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Carotenoid-Producing Yeasts: Selection of the Best-Performing Strain and the Total Carotenoid Extraction Procedure

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Abstract: Yeasts are considered an extraordinary alternative source of natural carotenoids and pigmented terpenoids with multiple applications. Production of carotenoids by yeast fermentation technology has many benefits; it is cost-effective, easily scalable, and safe. The aim of this research is the isolation of yeasts from natural resources and selection of the most potent bioagent for carotenoid production. Additionally, an upgraded carotenoid extraction protocol we established, which implies the testing of four methods for cell lysis (hydrochloric acid treatment, ultrasound treatment, milling treatment, and osmotic pressure treatment), three extraction methods (conventional extraction, ultrasound extraction, and conventional + ultrasound extraction), and three extraction solvents (acetone, isopropanol/methanol (50:50), and ethanol). For the first time, the obtained results were further modeled by an artificial neural network (ANN). Based on the obtained maximal carotenoid yield (253.74 ± 9.74 mg/100 g d.w) for the best-performing *Rhodotorula mucilaginosa*, the optimized extraction procedure involving milling treatment (for cell lysis) and conventional extraction with acetone (for carotenoid extraction) convincingly stood out compared to the other 35 tested protocols. Therefore, the selected carotenoid extraction protocol was verified with respect to its universality for all other yeast isolates, demonstrating its simplicity and effectiveness.

Keywords: carotenoid-producing yeasts; *Rhodotorula*; carotenoid extraction; red-pigmented yeasts; total carotenoid; artificial neural network

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1. Introduction

Carotenoids belong to a class of terpenoid-pigmented, lipid-soluble compounds and are important biomolecules for human health; in addition to their use as coloring agents, they exhibit anticancer and antioxidant activity, as well as pro-vitamin A function [1]. Their roles have already been established in phytomedicine, chemical, pharmaceutical, cosmetic, food, and feed industries, and demand for their production has only increased. Carotenoids are found in a wide variety of plants and microorganisms. Most of carotenoids are currently extracted either from plants, such as annatto, tomato, carrot, paprika, and grapes, which entails seasonal and geographic variabilities, or are chemically produced, generating hazardous waste [2].

Considering this high demand for carotenoids in various industries, emphasis is also placed on optimization of extraction methods. The chosen method is important because there are many diverse carotenoids with different levels of polarity and physical and chemical barriers in the food matrices, in addition to their sensitivity to excess heat, light, acids, and long extraction times. Non-polar solvents, such as hexane, are usually a good choice for extraction of non-polar carotenes or esterified xanthophylls, whereas polar solvents, such as methanol, ethanol, and acetone, are more suitable for extraction of polar carotenoids [3]. The numerous extraction methods can be classified into four main categories: ultrasound (UAE), pressurized liquid extraction (PLE), supercritical fluid

extraction (SFE), and enzymatic extraction (EE). Additionally, certain pretreatments in microbial biotechnology production are required to extract the targeted product, such as cell lysis (if the product is intracellularly located) [4]. Conventional extraction procedures are based on the extractive capabilities of different solvents and involve heating or mixing, such as the classical use of solvents in combination with a vortex apparatus, which is regularly used to mix samples of interest with dilutant, which helps with homogenization of the solution, followed by centrifugation with a supernatant [5]. Ultrasound extraction (UAE) is a modern, rapid, and inexpensive method meant to improve the extraction yield [6]. The main effect of ultrasound is acoustic cavitation, which causes cell rupture and increases the mass transfer of extractants. UAE is performed at relatively low temperatures, allowing for the preservation of thermolabile carotenoids. However, the optimized range of ultrasound intensity is a crucial parameter to obtain the highest yield of carotenoids without a significant increase in temperature causing damage to their structure [4]. In comparison to conventional techniques, UAE is considered to be more sensitive and selective. As carotenoids are intracellular molecules, their extraction is difficult; therefore, UAE should be combined with conventional methods, such as maceration and homogenization [6].

Although chemically synthesized carotenoids still dominate the market, this is starting to change, and considering the interest in green and sustainable industrial engineering, increasing efforts are being devoted to the search for natural sources and methods of carotenoid production. The challenge associated with the mentioned methods for mass production of carotenoids is that they are limited by low volume and high production costs, so there is an obvious need for an alternative production method, such as microbial biotechnological carotenoid biosynthesis. This alternative means of obtaining carotenoids has shown achieved improved yields with the possibility of using agro-industrial waste as cheap substrates and can directly affect the environmental and economic aspects of production. The product can also be obtained relatively quickly, at any time of the year [5], although pretreatments, such as cell lysis, are needed to extract the final product, as carotenoids present with intracellular metabolites [7].

The mentioned group of natural pigments can be produced by a diverse range of microorganisms, although not all are industrially or economically feasible. Therefore, microorganisms with carotenoid-producing characteristics, such as bacteria, archaea, algae, and fungi, are abundant in the ecosystem. Industrial production microorganisms include *Streptomyces chrestomyceticus*, *Blakeslea trispora*, *Phycomyces blakesleeanus*, *Flavobacterium* sp., *Phaffia* sp., *Actinomycetes*, and *Rhodotorula* sp. [8]. In recent years, microalgae have been used as a source for industrial carotenoid production, although there are other promising candidates. For example, yeasts have the potential to produce significant amounts of carotenoids, such as lycopene, β-carotene, astaxanthin, torulene, torularhodin, etc., through fermentation using numerous agricultural products and byproducts. They are mainly represented by the genera *Rhodotorula* sp., *Rhodosporidium* sp., *Sporobolomyces* sp., and *Xanthophylomyces* sp. [9]. These yeast species grow as pigmented colonies, which is why they are known as "red yeasts". Red yeasts have shown good potential as biocatalysts due to their biotransformation of carbon sources into a variety of primary and secondary metabolites. As such, species belonging to genus *Rhodotorula*, among others, have piqued the interest of many researchers with respect to carotenoid production. Their tolerance to inhibitory compounds occurring in natural substrates and high yields of various carotenoids, combined with still quite unexplored metabolic pathways, motivates a search for innovative biotechnological production of carotenoids [2].

In this research, yeasts isolated from natural sources were identified and characterized, and the most potent representative bioagent for carotenoid production was selected based on the widest range of biochemical and physiological characteristics. The main aim of the research was to establish an upgraded carotenoid extraction protocol, which implies testing four different methods for cell lysis (hydrochloric acid treatment, ultrasound treatment, milling treatment, and osmotic pressure treatment), three extraction methods (conventional extraction, ultrasound extraction, and conventional + ultrasound extraction), and three

extraction solvents (acetone, isopropanol/methanol (50:50), and ethanol). The obtained results were modelled by an artificial neural network and the defined best protocol for carotenoid extraction from yeast cells was further verified based on its universality for all other yeast strains from the *Rhodotorula* genus.

