodococcus erythropolis*. *Appl. Biochem. Microbiol.* **2011**, *47*, 270–273. [CrossRef]
28. Delegan, Y.; Yachkula, A.; Antipova, T.; Vainshtein, M. Evaluation of red-colored carotenoids in yeasts by the biomass color. *Folia Microbiol.* **2021**, *66*, 615–622. [CrossRef]
29. Kot, A.M.; Błażejak, S.; Gientka, I.; Kieliszek, M.; Bryś, J. Torulene and torularhodin: "new" fungal carotenoids for industry? *Microb. Cell Fact.* **2018**, *17*, 49. [CrossRef]
30. Li, N.; Han, Z.; O'Donnell, T.J.; Kurasaki, R.; Kajihara, L.; Williams, P.G.; Tang, Y.; Su, W.W. Production and excretion of astaxanthin by engineered *Yarrowia lipolytica* using plant oil as both the carbon source and the biocompatible extractant. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 6977–6989. [CrossRef]
31. Verwaal, R.; Wang, J.; Meijnen, J.P.; Visser, H.; Sandmann, G.; van den Berg, J.A.; van Ooyen, A.J. High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. *Appl. Environ. Microbiol.* **2007**, *73*, 4342–4350. [CrossRef]
32. Sharpe, P.L.; Di Cosimo, D.J. Construction and utilization of carotenoid reporter systems: Identification of chromosomal integration sites that support suitable expression of biosynthetic genes and pathways. *Methods Mol. Biol.* **2012**, *892*, 219–243.
33. Wang, C.; Zhao, S.; Shao, X.; Park, J.B.; Jeong, S.H.; Park, H.J.; Kwak, W.J.; Wei, G.; Kim, S.W. Challenges and tackles in metabolic engineering for microbial production of carotenoids. *Microb. Cell Fact.* **2019**, *18*, 55. [CrossRef] [PubMed]
34. Ichiyama, S.; Shimokata, K.; Tsukamura, M. Relationship between mycobacterial species and their carotenoid pigments. *Microbiol. Immunol.* **1988**, *32*, 473–479. [CrossRef]
35. da Costa Cardoso, L.A.; Feitosa Kanno, K.Y.; Karp, S.G. Microbial production of carotenoids—A review. *African J. Biotechnol.* **2017**, *16*, 139–146.
36. Barbosa-Filho, J.M.; Alencar, A.A.; Nunes, X.P.; de Andrade Tomaz, A.C.; Sena-Filho, J.G.; Athayde-Filho, P.F.; Silva, M.S.; de Souza, M.F.V.; da-Cunha, E.V.L. Sources of alpha-, beta-, gamma-, delta- and epsilon-carotenes: A twentieth century review. *Rev. Bras. Farmacogn.* **2008**, *18*, 135–154. [CrossRef]
37. Mussagy, C.U.; Winterburn, J.; Santos-Ebinuma, V.C.; Pereira, J.F.B. Production and extraction of carotenoids produced by microorganisms. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 1095–1114. [CrossRef] [PubMed]
38. Schweiggert, R.M.; Carle, R. Carotenoid Production by Bacteria, Microalgae, and Fungi. In *Carotenoids: Nutrition, Analysis and Technology*; Kaczor, A., Baranska, M., Eds.; John Wiley & Sons, Ltd.: Chichester, UK, 2016; pp. 217–240.
