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Research article

Effect of initial pH of medium with potato wastewater and glycerol on protein, lipid and carotenoid biosynthesis by *Rhodotorula glutinis*

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ABSTRACT

Background: Rhodotorula glutinis is capable of synthesizing numerous valuable metabolites with extensive potential industrial usage. This paper reports the effect of initial culture medium pH on growth and protein, lipid, and carotenoid biosynthesis by *R. glutinis*.
Results: The highest biomass yield was obtained in media with pH 4.0–7.0, and the value after 72 h was 17.2–19.4 $g_{d.w.}$/L. An initial pH of the medium in the range of 4.0–7.0 has no significant effect on the protein (38.5–41.3 g/100 $g_{d.w.}$), lipid (10.2–12.7 g/100 $g_{d.w.}$), or carotenoid (191.7–202.9 μg/$g_{d.w.}$) content in the biomass or on the profile of synthesized fatty acids and carotenoids. The whole pool of fatty acids was dominated by oleic (48.1–53.4%), linoleic (21.4–25.1%), and palmitic acids (13.0–15.8%). In these conditions, the yeast mainly synthesized torulene (43.5–47.7%) and β-carotene (34.7–38.6%), whereas the contribution of torularhodin was only 12.1–16.8%. Cultivation in medium with initial pH 3.0 resulted in a reduction in growth (13.0 $g_{d.w.}$/L) and total carotenoid (115.8 μg/$g_{d.w.}$), linoleic acid (11.5%), and torularhodin (4.5%) biosynthesis.
Conclusion: The different values of initial pH of the culture medium with glycerol and deproteinized potato wastewater had a significant effect on the growth and protein, lipid, and carotenoid biosynthesis by *R. glutinis*.

1. Introduction

Microorganisms are capable of biosynthesizing many valuable metabolites such as lipids (Single Cell Oil), microbial proteins (Single Cell Protein), and carotenoids. The ability to synthesize these cellular compounds is characteristic of different microorganisms, including *Rhodotorula* yeast [1,2]. The main representative of this genus is *Rhodotorula glutinis*, an anamorphic form of *Rhodosporidium toruloides* [3].

R. glutinis belongs to the group of so-called oleaginous microorganisms, which are defined as those that are capable of producing and accumulating over 20% of lipids in dry cellular substances [3]. The lipid content in their biomass can reach up to 60% [4]. This yeast primarily synthesizes palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acids (C18:3). In comparison to the production of vegetable and animal fats, microbial production is independent of climate and season. Production cycle is short, owing to the rapid growth rate exhibited by the microorganisms. Depending on the composition, microbial lipids can be used in the food industry as components of food and supplements or in the fuel industry as the substrate for third-generation biodiesel production [3].

Carotenoids belong to a group of natural pigments found in fruits, vegetables, fish, eggs, and oil. These compounds can also be synthesized by microorganisms including *R. glutinis* [3]. Therefore, this yeast is known as "red yeast." In addition, these compounds include β-carotene, torulene, and torularhodin [5]. β-carotene, the vitamin A precursor, is used in the food industry as a pigment and a component of food supplements [6]. Torulene ($C_{40}H_{54}$) and torularhodin ($C_{40}H_{52}O_2$) are not currently used in the industry. However, because of their strong antioxidant and antimicrobial properties [7] and color, they may in the future be used as components in cosmetics, food [8], and medications [9]. *Rhodotorula* biomass can also be a valuable addition to animal feed [10,11,12].

The biotechnological method for the production of yeast biomass can manage burdensome industrial waste. In addition, the removal

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of side products from different technological processes usually constitutes a serious issue for the production plant and for the natural environment. For biotechnology processes, glycerin formed during biodiesel production can serve as the carbon source in the culture medium [4], whereas the potato wastewater, a side product of potato starch production, can be utilized as a source of nitrogen and mineral components [13]. The growth and biosynthesis of cellular components by *R. glutinis* is influenced by numerous environmental factors. One of the most important factors is the culture medium pH value [14]. For the biomass production process to be applied in industry, it is necessary to reduce the costs of microbiological media and optimize the culture conditions to obtain high levels of efficiency.

The objectives of this research were (1) to determine the effect of initial culture medium pH on the growth of *R. glutinis*; (2) to determine the influence of initial culture medium pH on the biosynthesis of protein, lipids, and carotenoids by *R. glutinis* and composition of fatty acid and carotenoid fraction; and (3) to access the possibility of simultaneous biodegradation of potato wastewater and glycerol during the cultivation of *R. glutinis*. This screening experiment, focused on the medium composition optimization, was performed in Erlenmeyer flasks.