The innovation of this study is reflected in application of advanced mathematical tools for simplification and rapid evaluation of different experimental steps to obtain the best-performing yeast strain for carotenoid production, optimization of carotenoid extraction protocol, and validation of the universality of the selected extraction operating parameters. In addition to the main advantages of the proposed study, the following steps provide an improved understanding of the differences between the tested extraction protocols for carotenoid-producing yeast cells and utilization of time, energy, and other sources in a microbiological laboratory, representing the first step in further scale-up and utilization of alternative conditions for biotechnological production of carotenoids.

2. Materials and Methods

2.1. Yeast Isolates and Their Characteristics

Isolation was performed at the Laboratory for Testing Food Products (Department of Microbiological Analysis), Faculty of Technology, Novi Sad. Red yeast originates from food-related sources (i.e., food samples, food byproducts, agricultural soil from crops, etc.). In order to isolate red-pigmentated yeasts, a total of more than 100 samples were tested, which can be dived in the following groups: fresh fruit and vegetables (apples, cherries, cucumbers, Jerusalem artichokes, carrots, beets, tomatoes, cherry tomatoes, peppers, potatoes, grapefruits, lettuces, etc.), processed fruits (figs, plums, apricots, pineapples, watermelons, mangos, bananas, oranges, etc.), dairy products (butter, cheese, natural and processed cheese products, yogurt, sour cream, dips, ice cream, frozen desserts made with dairy ingredients, milk powder, etc.), juices and sugar-rich solutions (orange juice, apple juice, carrot juice, beet juice, cherry juice, elder juice, tomato juice, sugar cane, dextrose solutions, high-fructose corn syrup, malt syrup, etc.), and agricultural soil. All food samples were subjected to conventional microbiological analysis for isolation and determination of yeast and aliquots of the processed sample streaked and incubated on Sabouraud maltose agar (SMA) (HiMedia, Mumbai, India). Briefly, a series of dilutions was prepared for each sample, and aliquots were streaked on the mentioned nutrient agar. After incubation (48 h at 30 °C), all pure colonies with the targeted macromorphology (orange or red color) were selected, and the pure red-pigmented cultures were obtained using a new batch of sterile nutrient agar plates.

In order to identify selected red yeast isolates, an API 20C AUX yeast identification system (BioMérieux, Marcy-l'Étoile, Lyon, France) was used according to the manufacturer's recommendation and scientific literature [10–12].

For permanent storage, the selected red-pigmented yeast strains were deposited in the Collection of Microorganisms at the Faculty of Technology, University of Novi Sad, and stored in a deep freezer at −80 °C (with addition of glycerol as cryoprotectant). Prior to use, cultures were incubated in SMA medium at 30 °C for 48 h.

The growth curve was determined during incubation of all yeast isolates in Sabouraud maltose broth (HiMedia, Mumbai, India) for 216 h. The initial concentration of cells was about 4 log CFU/mL (obtained after overnight incubation and preparation of a dilation series) in the nutrient medium, with sampling at 0 h and every 12 h during the first 3 days and every 24 h until the end of incubation period at 30 °C. The total number of living yeast cells (after biomass collection) was determined by the plate counting method using SMA as a nutrient medium. Cell number is expressed as log CFU. In addition, kinetic modelling was performed to determine and predict yeast behavior during incubation (see Section 2.6.1).

In order to define biokinetic zones for growth, yeast isolates were incubated on a solid YPD medium at various temperatures (20, 30, 37, and 44 °C) and in a liquid YPD medium with different pH values (3, 5, 7, and 9) for 120 h with visual observation of growth every

24 h. The absence of growth was categorized as a negative result (0), whereas the presence of growth was categorized as either low (1), medium (2), or high (3). Non-inoculated media were used as controls (blanks). Mathematical analysis was performed to interpret the obtained results according to the methodology reported by Šovljanski et al. [13] (see Section 2.6.2).

The obtained results of assimilation of different carbon sources during API identification were additionally used for mathematical ranking of yeast strains (see Section 2.6.3). For this step of selection of the most improved yeast isolate, isolates were additionally tested in terms of extracellular enzyme production, which can be decisive during further biowaste fermentation. Briefly, lipase activity was detected in tributyrin agar medium (48 h at 30 °C), and a positive reaction was indicated by a halo zone around the colony. The protease activity was tested using skim-milk-supplemented nutrient agar (HiMedia, Mumbai, India) during 48 h of incubation at 30 °C. For both mentioned tests, a positive reaction is indicated by a halo zone around colonies. Cellulase activity was observed after incubation for 3 days at 30 °C on YPD with 20 g L^{-1} carboxymethylcellulose (CMC, Sigma Aldrich, St. Louis, MO, USA). YPD medium contains 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 10 g L^{-1} glucose. A positive reaction is indicated by a halo zone following Congo red staining.

2.2. Biomass Production

Yeast cells obtained by multiplying a pure laboratory culture of the mentioned red yeast isolates were used to obtain biomass. After the first cell passage (SMA, 30 °C for 72 h), the obtained biomass was used for the second passage in YP nutrient medium (20 g L^{-1} yeast extract, 10 g L^{-1} glucose, and 10 g L^{-1} peptone). The mentioned nutrient medium and conditions were selected for simultaneous production of yeast biomass and carotenoids according to the recommendation of multiple scientific groups [14–18]. Incubation was performed at 30 °C for 120 h hours in the dark. The obtained biomass was collected and centrifuged twice on an ultracentrifuge at 6000 rpm for 10 min (ROTINA 380R, Hettich, Tuttlingen, Municipality, Germany). Between centrifugations, the supernatant was discarded, and a pellet was lyophilized by a laboratory lyophilizator (Alpha 2–4 LSC, Martin Christ, Osterode, Germany) for 28 h.

2.3. Experimental Design for Extraction of Total Carotenoids from Yeast Cells

In order to define an improved protocol for extraction of total carotenoids from yeast biomass, the experimental design involved three qualitative variables: a method for cell lysis (X1), a carotenoid extraction method (X2), and a solvent extraction method(X3). According to the principle of varying the variables relative to one another, the experimental design involved:

- Methods for cell lysis: chemical (hydrochloric acid treatment and sodium chloride treatment) and mechanical (ultrasound wave treatment and milling) methods;
- Carotenoid extraction methods: conventional extraction (CE), ultrasound extraction (USE), and a combination of conventional and ultrasound extraction (CUSE); and
- Solvent extraction methods: acetone, isopropanol: methanol (50:50), and ethanol extraction.