39. Ribeiro, B.D.; Barreto, D.W.; Coelho, M.A.Z. Technological aspects of β-carotene production. *Food Bioprocess. Technol.* **2011**, *4*, 693–701. [CrossRef]
40. Daraseliia, G.I.; Khachapuridze, D.O.; Gagelidze, N.A.; Amiranashvili, L.L. The kinetics of *Mycobacterium rubrum* growth under the conditions of batch cultivation. *Mikrobiologiia* **1989**, *58*, 596–601. (In Russian)
41. Zhao, A.; Zhang, X.; Li, Y.; Wang, Z.; Lv, Y.; Liu, J.; Alam, M.A.; Xiong, W.; Xu, J. *Mycolicibacterium* cell factory for the production of steroid-based drug intermediates. *Biotechnol. Adv.* **2021**, *53*, 107860. [CrossRef]
42. Wang, N.; Peng, H.; Yang, C.; Guo, W.; Wang, M.; Li, G.; Liu, D. Metabolic engineering of model microorganisms for the production of xanthophyll. *Microorganisms* **2023**, *11*, 1252. [CrossRef]
43. Mata-Gómez, L.C.; Montañez, J.C.; Méndez-Zavala, A.; Aguilar, C.N. Biotechnological production of carotenoids by yeasts: An overview. *Microb. Cell Fact.* **2014**, *13*, 12. [CrossRef]

44. Elamin, O.; Marwa, C.; Koraichi, S.I.; Iraqui, H.M. Lycopene production in *Mycobacterium smegmatis* by expression of *crt* genes from *Mycobacterium aurum* and protective effect of lycopene in vivo and in vitro against UV radiation. *Int. J. Pharm. Pharm. Sci.* **2018**, *10*, 49–53. [CrossRef]
45. Khaliq, S.; Akhtar, K.; Afzal Ghauri, M.; Iqbal, R.; Mukhtar Khalid, A.; Muddassar, M. Change in colony morphology and kinetics of tylosin production after UV and gamma irradiation mutagenesis of Streptomyces fradiae NRRL-2702. *Microbiol. Res.* **2009**, *164*, 469–477. [CrossRef] [PubMed]
46. Brabender, M.; Hussain, M.S.; Rodriguez, G.; Blenner, M.A. Urea and urine are a viable and cost-effective nitrogen source for *Yarrowia lipolytica* biomass and lipid accumulation. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2313–2322. [CrossRef] [PubMed]
47. 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. *Bioresour. Technol.* **2021**, *326*, 124816. [CrossRef] [PubMed]
48. Siziya, I.N.; Yoon, D.J.; Kim, M.; Seo, M.J. Enhanced production of C_{30} carotenoid 4,4′-diaponeurosporene by optimizing culture conditions of *Lactiplantibacillus plantarum* subsp. *plantarum* KCCP11226^{T}. *J. Microbiol. Biotechnol.* **2022**, *32*, 892–901. [CrossRef] [PubMed]
49. Ojha, S.; Kapoor, S.; Mishra, S. Carotenoid production by a novel isolate of *Microbacterium paraoxydans*. *Indian. J. Microbiol.* **2018**, *58*, 118–122. [CrossRef]
50. 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]
51. Ni, H.; Chen, Q.H.; Ruan, H.; Yang, Y.F.; Li, L.J.; Wu, G.B.; Hu, Y.; He, G.Q. Studies on optimization of nitrogen sources for astaxanthin production by *Phaffia rhodozyma*. *J. Zhejiang Univ. Sci. B* **2007**, *8*, 365–370. [CrossRef]
52. El-Banna, A.A.; El-Razek, A.M.A.; El-Mahdy, A.R. Some factors affecting the production of carotenoids by *Rhodotorula glutinis* var. *glutinis*. *Food Nutr. Sci* **2012**, *3*, 64–71.
53. Baughn, A.D.; Rhee, K.Y. Metabolomics of central carbon metabolism in *Mycobacterium tuberculosis*. *Microbiol. Spectr.* **2014**, 2. [CrossRef]
54. Paniagua-Michel, J.; Olmos-Soto, J.; Ruiz, M.A. Pathways of carotenoid biosynthesis in bacteria and microalgae. *Methods Mol. Biol.* **2012**, *892*, 1–12. [PubMed]
55. 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] [PubMed]
56. Prabhu, S.; Rekha, P.D.; Young, C.C.; Hameed, A.; Lin, S.Y.; Arun, A.B. Zeaxanthin production by novel marine isolates from coastal sand of India and its antioxidant properties. *Appl. Biochem. Biotechnol.* **2013**, *171*, 817–831. [CrossRef]
57. Takaichi, S.; Ishidsu, J. Influence of growth temperature on compositions of carotenoids and fatty acids from carotenoid glucoside ester and from cellular lipids in *Rhodococcus rhodochrous* RNMSI. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1886–1889. [CrossRef]
58. Xiong, L.B.; Liu, H.H.; Song, L.; Dong, M.M.; Ke, J.; Liu, Y.J.; Liu, K.; Zhao, M.; Wang, F.Q.; Wei, D.Z. Improving the biotransformation efficiency of soybean phytosterols in *Mycolicibacterium neoaurum* by the combined deletion of *fbpC3* and *embC* in cell envelope synthesis. *Synth. Syst. Biotechnol.* **2021**, *7*, 453–459. [CrossRef]
59. Thanapimmetha, A.; Suwaleerat, T.; Saisriyoot, M.; Chisti, Y.; Srinophakun, P. Production of carotenoids and lipids by *Rhodococcus opacus* PD630 in batch and fed-batch culture. *Bioprocess. Biosyst. Eng.* **2017**, *40*, 133–143. [CrossRef]