2. Materials and methods

2.1. Deproteinized potato wastewater

Potato wastewater was prepared in laboratory conditions following a methodology elaborated on the basis of the industrial potato starch production process. Cleaned potatoes (Irga variety) were crushed using a juicer (Smith XXL, Moulinex). The juice separated from the precipitate was centrifuged for 10 min at 3000 × g (Centrifuge 5810, Eppendorf), and then the supernatant was acidified to pH 5.0 using sulfuric acid. The potato wastewater was sterilized at 117°C for 10 min (HICLAVE HG-80, HMC Europe). The precipitated protein was separated by filtration, and the filtrate (deproteinized potato wastewater) was used to prepare the experimental media.

The potato wastewater contained 1.29% total protein, determined by the Kjeldahl method, and the protein concentration using the Lowry method was 0.43% (Table 1); approximately 66% of nitrogenous substances occurred in the form of nonprotein compounds, probably in the inorganic form. Reducing sugar content determined by the Miller method was 1.38%.

2.2. Biological material and culture conditions

The yeast strain used in this study was *R. glutinis* var. *rubescens* LOCKR13 obtained from Technical University of Lodz, Poland [15].

The inoculum was prepared by the inoculation of the YPD liquid medium (2% glucose, 2% peptone, 1% yeast extract, pH 5.0) using material collected from agar slants. The cultivation was performed at 28°C for 24 h (200 rpm/min, SM-30 Control, Edmund Bühler).

All experimental media were prepared from potato wastewater supplemented with 5% (w/v) glycerol (Avantor™ Performance Materials). Six variants of media with different initial pH (2.0, 3.0, 4.0, 5.0, 6.0, and 7.0) were used. In this media, the molar ratio of C/N was equal to 4.2/1 (Table 2). The inoculum size was 10% (v/v). The cultivation of yeast was performed in 500-mL flat-bottom flasks containing 100 mL of the medium on a reciprocating shaker (200 rpm/min) at 28°C for 72 h.

2.3. Cellular biomass yield

The yeast growth during cultivation in experimental media was evaluated based on the change of cellular biomass, determined by the dry weight method. A total of 10 mL of the culture medium was centrifuged for 10 min at 6000 × g (Centrifuge 5804R, Eppendorf); the supernatant was removed, and the biomass was washed with deionized water. The wet cellular biomass was dried at 105°C until constant weight was obtained.

2.4. Analysis of postculture medium composition

Glycerol content, total protein content, pH value, and the value of the chemical oxygen demand (COD) ratio were determined for the postculture media. Glycerol content was determined using a chemical method [16]. Total protein content was determined by the Kjeldahl method. The COD ratio was determined through the dichromate method using Hach Lange cuvette tests (LCK014). To determine the pH of culture media, potentiometric method was used (Conbest CP-501). The level of glycerol, total protein content reduction, and COD ratio were calculated.

2.5. Analysis of cellular biomass composition of R. glutinis

The total protein content in the cellular yeast biomass was determined by the Kjeldahl method using a conversion factor 6.25 [17].

The intracellular lipid content was determined by the modified Bligh and Dyer method [18]. Acid hydrolysis of the dry cellular substance was performed before lipid extraction. Ten milliliters of 1 M HCl was added to 200 mg of dry yeast biomass and incubated in a water bath at 60°C for 2 h (Memmert WNB14, Schwabach). Lipids were extracted using a mixture of 10 mL chloroform and 10 mL methanol. To achieve phase separation, 5 mL of 20% NaCl was added to the solution and centrifuged (3500 × g/10 min). The bottom phase was collected into a weighed tube, chloroform was evaporated in a nitrogen atmosphere, and the lipid content in the yeast biomass was determined gravimetrically.

The total fatty acids composition of lipid fraction extracted with the mixture of chloroform and methanol (according to the above-explained procedure) was determined through gas chromatography equipped with a flame ionization detector (GC-FID, TRACE™ 1300, Thermo Fisher Scientific, USA). Extracted lipids were dissolved in 2 mL of hexane. Fatty acid methyl esters (FAMEs) were prepared by transesterification. A volume of 0.5 mL of 2 M methanolic KOH was added, and samples were incubated at 37°C overnight (16 h). After incubation, 0.5 mL of hexane layer was collected for GC analysis. FAME separation was performed using an RTX-2330 capillary column (60 m × 0.25 mm × 0.2 μm, Restek, USA). The oven temperature was set at 50°C (3 min); the temperature increase rate was 3°C/min up to 250°C (5 min). Nitrogen (1.6 mL/min) was the carrier gas. The temperatures of the injector and detector were set at 230°C and 260°C, respectively. Identification of individual fatty acids was performed on the basis of the retention times

Table 1
Analysis of results of potato wastewater composition.