Carotenoid yield was determined as the output of the experimental procedure using the spectrophotometric method [19] with microtiter plates. Briefly, 200 µL sample aliquots were applied in wells, and absorbances were immediately analyzed at wavelengths of 663, 645, 505, and 453 mm (with the application of an extraction solvent as a blank). Carotenoid yield (mg/100 mg d.w.) was determined based on Equation (1). All analyses were performed in triplicate, and the obtained results are presented as mean values with standard deviations.

$$\text{Carotenoid yield} = 0.216 \cdot A_{663} - 1.22 \cdot A_{654} - 0.304 \cdot A_{505} + 0.452 \cdot A_{453} \quad (1)$$

2.4. Methods for Cell Lysis

Cell lysis by hydrochloric acid was performed using a 4 M acid solution. The biomass-to-acid solution ratio was 1:5, and the mix was subjected to agitation at 150 rpm for 5 min at 35 °C. Cell lysis by ultrasound was performed done using a T 25 ULTRA-TURRAX instrument (IKA Werke, Staufen im Breisgau, Germany). Briefly, 60 g of biomass and 300 mL of NaCl solution were subjected to 4 ultrasonic cycles (50 Hz, 11,000 rpm, 20 °C) for 10 min in dark glass. Cell lysis by osmotic pressure treatment was performed with sodium chloride solution in a 1:5 ratio. The mixture was subjected to a temperature of 40 °C for 24 h. For all three techniques, the obtained precipitate after the centrifugation process (10,000 rpm, 10 min) was double-washed using distilled water to eliminate chemical residues. For milling treatment, biomass was subjected to grinding (1 min, 5000 rpm) with a batch mill tube mill control system (LLG, Meckenheim, Germany).

2.5. Methods for Carotenoid Extraction

The extraction process was performed with conventional and ultrasound methods, as well as a combination of conventional and ultrasound methods. These methods were chosen due to their differing mechanisms for extraction of targeted carotenoids, as well as the possibility of preventing the loss of the bioactivity of the targeted compounds. The conventional method is commonly used for evaluation of other methods; however, with a very high recovery rate of carotenoids, it can be time-consuming costly, using a large amount of solvent [4]. On the other hand, ultrasound, as a greener method, which includes an acoustic cavitation base for extraction, can be applied for many processes, including intracellular metabolite extraction, as well as microbial inactivation [4]. Three solvents were used for each extraction method, as previously mentioned. Briefly, conventional extraction (CE) involved the addition of the solvent in a ratio of 1:2.5 (m/V) and mixture in a vortex for 2 min. After the addition of the same ratio of sample: solvent as for conventional extraction, ultrasound extraction (USE) was performed in an ultrasound bath for 20 min. Consequently, the combination of conventional and ultrasound extractions (CUSE) involved, first, the use of a vortex and then an ultrasonic bath procedure. Following the extraction process, all samples were centrifugated at 8000 rpm for 5 min, and supernatants were separated for further analysis.

In addition to experience with extraction technologies, we followed the recommendations of Mata-Gomez et al. [20], Lopez et al. [21], Michelon et al. [22], and Nemer et al. [23] for the selection and formation of the presented cell lysis and carotenoid extraction methods.

2.6. Statistical Analysis

2.6.1. Growth Kinetics Modelling

In this investigation, time-kill kinetics modelling was implemented by applying the four-parameter sigmoidal computational model, which is convenient for biological systems and described in detail by Brlek et al. [24]. The projected data should be shaped in an S-shaped curve, and the model can be expressed in the form of Equation (2).

$$y(t) = d + \frac{a - d}{1 + \left(\frac{t}{c}\right)^b} \quad (2)$$

The yeast isolate number (log CFU/mL) during incubation (hours) is denoted as $y(t)$, whereas the regression coefficients are denoted as follows: a, minimum of the experimentally obtained values (at t = 0); d, the maximally acquired value (at $t = \infty$); c, the inflection point (the point between a and d); and b, the Hill's slope (the steepness of the inflection point (c)).

2.6.2. Cluster Analysis of Temperature and pH Growth Profiles of Yeast Isolates

To predict the distribution of yeast isolates as a function of environmental parameters, a cluster analysis was performed. Cluster analysis (CA) was used to determine the possible

correlations among different yeast isolates and to classify objects according to temperature and pH growth profiles of selected yeast isolates. The complete linkage algorithm and city block (Manhattan) distances were used to explain the temperature and pH growth profile of the natural isolates grouped it in different clusters.

2.6.3. Ranking Procedure

Yeast isolates were ranked based on their assimilation/enzyme production capabilities [25], which were tested in the primary step of this study (see Section 2.1). The obtained scores of yeast isolates for utilization of different C and N sources were ranked in the following order: D-glucose, glycerol, calcium, 2-keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-α-D-glucopyranoside, N-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-sucrose, D-trehalose, D-melesitosis, and D-raffinose. With respect to the production of different enzymes, the isolates were ranked in the following order: protease, lipase, and cellulase. A negative reaction was coded as "0", whereas a positive reaction of the yeast isolates was coded as "1". Several quality parameters were calculated within the standard score analysis for each yeast isolate: the sum of utilization scores, the sum of production scores, the total sum of scores, the number of positive reactions, and the number of negative reactions.

2.6.4. Artificial Neural Network (ANN) Optimization

A multilayer perceptron model (MLP) with three layers was used for modelling. This model construction was proven accurate in approximating non-linear functions [26,27]. The experimental database for ANN was randomly divided into training, cross-validation, and testing data (60%, 20%, and 20% of experimental data, respectively). The Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm was applied as an iterative method to solve unconstrained non-linear optimization during ANN modelling. The number of repeating steps during ANN construction was 100,000. Coefficients associated with the hidden and output layer (weights and biases) were grouped in matrices W_1 and B_1 and W_2 and B_2, respectively, as represented in Equation (3).

$$Y = f_1(W_2 \cdot f_2(W_1 \cdot X + B_1) + B_2) \tag{3}$$

Weight coefficients were determined during the ANN learning cycle to minimize the error between network results and experimental values [28,29]. The developed models were numerically verified using reduced chi-square (χ^2), root mean square error (RMSE), coefficient of determination (r^2), mean bias error (MBE), and mean percentage error (MPE). These parameters can be calculated using Equations (4)–(7).

$$\chi^2 = \frac{\sum_{i=1}^{N} (x_{\exp,i} - x_{pre,i})^2}{N - n} \tag{4}$$

$$RMSE = \left[\frac{1}{N} \cdot \sum_{i=1}^{N} (x_{pre,i} - x_{\exp,i})^2 \right]^{1/2} \tag{5}$$

$$MBE = \frac{1}{N} \cdot \sum_{i=1}^{N} (x_{pre,i} - x_{\exp,i}) \tag{6}$$

$$MPE = \frac{100}{N} \cdot \sum_{i=1}^{N} \left(\frac{\left| x_{pre,i} - x_{\exp,i} \right|}{x_{\exp,i}} \right) \tag{7}$$

where $x_{\exp,i}$ represents the experimental values, and $x_{pre,i}$ represents the predicted values obtained by calculating the model for these measurements. N and n are the number of observations and constants, respectively.