Dry substance (g/L)	Protein content — lowry method (g/L)	Total protein — Kjeldahl method (g/L)	Reducing sugars (g/L)	COD (g/L)
35.5 ± 0.5	4.3 ± 0.4	12.9 ± 0.5	13.8 ± 0.2	35.12 ± 3.7

Table 2
Characteristics of the tested media.

Medium compound	Concentration (%)	Molar mass (g/mol)	Molar concentration of source (mol/L)	Molar ratio C:N
Glycerol	5.000	92.090	0.543	4.2:1
Reducing sugars (calculated per glucose unit)	1.380	180.160	0.076	
Nitrogen	0.206	14.007	0.147	

of Nu-Chek-Prep Inc. (USA) external reference standards present in the GLC 461 solution (32 FAMEs from C4:0 to C24:0). The percentage of individual fatty acids in lipid fraction was calculated on the basis of participation of individual FAME peak area in the sum of peaks areas (100%) of all identified FAMEs on the chromatogram.

The total carotenoid content was determined spectrophotometrically according to Cutzu et al. [19]. The carotenoid pigment extraction was preceded by yeast cell wall disintegration, which was performed using dimethyl sulfoxide and zirconia beads (BioSpec Products, 0.5 mm). The measurement of the colored ether fraction was performed at $\lambda = 457$ nm, and the carotenoid content was calculated using the model curve prepared for the pure β-carotene (Sigma-Aldrich).

Carotenoids were identified using high-performance liquid chromatography (HPLC) with a UV/Vis detector (Agilent 1200 Series, Palo Alto, CA, USA). The separation was performed on a C18 analytical column (Luna HILIC-Phenomenex, 250 mm × 4.6 mm, 5 μm). The column thermostat was set at 25°C. The mobile phase consisted of a mixture of acetonitrile, isopropanol, and ethyl acetate in the proportion 4:4:2. The flow rate was established at 0.7 mL/min (isocratically), and the detector operated at a wavelength of 457 nm [20]. The β-carotene was identified on the basis of the external standard retention time (Sigma-Aldrich). Torulene and torularhodin were identified on the basis of retention times of external standards separated using thin-layer chromatography (TLC). Fractionation of the crude carotenoid from *R. glutinis* was performed on silica gel plates (5 × 20 cm, Kieselgel 60 F_{254}, Fulka). The extracted carotenoids were loaded on the TLC plates with a mixture of acetone and hexane (3:7, v/v). After development, four fractions were found, dark pink (fuchsia), pink, orange, and yellow, for which the R_f coefficients were 0.40, 0.42, 0.89, and 0.93, respectively. The bands were scraped in acetone and separated by centrifugation, and retention time of this external standards was determined by HPLC-UV/Vis [21].

2.6. Statistical analysis

The results obtained in three independent experimental series were subjected to a statistical analysis using R software (version i386 2.15.3) in the RCommander tab. The normal distribution of the data was performed using the Shapiro–Wilk test, and the variance homogeneity was tested using the Levene test. To determine the significance of differences between the means, a single-variant ANOVA and Tukey's test were performed. All analyses were performed at a significance level of $\alpha = 0.05$.

3. Results and discussion

3.1. Yeast growth and postculture medium composition characteristics

During the 72 h of cultivation, the yeast growth, glycerol, total protein uptake, and culture medium pH were monitored, and the results of the analyses are presented in Table 3 and Fig. 1. It was determined that the initial pH value of the media with potato wastewater and addition of 5% glycerol influenced the growth of *R. glutinis*. A decrease in medium pH to 2.0 completely inhibited the growth of *R. glutinis*. During the cultivation of yeast in medium with pH 3.0, a significant reduction of growth was determined, and the biomass yield after 72 h was 13.0 $g_{d.w.}$/L. In the media with initial pH 4.0, 5.0, 6.0, and 7.0, the growth of *R. glutinis* was similar. In all cases, the highest increase of biomass yield was observed after 24 h of cultivation, and in the next 2 d, its value was slightly increased. The highest value of the cellular biomass yield after 72 h was observed in medium with initial pH 5.0 (19.4 $g_{d.w.}$/L). Slightly lower values were obtained during cultivation in media with pH 4.0 (17.3 $g_{d.w.}$/L), 6.0 (18.0 $g_{d.w.}$/L), and 7.0 (17.2 $g_{d.w.}$/L); however, the statistical analysis did not demonstrate significant differences between these results and those obtained in medium with initial pH 5.0 (Table 3). Johnson et al. [22] also observed a reduction in the growth of *R. glutinis* IIP-30 in medium with pH 3.0 (9.5 $g_{d.w.}$/L), whereas the highest value of the biomass yield was observed in medium with pH 4.0 (22.3 $g_{d.w.}$/L). In media with pH 5.0 and 6.0, the values of the index were considerably lower, i.e., 18.0 and 15.2 $g_{d.w.}$/L, respectively. A similar relationship was determined by Dias et al. [14]. The highest biomass yield of *R. glutinis* NRRL Y-1091 was observed in medium with pH 4.0 (5.9 g/L). However, for cultivation in media with pH from 4.5 to 6.0, values were not as low (4.3–4.9 g/L) compared to the medium with pH 4.0, as was the case in the study by Johnson et al. [22].