2.7. Experimental Validation of the Universality of the Chosen Carotenoid Extraction Procedure

The chosen extraction procedure was experimentally verification in a microbiology laboratory in order to demonstrate its accuracy. The best experimental procedure was tested for all other yeast strains investigated in this study, and the obtained results were compared with the best-performing yeast isolate (based on the ranking procedure described in Section 2.6.3). All yeasts were grown under the same conditions, as described in Section 2.2.

3. Results and Discussion

Yeasts were isolated from food and food-related samples, and the obtained average number of yeast in samples was between 34 and 117 CFU/g. According to the targeted macromorphology (orange–red-pigmented colonies), the total number of potential carotenoid-producing isolates was five. All five isolates were obtained from the different samples (Jerusalem artichoke, tomato juice, sugar cane, agricultural soil, and yogurt) and designated as top 30, KV1105, CRV, 4/34, and FK3, respectively. Their macromorphology after 72 h of incubation at 30 °C is shown in Figure 1. The obtained colonies have a typical orange color, but size, shape, surface appearance, and texture differ the strain level. Therefore, the isolates were identified with an API identification system. Four isolates represent *Rhodotorula mucilaginosa* strains (top 30, KV1105, CRV, and 4_34), whereas only one is *Rhodotorula glutinis* (strain FK3). The *Rhodotorula* genus represents comprises environmental basidiomycetous yeasts, and species that are commonly isolated from soil, as well as fermented food, fruit juice, milk, and food waste [30].

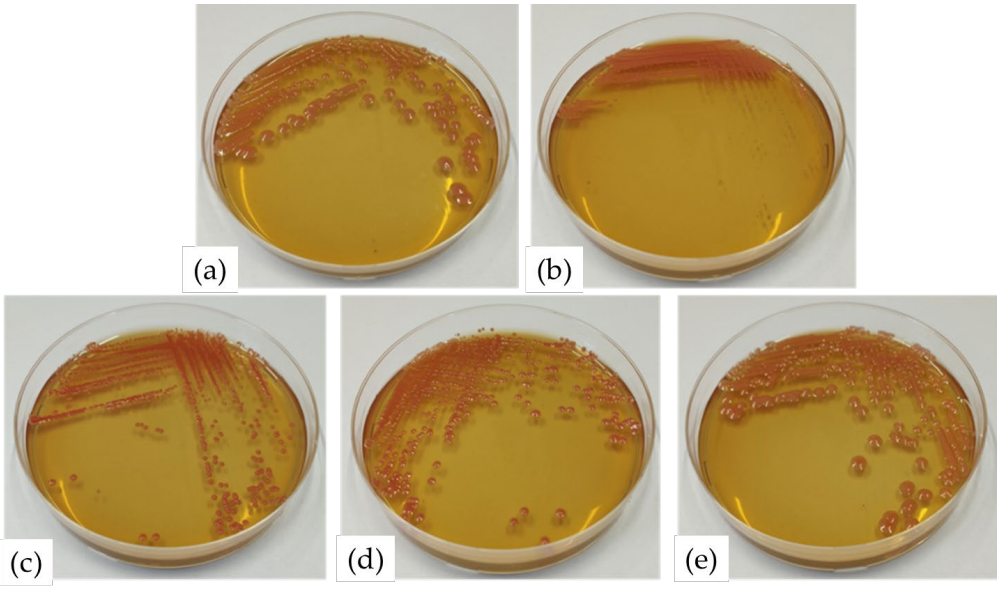

Figure 1. *Rhodotorula* isolates on a solid growth medium after 72 h of incubation: (**a**) top 30; (**b**) KV1105; (**c**) CRV; (**d**) 4_34; (**e**) FK3.

However, the significance of *R. mucilaginosa* and *R. glutinis* lies in their ability to produce carotenoids, which has been described in numerous literature reports. Briefly, representatives of this genus are capable of synthesizing carotenoids, such as β-carotene, astaxanthin, torulene, and torularhodin [30–32]. Their colonies vary from coral red/salmon to slightly orange in color (depending on the nutrient medium and incubation conditions), with mucoid to slightly tough, smooth to wrinkled, or highly glossy to semi-glossy surface texture [33], which is in agreement with the colonies observed in this study (Figure 1). Figure 1a–d depicts the macromorphological details of *R. muciliginosa* strains, whereas Figure 1e correspond to the only *R. glutinis* strain isolated in this study.

As the next experimental step, the growth curve (growth as a function of incubation time) was determined for all selected *Rhodotorula* isolates, as shown in Figure 2.

Dyaa et al. [34] reported that the optimal temperature for carotenoid production in *Rhodotorula* strains is around 30 °C, and the carotenoid rate was reduced at higher temperatures. Therefore, growth was monitored at 30 °C. During biomass determination (Figure 2), the cells multiplied intensively for all isolates, following the sigmoidal shape of the curve.

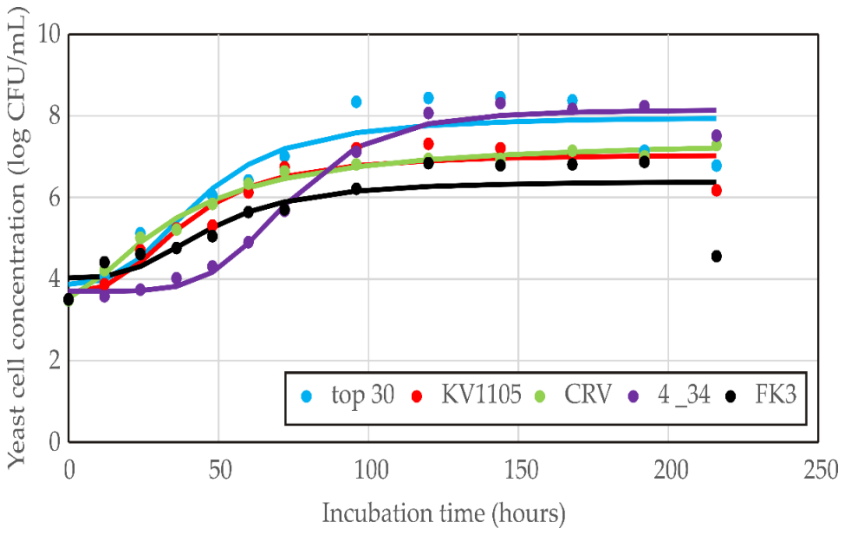

Figure 2. Growth curves for *Rhodotorula* isolates (markers signify the experimental data; lines indicate predictive results).

A growth adjustment phase (lag phase) was observed during the first 12 h. A moment of transition from the exponential phase (log phase) to the stationary growth phase was observed after 4 days (96 h) for all strains, except for *R. glutinis* strain 4_34, for which the starting point of the stationary phase was observed after approximately 120 h. According to Figure 2, the highest cell concentration (approx. 8 log CFU/mL) was achieved for the strains *R. glutinis* 4_34 and *R. mucilaginosa* top 30. In the case of all other examined yeasts, the maximum cell concentration was 1–2 log CFU/mL lower.