The level of glycerol utilization from the experimental media depended on the yeast growth rate. In media with initial pH 4.0, 5.0, 6.0, and 7.0, the glycerol consumption was similar and amounted to approximately 50%, whereas in medium with pH 3.0, this value was only 22% (Table 3). In our previous study [23], after the cultivation of *R. glutinis* in medium with potato wastewater and 5% glycerol, the level of this carbon source uptake after 72 h was 70%. These differences in the obtained values originate from the variable simple sugar content in potato wastewater. In our previous work, potato wastewater contained a low amount of reducing sugars (0.30%), whereas the wastewater used in this study contained 1.38%. These compounds constitute a more favorable carbon source in terms of energy; they are the first to be metabolized, and glycerol is used when they are exhausted.

Table 3
Cellular biomass yield values and experimental culture medium parameters after 72 h of cultivation.

Initial medium pH	Biomass yield ($g_{d.w.}$/L)*	Final medium pH*	Level of glycerol usage (%)	Level of total protein usage (%)	Level of COD index reduction (%)
2.0	0.7 ± 0.1^c	2.0 ± 0.1^c	0	0	**
3.0	13.0 ± 1.5^b	4.6 ± 0.2^b	22.0	26.2	18.9
4.0	17.3 ± 0.8^a	8.6 ± 0.3^a	49.8	40.2	44.9
5.0	19.4 ± 0.6^a	8.6 ± 0.2^a	52.9	41.9	46.4
6.0	18.0 ± 0.5^a	8.6 ± 0.1^a	49.5	38.7	47.6
7.0	17.2 ± 1.4^a	8.5 ± 0.1^a	47.6	40.3	43.2

* a, b, c–indices mark homogeneous groups; ** not determined.

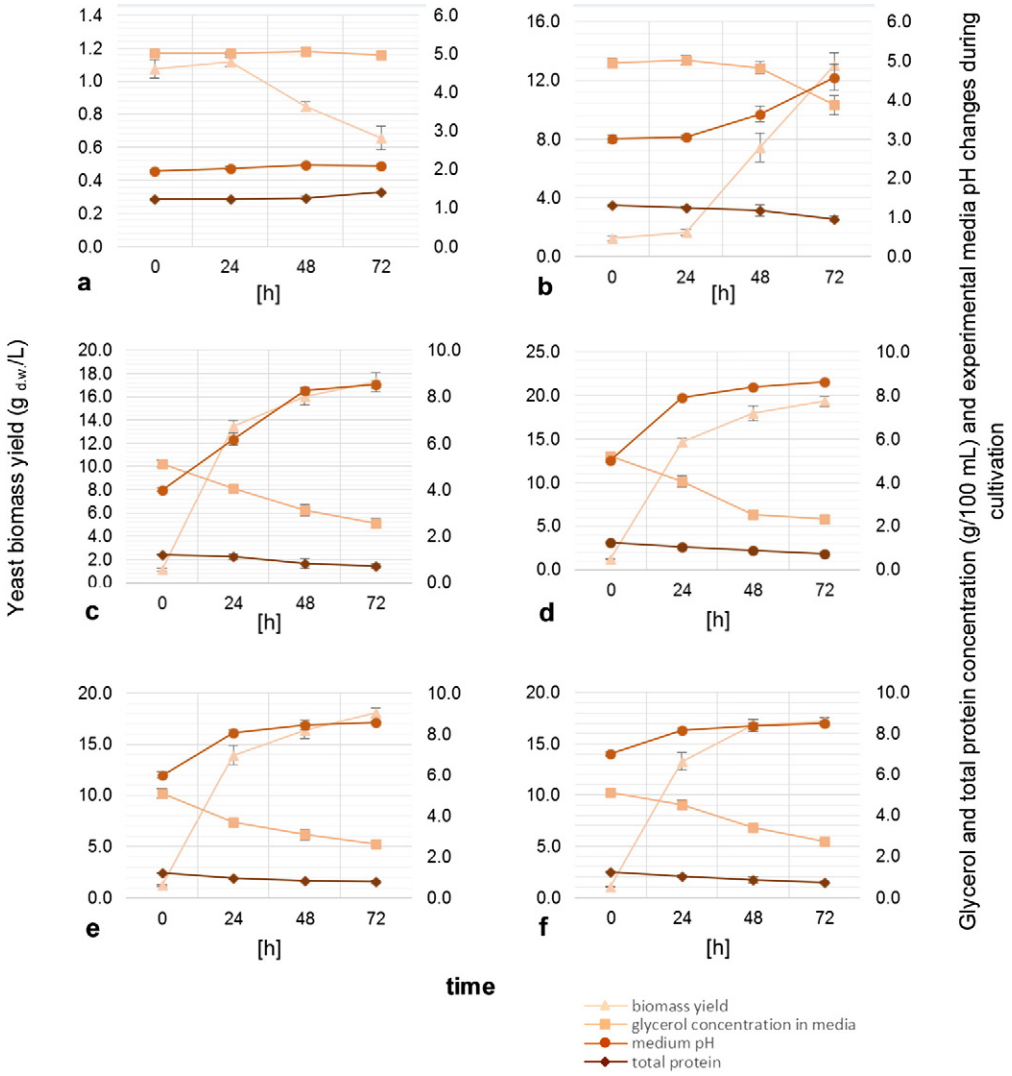

Fig. 1. Changes in the yeast cellular biomass yield, glycerol, total protein content, and media pH during the cultivation of *Rhodotorula glutinis*: (a) pH 2.0, (b) 3.0, (c) 4.0, (d) 5.0, (e) 6.0, and (f) 7.0.

The maximum reduction of total protein from the potato wastewater was detected in experimental media with initial pH 4.0, 5.0, 6.0, and 7.0. After 72 h of cultivation over 0.7 g/100 mL of total protein still remained, and the degree of reduction was estimated at approximately 40%. In medium with initial pH 3.0, the content of these components was higher (0.96 g/100 mL), which resulted from the reduced growth rate (Fig. 1).

For economic reasons, the pH of the experimental media was adjusted initially and was not controlled during the cultivation of *R. glutinis*. Experimental medium pH changes depended on the growth of the *R. glutinis*. In the medium with initial pH 3.0, the value reached pH 4.6 after 72 h. The course of changes of pH was similar in the remaining media (4.0, 5.0, 6.0, and 7.0). Alkalinization of the media was observed after 24 h, and pH increased to approximately 8.5 after 72 h (Fig. 1). Strong alkalinization of culture medium has also been observed during the cultivation of filamentous fungi in medium with deproteinized potato wastewater [24]. The initial value of the medium pH was established at 5.35–5.45. For *A. oryzae* 448 and *A. niger* 334, the pH increased 9.4 after 72 h, and in the *R. oligosporus* 2710 culture, it reached to 7.8.

The COD index is a significant parameter, which characterizes the level of waste purification. The addition of 5% glycerol to deproteinized potato wastewater increased the value of the index from 35.1 to 95.2–97.8 g O_2/L. After 72 h of *R. glutinis* cultivation, values for the COD index in experimental media with initial pH 4.0–7.0 were similar, and their values remained within the range from 48.6 to 52.3 g O_2/L (a reduction level of approximately 45%, Table 3). High COD index values resulted from the presence of residues of nonmetabolized glycerol. After cultivation in medium with pH 3.0, the COD index reduction was insignificant and amounted to only 18.9%.

3.2. Protein, lipid, and carotenoid biosynthesis by R. glutinis

The biomass of yeast obtained after 72 h of cultivation in media containing potato wastewater and 5% glycerol at different initial pH was characterized by similar intracellular protein contents (38.5–41.3 g/100 $g_{d.w.}$), and these results did not differ significantly. After the inclusion of the cellular biomass yield, the highest titer of microbial protein (7.83 g/L) was obtained after cultivation in medium with initial pH 5.0. Zheng et al. [2] reported that waste originating from the industrial production of monosodium glutamate was used as the medium for a mixed culture of *Candida halophila* GZ991 and *R. glutinis* GZ996. The obtained biomass had a high protein content (56 g/100 $g_{d.w.}$) and a very low lipid content (0.4 g/100 $g_{d.w.}$). An analysis of the composition of the separated protein demonstrated that it was rich in leucine (5.7%), isoleucine (4.4%), valine (4.2%), and lysine (4.1%). Martelli et al. [25] used glycerol as the carbon source for

Rhodotorula lactosa. After 48 h of cultivation, the protein content in the biomass was 36–38 g/100 $g_{d.w.}$ Because of its high intracellular protein content, yeast biomass from *Rhodotorula* may be a valuable addition to animal feed.