To better understand the growth profile of the tested yeasts, a growth kinetic model was developed. In Figure 2, markers indicate the experimentally obtained values of cell concentration at a given point in time, whereas lines indicate predictive results of the developed growth kinetic model. According to the regression coefficients of the obtained models for yeast isolate number during incubation (Table 1) and the goodness of fit between experimentally and model-obtained (predicted) results (Table 2), it can be concluded that the developed models for yeast growth were accurate, with high coefficients of determination (up to 0.989) and proposed models that fit well with experimental data. It can be concluded that the obtained kinetic models for the growth of yeast isolates can be used for the prediction of yeast isolate number during the whole incubation period.

Table 1. Regression coefficients for kinetic models of the growth of yeast isolates.

Regression Coefficient	Yeast Isolate				
	Top 30	**KV1105**	**CRV**	**4_34**	**FK3**
d	7.977	7.059	7.421	8.152	6.393
a	3.872	3.670	3.525	3.698	4.021
c	43.335	38.766	35.446	73.525	46.521
b	2.829	2.654	1.571	5.041	3.016

Table 2. "Goodness of fit" of the kinetic models for the growth of yeast isolates.

Parameter	Yeast Isolate				
	Top 30	**KV1105**	**CRV**	**4_34**	**FK3**
Reduced chi-square (χ^2)	0.362	0.135	0.017	0.060	0.406
Root mean square error (RMSE)	0.578	0.353	0.124	0.235	0.612
Mean bias error (MBE)	0.000	0.000	0.000	0.000	0.000
Mean percentage error (MPE)	7.551	4.586	1.791	3.099	8.242
Coefficient of determination (r^2)	0.872	0.922	0.989	0.985	0.680
Skewness (Skew)	−0.382	−1.127	−0.863	−1.296	−2.090
Kurtosis (Kurt)	−0.712	1.240	0.514	2.523	5.373
Mean of residuals (Mean)	0.000	0.000	0.000	0.000	0.000
Standard deviation of residuals (StDev)	0.601	0.368	0.129	0.245	0.637
Variance of residuals (Var)	0.362	0.135	0.017	0.060	0.406

The next steps involved a comprehensive study of biokinetic zones with respect to temperature and pH values for the growth of yeasts. The elementary goal of all in vitro bioprocesses is to create an environment that, for each set of parameters, simulates the optimal and specific environment for initiation of production of the desired metabolite. The growth of microorganisms is a function of environmental factors, of which the most significant are the temperature and pH values of the environment. Temperature and pH are the most important environmental factors influencing the growth of microorganisms [35]. Temperature tolerance is an essential parameter for biotechnology processes due to significant changes in this factor [13,36]. Therefore, it is imperative to investigate the behavior of natural isolates at varying incubation temperatures. On the other hand, a similar dependence can be observed for pH values as one of the most influential factors with respect to the growth of yeasts [37]. Yeasts exhibiting pH tolerance are considered potentially beneficial candidates for efficient biotechnology production, whereas most yeasts as bioagents show high metabolic activity in neutral or acidic environments. Therefore, it is necessary to evaluate the effect of pH values on yeast growth in a wide range (between 3 and 9) [38]. To determine the optimal growth rate, biomass production was monitored for 5 days at temperatures of 20, 30, 37, and 44 °C and pH values of 3, 5, 7, and 9. A summary statistical analysis of growth tolerance at different incubation temperatures and pH values is presented in Supplementary Table S1. For most of the selected strains, the optimal growth temperature is between 20 and 30 °C. On the other hand, a temperature of 44 °C is beyond the biokinetic zone for all strains. The majority of *R. mucilaginosa* and *R. glutinis* strains are considered mesophilic [32], in agreement the results obtained in this study. The optimal pH value for the growth of *Rhodotorula* strains is 7, although growth can occur in a more expansive biokinetic zone (pH value range of 3–7) after the total incubation time for all isolates. In terms of the production of carotenoids by *Rhodotorula* representatives, many researchers have indicated that a pH value of 7 is optimal not only for growth but also for carotenoid production [39,40].

We further analyzed the behavioral similarity between natural yeast isolates by cluster analysis of temperature and pH growth. According to results presented in Supplementary Table S1, two dendrograms of temperature and pH growth profiles of yeast isolates are plotted in Figure 3, using complete linkage as an amalgamation rule and a city block (Manhattan) distance as a measure of the proximity between yeast isolates. Two dendrograms based on the growth profile over time showed a proper distinction between yeast isolates. According to the temperature profile, yeast isolates *R. mucilaginosa* top 30 and *R. glutinis* 4_34 showed a similar growth profile, as did yeast isolates *R. mucilaginosa* CRV and *R. mucilaginosa* FK3. Based on the pH profile, yeast isolates *R. mucilaginosa* top 30 was similar to *R. mucilaginosa* FK3, whereas sample *R. glutinis* 4_34 corresponded most closely

to the *R. mucilaginosa* CRV isolate. As shown in Figure 3, the *R. mucilaginosa* KV1105 strain is the least similar to the other tested strains in both cases.

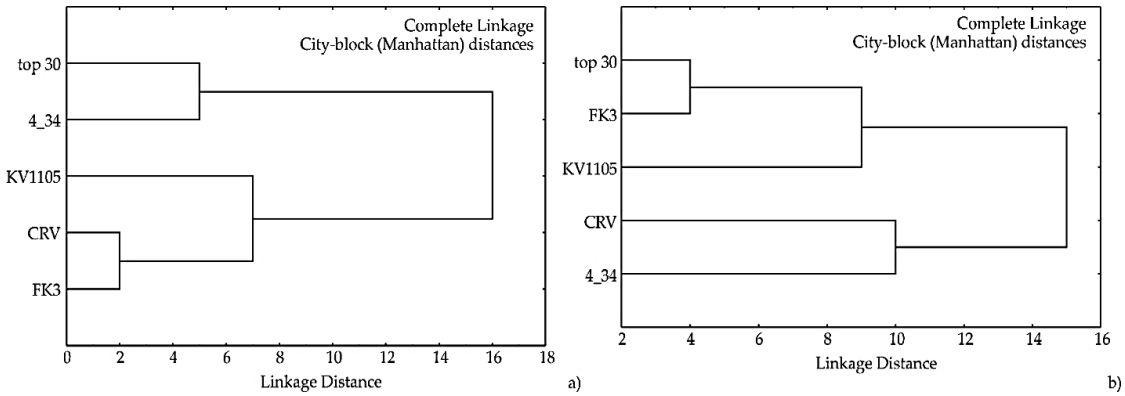

Figure 3. Dendrograms of cluster analysis of (**a**) temperature and (**b**) pH growth profiles of yeast isolates.