It was determined that in all cases, the intracellular lipid content in the yeast biomass was similar, i.e., 10.2–12.7 g/100 $g_{d.w.}$ It has been previously reported [22] that lipid accumulation in *R. glutinis* IPP-30 is strongly influenced by pH, with yields of 12 g/100 $g_{d.w.}$ at pH 3, 48 g/100 $g_{d.w}$ at pH 5, and 44 g/100 $g_{d.w.}$ at pH 6. These differences probably resulted from the type of culture medium. Johnson et al. [22] used mineral medium with glucose (3%) as a carbon source and low dose of ammonium sulfate (0.2%). The content of nitrogen source in their medium was almost seven-fold lower than that used in this study.

In the tested conditions, the examined strain of *R. glutinis* did not fulfill the criteria for oleaginous microorganisms, which are defined as being capable of producing and accumulating over 20% of lipids in a dry cellular substance [26]. The reason for the low lipid content in the cellular biomass of the *R. glutinis* obtained in the present study could be the excessive initial nitrogen compound content in the medium, which was not metabolized in whole during the cultivation (Table 3). In general, intracellular lipid biosynthesis in yeast begins when the nitrogen source is exhausted in the culture medium and the carbon source remains in surplus [27].

Despite the low total intracellular lipid content in the *R. glutinis* biomass, the microbial lipid amounted to between 1.5 and 2.2 g/L (Table 4) because of the high cellular biomass yield (Table 3). A considerably higher intracellular lipid content in the *Rhodotorula* biomass after cultivation in media containing glycerol as the carbon source was obtained by Yen et al. [28]. In their study, thin stillage was used as the source of nitrogen, and glycerol was the source of carbon at a 3% dose. After the cultivation, the lipid content in the cellular biomass of *R. glutinis* BCRC 22360 was 36.5%.

Oleic acid had the highest contribution in the lipid extracted from the *R. glutinis* biomass. The highest content of this acid was observed in medium with initial pH 3.0 (60%), whereas in the remaining cases (pH 4.0, 5.0, 6.0, and 7.0), its amount was considerably lower, i.e., 48.1–53.4%. The second most common fatty acid present in the lipids of yeast grown in medium with pH 3.0 was palmitic acid (15.2%), whereas linoleic acid occurred in a lower amount (11.5%). After cultivation in the remaining experimental media (pH 4.0, 5.0, 6.0, and 7.0), linoleic acid had a higher contribution (21.4–25.1%), whereas the palmitic acid content (13.0–15.8%) was similar to the content determined in medium with initial pH 3.0 (Fig. 2). The higher content of oleic acid after the cultivation of yeast in medium with initial pH 3.0 probably resulted from a lower activity of enzymes converting oleic acid to linoleic acid. The remaining fatty acids that were identified in the lipids synthesized by *R. glutinis* occurred in significantly lower amounts. A similar fatty acid profile was obtained by Saenge et al. [4] during the cultivation of *R. glutinis* TISTR 5159 in medium with glycerol at a concentration of 8.5%. Oleic acid (45.75%) was dominant, and its amount was comparable to the amount obtained in media with initial pH 5.0, 6.0, and 7.0 (48.1–49.5%) in our study. Another acid synthesized by *R. glutinis* TISTR 5159 was linoleic

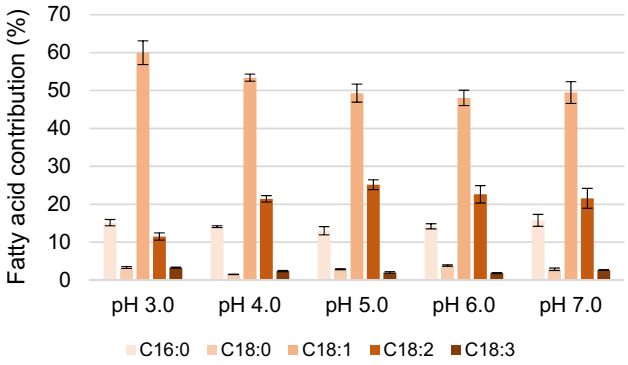

Fig. 2. Fatty acid profile after 72 h of cultivation of *R. glutinis* in media with different initial pH.

acid (C 18:2), and its content was 17.92%. The percentage contribution of this acid in the lipids synthesized by *R. glutinis* in media with potato wastewater and 5% glycerol at initial pH 4.0–7.0 was slightly higher and amounted to 21.4–25.1%. The palmitic acid (C 16:0) content determined by Saenge et al. [4] was 16.80%, and this was comparable to the results obtained in the present study (13.0–15.8%).