For further selection of the best-performing yeast isolate with respect to application in engineered bioprocesses, the assimilation of different C and N sources, as well as enzyme production, was tested (Figure 4). The data are indicated as positive reactions (green circle) or negative reactions (red circle). Interestingly, the tested yeast isolates do not utilize inositol, methyl-α-D-glucopyranoside, N-acetyl-glucosamine, or D-lactose. It has been proven that inositol has a positive effect on fermentation performance, cell growth, and tolerance to negative environmental conditions in the case of *Saccharomyces cerevisiae* [41], but the tested strains in this study remain neutral to this source, as was the case for strains M14, M22, M23, and M24 in a study by Allahkarami et al. [42]. The negative response of the tested strains was also confirmed in the case of methyl-α-D-glucopyranoside. Taking into account that methyl-α-D-glucopyranoside is usually added to the production medium as an inducer of amylases in microorganisms [43], it can be concluded that the yeasts used in the present study do not have the ability to produce amylase. The same negative results were obtained for N-acetyl-glucosamine and D-lactose, indicating that these sources of C and N atoms cannot be utilized by the tested yeasts. On the contrary, according to Figure 4, there are three common sources of C atoms: D-glucose, D-sucrose, and D-raffinose, all of which were found to be fermented by all tested yeast strains.

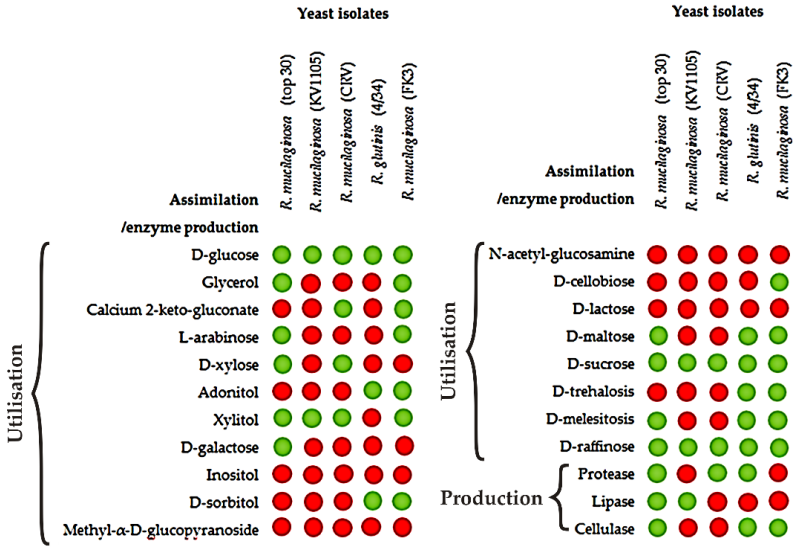

Figure 4. Biochemical and physiological tests (red color indicates a negative result, whereas green color indicates a positive result).

In order to determine the best-performing strain, tested yeasts were statistically ranked based on the mentioned characteristics presented in Figure 4. We evaluated the response of the yeast strains to the assimilation of different C and N sources, as well as enzyme production. In yeast biotechnology, it is believed that wild isolates with high fermentation capabilities in terms of different sources of C and N atoms are a potentially suitable choice for biotechnological processes at the industrial level [44]. The ranking procedure was performed as a method of selection based on the maximum of defined functions (the sum of utilization scores, the sum of production scores, the total sum of scores, and the sum of utilization scores), representing the optimal processing parameters. If the value of the specific function reaches its maximum for a specific yeast isolate, it shows indicates the tendency of the tested parameters to be optimal.

The ranking procedure was based on the discrimination criteria described in Figure 4, and the ranking results are presented in Table 3. According to the ranking procedure, yeast isolate *R. mucilaginosa* top 30 achieved the best performance, showing the highest number of positive reactions (13) and the fewest negative reactions (9). Positive reactions for yeast isolate *R. mucilaginosa* top 30 were observed for D-glucose, glycerol, L-arabinose, D-xylose, xylitol, D-galactose, D-maltose, D-sucrose, D-melesitosis, and D-raffinose assimilation, as well as protease, lipase, and cellulase production. Similar results were obtained for isolate *R. glutinis* 4_34 (which showed 11 positive and 11 negative reactions) and isolate *R. mucilaginosa* FK3 (which showed 12 positive and 10 negative reactions). The scoring of yeast isolates coincides with the cluster analysis (Figure 3).

Table 3. Ranking procedure results.

Yeast Isolate	Sum of Utilization Scores	Sum of Production Scores	Total Sum of Scores	Number of Positive Reactions	Number of Negative Reactions
top 30	10	3	13	13	9
KV1105	4	1	5	5	17
CRV	6	1	7	7	15
4_34	9	2	11	11	11
FK3	11	1	12	12	10

Therefore, *R. mucilaginosa* top 30 was selected as the best-performing yeast for further biotechnological application and used for the experimental design to establish a carotenoid extraction procedure. In this study, 36 cell lysis method–extraction method–solvent combinations were tested to determine the maximum carotenoid yield (Figure 5). Additionally, ANN modelling (Figure 6 and Table 4) was performed to verify the best extraction procedure according to the predicted maximum.