R. glutinis had the ability to biosynthesize carotenoids during cultivation in the experimental media with potato wastewater and glycerol. The content of these compounds in the yeast biomass was at its lowest level (115.8 μg/$g_{d.w.}$) after cultivation in medium with pH 3.0. In the remaining culture variants (pH 4.0, 5.0, 6.0, and 7.0), the carotenoid content was significantly higher (191.7–202.9 μg/$g_{d.w.}$), and the total titer was 3.4–3.7 mg/L (Table 4). A comparison of efficiency of carotenoid production yield between *R. glutinis* LOCKR13 and other yeasts belonging to this species is shown in Table 5. The tested yeast strain synthesized a similar amount of carotenoid pigments as *R. glutinis* ATCC 15125 cultivated on a medium containing sweet potato extract as the carbon source [1] or *R. glutinis* DFR-PDY in mineral medium with dextrose and sodium nitrate [29]. Almost two-fold higher titer of carotenoid production (6.5 mg/L) was obtained by Kanzy et al. [30]. In their study, cultivation of *R. glutinis* RCMB 028001 was performed in a medium containing cheese whey and 3% NaCl. An application of higher dose of chloride sodium caused osmotic stress and enhanced the biosynthesis of carotenoids in *R. glutinis*. In medium containing 1% NaCl titer of carotenoid production was significant lower and amounted to 1.47 mg/L.

The profile of carotenoids synthesized by *R. glutinis* was also analyzed. The separation of pigments extracted from biomass has been performed on the basis of their different polarity with the use of TLC on silica gel plates. As a result of the separation, three main fractions were found, dark pink (fuchsia), orange and yellow, for which the R_f coefficients were 0.40, 0.89, and 0.93, respectively. The yellow fraction (R_f 0.93) was β-carotene, which was confirmed using the standard (Sigma-Aldrich). On the basis of the color and R_f value determined under these same conditions [21], the remaining compounds were identified as torularhodin (R_f 0.40) and torulene (R_f 0.89). Furthermore, a fourth fraction was separated directly above torularhodin, a light pink

Table 4
Protein, lipid, and carotenoid content in *R. glutinis* biomass and their titers after 72 h of cultivation.

Initial medium pH	Protein content in biomass (%)*	Total protein titer (g/L)	Lipid content in biomass (%)*	Total lipid titer (g/L)	Carotenoid content in biomass (μg/$g_{d.w.}$)**	Total carotenoid titer (mg/L)
3.0	41.2 ± 1.9	5.4	11.9 ± 1.0	1.5	115.8 ± 17.2[b]	1.5
4.0	40.4 ± 1.3	7.0	10.2 ± 2.1	1.8	191.7 ± 28.9[a]	3.4
5.0	40.5 ± 2.0	7.8	10.8 ± 0.7	2.1	194.1 ± 11.2[a]	3.7
6.0	39.9 ± 2.2	7.2	11.3 ± 1.3	2.0	188.6 ± 21.4[a]	3.4
7.0	39.5 ± 1.1	6.8	12.7 ± 1.4	2.2	202.9 ± 9.5[a]	3.5

* ANOVA analysis demonstrated that the initial culture media pH did not have a significant effect on the protein and lipid content in *R. glutinis* biomass; ** a, b — indices mark homogeneous groups.

Table 5
Comparison of efficiency of carotenoid production by different strains of *R. glutinis*.

Microorganisms	Carbon and nitrogen source	Conditions	Time (h)	Carotenoids production (mg/L)	References
R. glutinis NCIM 3353	Dextrose + yeast extract	pH 6.0, 28°C	72	2.2	[20]
R. glutinis ATCC 15125	Dextrose + yeast extract	30°C	216	1.247	[32]
R. glutinis DBVPG 3853	Concentrated rectified grape must	30°C	120	5.95	[33]
R. glutinis RCMB 028001	Cheese whey	+ 3% NaCl, pH 6.6, 30°C	120	6.5	[30]
R. glutinis DFR-PDY	Dextrose + sodium nitrate	29–32°C	288	3.5	[29]
R. glutinis ATCC 15125	Hydroyzed mung bean waste flour + sweet potato extract	pH 5.91, 30.3°C	94.78	3.48	[1]
R. glutinis var. *rubescens* LOCKR13	Glycerol + potato wastewater	pH 4.0–7.0, 28°C	72	3.4–3.7	This study

fraction (R_f = 0.42), which probably constituted an intermediate metabolite of the torularhodin biosynthesis. Torularhodin is produced in the *R. glutinis* cells as a result of the transformation of torulene, consisting hydroxylation and oxidation. As a result of these reactions, intermediate metabolites of the cycle are produced: torularhodin alcohol and torularhodinaldehyde [3]. The percentage contribution of torularhodin, torulene, and β-carotene in the total composition of the carotenoids extracted from the *R. glutinis* biomass is presented in Fig. 3.