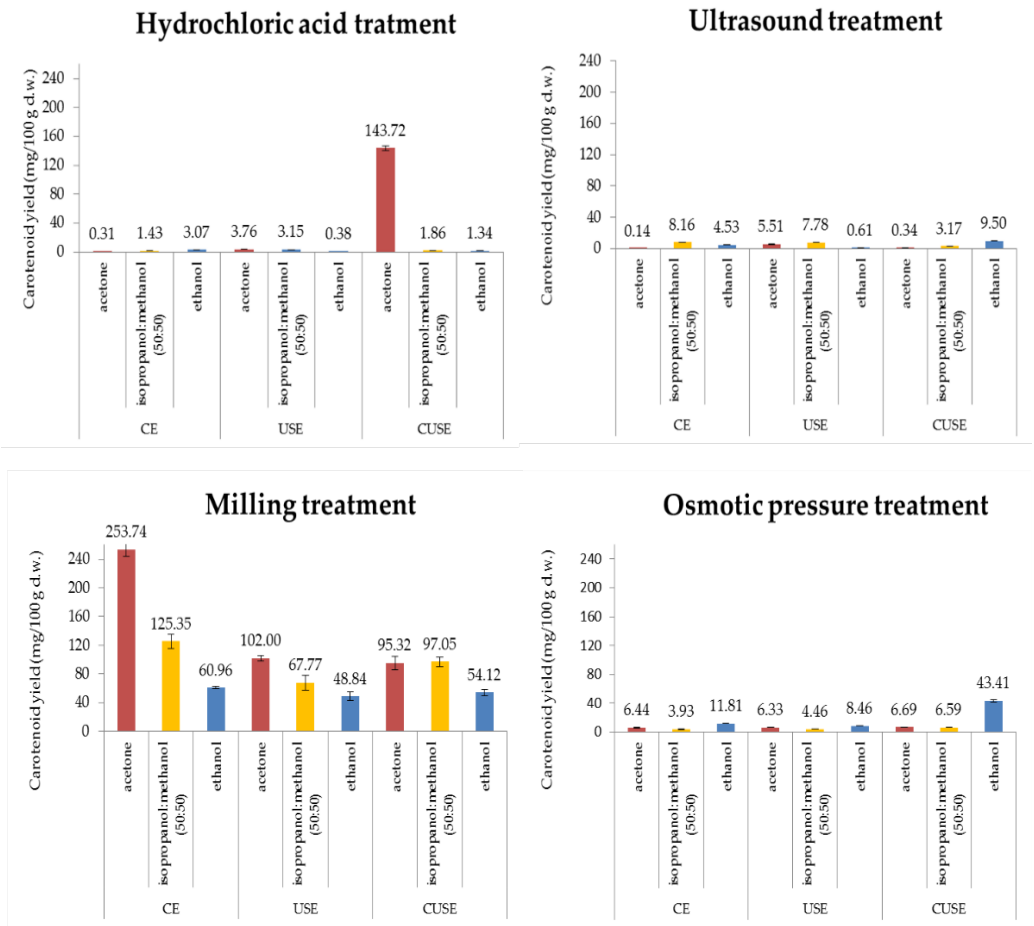

Figure 5. The obtained carotenoid yield (mg/100 g d.w.) using different carotenoid extraction procedures (acetone—red colour; isopropanol:methanol (50:50)—yellow colour; ethanol—blue colour).

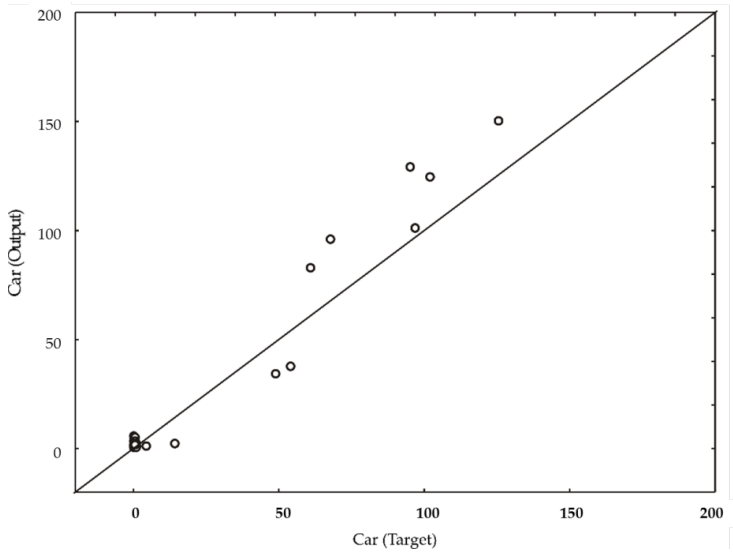

Figure 6. Experimental and predicted values of carotenoid yield for the *R. mucilaginosa* top 30 strain.

Prior to extraction procedure, the obtained biomass was lyophilized. According to Saini and Keum [4], this step enables a high water content in the system (from yeast cells). Because a water environment can be unfavorable for efficient extraction of carotenoids due to their hydrophobic nature, dehydration of samples is an efficient step in yield improve-

ment. Additionally, thermal degradation and isomerization of some dehydration process can be harmful to carotenoids; thus, lyophilization is the most adequate pretreatment for carotenoid extraction.

The experimentally obtained and calculated maximum for carotenoid yield was 253.74 mg/100 g d.w. The optimal output was obtained using milling treatment as a method for cell lysis, conventional extraction as the extraction method, and acetone as the extraction solvent. Cell disruption might be the key step in the production and purification of intracellular compounds from yeast cells, as it exerts important effects on their recovery and quality [45,46], as well as for analytical aims. Owing to its large size and varying cell wall structure, disruption of the ascomycetous yeast cell wall is generally easier than that of other microorganisms, such as bacteria. Various methods, such as mechanical, chemical, or enzymatic approaches, can be used to disrupt cell walls; the optimal method depends on both the yeast characteristics (culture time, specific growth rate, etc.) and the target substance [47–49].

As demonstrated by the results presented in Figure 5, among all chosen methods for cell lysis applied in the present study, the milling treatment showed a significantly higher carotenoid yield than the other methods. This mechanical treatment coupled with conventional extraction with acetone as the extraction solvent led to the highest carotenoid yield, i.e., 253.74 ± 9.74 mg/100 g d.w. According to Nemer et al. [23], a significant advantage of mechanical cell disruption techniques is that they can generally be scaled-up with relative ease, in addition to being quite effective. This is a crucial step for industrial applications, but special attention needs to be directed to potential heating during the process, as well as carotenoid degradation. Aksu and Eren [50] used a milling process and acetone-dependent extraction (specific procedure data not available) to obtain carotenoids from *Rhodotorula mucilaginosa* cells, achieving a yield of 69.8 mg L^{-1}, which is lower than that achieved in the present study.

In the case of all other tested methods for cell lysis, the carotenoid content was 10–1000 times lower in comparison to the milling treatment. In the present study, the conventional extraction method achieved better results than ultrasonic extraction and the combination of conventional and ultrasound extraction methods, possibly because during the ultrasound treatment, the temperature usually increased, and heat shocks may have a negative influence on carotenoid content [51]. The answer to this hypothesis might be a further crucial moment for optimizing the extraction time and combining classic and modern methods of extraction. Methods for cell lysis for the extraction of pigments in the available literature are divided between ultrasound and milling treatments, emphasizing that the obtained results may vary depending on the culture type, pigment, and experimental parameters [23,49]. Owing to differences in experimental setups and the used yeast cultures, it is impossible to compare results between studies. It is additionally difficult to compare the results reported in the literature owing to the fact that different commercial and alternative substrates with complex compositions are used for yeast cultivation, which significantly affects the yield of carotenoids.

As previously mentioned, ANN modelling was also performed for analysis of carotenoid yield during the tested extraction procedures, with all three categorical variables for ANN contributing to the anticipation of carotenoid content:

1. Cell lysis methods (hydrochloric acid treatment, ultrasound treatment, milling treatment, and osmotic pressure treatment);
2. Extraction methods (conventional extraction, ultrasound extraction, conventional + ultrasound extraction); and
3. Solvent extraction methods (acetone, isopropanol/methanol (50:50), and ethanol extraction).

The ANN technique proved to be a beneficial method for prediction of the yield of different microbiological products [51,52], but it has not been used to date for the selection of a total carotenoid extraction protocol based on the highest carotenoid yield from yeast cells. For the first time, the obtained results of different extraction protocols were involved

in this advanced mathematical analysis, and the optimal number of neurons in the hidden layer was four (network MLP 10-4-1) to obtain high values of coefficients of determination (overall r^2 is 0.86) and low values of the sum of squares (SOS). The obtained r^2 values for training, testing, and validation sequences of ANN modeling were 0.94, 0.98, and 1.00, respectively. BHGS 6 was used as a training algorithm, and the error function was SOS (sum of squares). The optimal hidden and output layer activation function was the logistic function. An ANN model was used to predict experimental variables reasonably well for a broad range of process variables (as shown in Figure 5, in which the experimentally measured and ANN-model-predicted values are presented). The accuracy of the ANN model was visually assessed by the dispersion of points in the diagonal line shown in the graphics presented in Figure 6.

The predicted values were very close to the desired values in most cases in terms of the value of the coefficient of determination [29]. The qualities of the model fit were tested in Table 4; the coefficient of determination (r^2) should be close to 1, whereas the values of other tests (χ^2, RMSE, MBE, and MPE) should be lower to achieve a good fit with the experimental values [53,54]. The obtained results indicate the possibility of using the ANN model in the presented experimental setup. This is in agreement with the results reported by Shafi et al. [55], who emphasized that using ANN modelling for analysis of natural sources of bioactive compounds is more flexible and accurate than the response surface methodology.

Table 4. "Goodness-of-fit" kinetics models for the yeast isolate number.

Yeast Isolate	χ^2	RMSE	MBE	MPE	r^2
***R. mucilaginosa* top 30**	3.1×10^4	17.49	−13.27	14.29	0.89
	Skew	**Kurt**	**Mean**	**StDev**	**Var**
	2.22	11.62	−13.27	17.68	3.1×10^4

Legend: Reduced chi-square (χ^2), root mean square error (RMSE), mean bias error (MBE), mean percentage error (MPE), coefficient of determination (r^2), skewness (Skew), kurtosis (Kurt), mean of residuals (Mean), standard deviation of residuals (StDev), variance of residuals (Var).

In order to reduce the time and reagents required for analysis, the universality of the milling treatment coupled with acetone as an extraction solvent using a conventional extraction procedure was validated for all other yeast isolates in terms of comparative achieved carotenoid yield. A simple, rapid, universally applicable, and reproducible procedure is indispensable in order to reduce laboratory time and cost. This is important for studies involving multiple isolates. Furthermore, spectrophotometric analysis of carotenoids was selected as the output. This method is among the cheapest and most useful methods for quantification of carotenoids [56]. Within the field of yeast biotechnology, experimental validation of the universality of some processes or procedures in a laboratory does not rely on the extensive use of expensive resources, but the obtained data confer multiplied benefits for further applications of bioagents in semi-industrial and industrial processing. The results presented in Figure 7 show that for all tested yeast strains, the accomplished carotenoid yield was significant (above 227 mg/100 g d.w.) under the same extraction conditions compared to *R. mucilaginosa* top 30. Slight variations in the carotenoid content were observed between strains, which positively correlated with the diversity in the results obtained in terms of the ranking of yeast isolates (Table 4.). Nevertheless, in this experiment, a similar carotenoid content was recorded across all tested yeast strains when the milling treatment was applied in combination with conventional extraction with acetone as the extraction solvent; therefore, the universality of the best extraction procedure in this study is acknowledged.

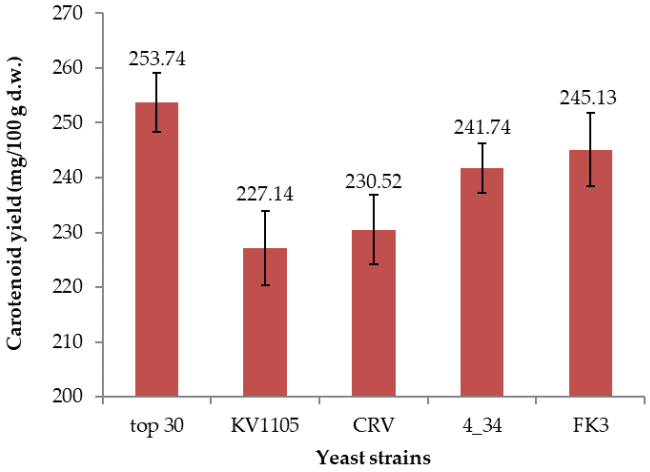

Figure 7. Experimental validation of the universality of the chosen carotenoid extraction procedure for all *Rhotodorula* strains.

Based on the results of the present study, it can be concluded that beneficial effects of carotenoids on human health and their widespread application in various industries have led to increased interest in biotechnological study of these substances. This work can contributes to current research oriented toward the identification, quantification, and optimization of microbial carotenoid production. Various possibilities for the isolation of carotenoid-producing yeasts and multiple sets of extraction procedures should be further explored in order to define the biological activity of effective substances in yeast resources for utilization in the biotechnological field. One such approach involves the use of advanced mathematical tools, as explored in the present study for the selection of yeast isolates by determination of growth characteristics, pH, and temperature profiles to optimize carotenoid production protocols and verify the universality of the selected procedures.

4. Conclusions

Following isolation and identification of five carotenoid-producing yeasts, the best-performing strain, identified as *Rhodotorula mucilaginosa* (strain name: top 30) was selected based on an advanced ranking procedure. Using this strain, we optimized an experimental design for carotenoid extraction. Based on the obtained maximal carotenoid yield (253.74 ± 9.74 mg/100 g d.w), this procedure, which involves milling treatment (for cell lysis) and conventional extraction with acetone, convincingly stood out compared to the other 35 tested protocols. Finally, we validated the universality of the optimized procedure.

Based on a statistical ranking of microorganisms, the universality of an experimental design to obtain an optimized procedure for carotenoid extraction from yeast cells was validated. This method represents an economically feasible calculation approach to an experimental setup involving multiple natural isolates and tested protocols.

Future research should investigate the use of agro-industrial waste as a substrate for biomass and carotenoid production by the best-performing yeast isolate, *R. mucilaginosa* top 30. Coupled with the optimized extraction procedure, this step will contribute to an economically and ecologically acceptable solution for carotenoid production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10091699/s1, Table S1: Temperature and pH growth profiles of *Rhodotorula* isolates during incubation.

Author Contributions: Conceptualization, O.Š. and A.S.; methodology, V.Š. and A.T.; validation, B.L., L.P. and V.Š.; formal analysis, A.S. and O.Š.; investigation, A.R., A.T. and D.C.; resources, S.M. and G.Ć.; data curation, B.L. and L.P.; writing—original draft preparation, O.Š. and A.T.; writing—review and editing, A.T.; visualization, B.L. and O.Š.; supervision, J.Č.-B. All authors have read and agreed to the published version of the manuscript.

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