Similar to fatty acids, no significant differences were observed in the contribution of individual carotenoids after *R. glutinis* cultivation in media with initial pH 4.0, 5.0, 6.0, and 7.0. Torulene (43.5–47.7%) and β-carotene (34.7–38.6%) had the highest contribution (34.7–38.6%), whereas torularhodin had the lowest contribution (12.1–16.8%). In strongly acidified medium (pH 3.0), an increase in torulene biosynthesis (56.2%) was determined, and the amount of torularhodin was significantly decreased (4.5%). The β-carotene content (36.8%) was similar to its value determined in the remaining media. The decrease in torularhodin biosynthesis by *Rhodotorula* in media with low pH was also observed by Cheng and Yang [31]. After the cultivation of *Rhodotorula mucilaginosa* R-1 in YM medium with initial pH 4.0, torularhodin content amounted to 20.1%, whereas this increased to 36.0% in medium with pH 7.0.

4. Conclusions

It was determined that glycerol and deproteinized potato wastewater can be used as the source of carbon and nitrogen in culture media used for the production of *R. glutinis* biomass. The different values of initial pH of the culture medium had a significant effect on the growth of the studied yeast strain. The cultivation of *R. glutinis* in media with initial pH 4.0, 5.0, 6.0, and 7.0 enabled the production of a high yield of cellular biomass (17.2–19.4 $g_{d.w.}$/L). In medium with pH 3.0, a significant reduction of growth was determined, and yeast biomass yield was 13.0 $g_{d.w.}$/L.

The yeast biomass obtained after 72 h of culture in the experimental media with initial pH 3.0–7.0 was characterized by a similar protein content (39.5–41.2 g/100 $g_{d.w.}$). In addition, the different initial medium pH did not have an effect on the intracellular lipid content in *R. glutinis* biomass (10.2–12.7 g/100 $g_{d.w.}$). The obtained results demonstrate that in the examined conditions, *R. glutinis* did not fulfill the criteria for oleaginous microorganisms, which probably originated from the high content of nitrogen compounds present in the potato wastewater and low value of C/N molar ratio. Low content of lipids in the yeast biomass can be also the result of the employed conditions such as a flask culture. During cultivation in the experimental media, the studied strain exhibited the ability to synthesize carotenoids. The carotenoid content in the yeast biomass was the lowest after cultivation in medium with pH 3.0 (115.8 μg/$g_{d.w.}$), whereas in the remaining variants (pH 4.0–7.0), the amount of carotenoids was almost two-fold higher (191.7–202.9 μg/$g_{d.w.}$). The chromatographic analysis of the lipid and carotenoid fraction composition demonstrated that in medium with initial pH 3.0, a reduction of linoleic acid and torularhodin biosynthesis occurred. In contrast, under these conditions, the yeast produced larger amounts of oleic acid and torulene in comparison to the remaining culture media.

During the submerged cultures, a partial utilization of the potato wastewater and glycerol occurred with the simultaneous production of *R. glutinis* biomass, which, owing to its composition, may constitute a valuable addition to animal feed. An initial pH value of the culture medium in the range of 4.0–7.0 did not have a significant influence either on the growth, intracellular lipid content, protein content or carotenoid content or on the profile of synthesized fatty acids and carotenoids. This is probably related to the strong alkalinization of the medium during the cultivation as its pH had increased to approximately 8.0 as early as in the 24th hour of the experiment. Further research will be performed in a laboratory bioreactor with the use of specific conditions such as fed-batch and two-phase culture and pH control to increase lipid and carotenoid biosynthesis.

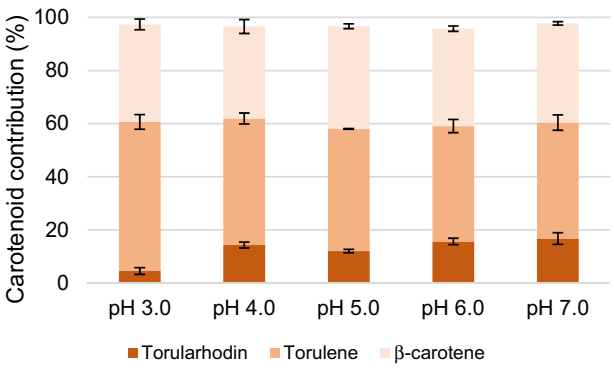

Fig. 3. Carotenoid profile synthesized by *R. glutinis* after 72 h of cultivation in media with different initial pH.

Conflict of interest

The authors declare that they have no conflict of interest.

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