RESEARCH ARTICLE

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Valorization of shalgam juice plant waste for the production of carotenoids by *Rhodotorula glutinis*

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Abstract

Food waste is an emerging global problem that should not be underestimated. One of the most abundant wastes in Türkiye and neighbour countries is the waste of shalgam juice plants (WSJP). In this study, WSJP was used as a growth medium for *Rhodotorula glutinis*. The effects of initial medium pH (3.4-5.4), carbon (0-60 g/L glycerol) and nitrogen sources (0-1 g/L urea) were investigated on biomass, medium pH, reducing sugar concentration, total lipid and carotenoid contents, as well as carotenoid composition (torulene, torularhodin, β-carotene). When crude waste extract (no additional nutrient) was used as growth medium (initial medium pH 3.4, 6.14 g/L sugar), biomass was relatively low (1.47±0.055 g/L), due to acidic pH and insufficient nutrients. However, these stress conditions increased the production of total lipid and carotenoid contents by *R. glutinis*. The highest lipid and carotenoid contents were obtained as 0.14±0.0004 g/g dry cell weight (dcw) and 1221.57±0.59 µg/g dcw, respectively, under these circumstances. As additional stress factor fermentation medium -crude WSJP extract- was illuminated for 72 h. This increased lipid content by 1.7-fold, while showing low impact on carotenoid content. Interestingly, illumination changed the carotenoid composition by decreasing torulene and β-carotene percentages, but increasing torularhodin percentage. On the other hand, tuning the initial pH to an ambient value (5.4) and the addition of carbon and nitrogen sources stimulated cell growth (4.67±0.07 g/L). This study presents the first time use of WSJP extract as a growth medium, without any additional nutrition, moreover, the simultaneous production of high-value added carotenoids.

Keywords: *Rhodotorula glutinis,* Waste, Shalgam juice, Lipid, Carotenoid

INTRODUCTION

Today, food waste is one of the major problems in the world. The amount of food waste has reached to almost billions of tones annually (De Souza Mesquita et al., 2019). In addition to the economic cost of the waste management, environmental pollution also creates a serious problem. Regarding this issue, worldwide meetings and conferences have been organized to take the attention of authorized organizations (Bröring et al., 2020; Hanedar et al., 2021). As a result of the increasing consciousness, 'zero waste' have gradually become the primary aim of the processes to prevent waste and ensure sustainability.

Fermented beverages are generally preferred due to various beneficial effects on human health. Especially the presence of microorganisms such as probiotics,

together with the healthful ingredients, have positive effects on the gastrointestinal system (Marsh et al., 2014). Shalgam juice (SJ) is one of the most popular non-alcoholic traditional beverages in Turkiye. Due to the interaction with the contiguous countries and exportation of the beverages, SJ has also gained attention around Middle East and Balkans region (Keskin & Güneş, 2021).

SJ is mainly produced by fermentation of two vegetables; black carrot (Daucus carota) and turnip (Brassica rapa). The other ingredients are wheat, sourdough, salt and water (Turker et al., 2004). However, some processes prefer to produce this drink using one of the vegetables, that is mostly black carrot. In 2020, the production of SJ in Turkiye was reported as 2,599 tones and showed an increasing trend every year (Omrak, 2021). Considering an average SJ production process, this value corresponds to almost 520 tones of fermented black carrot waste, that is quite high (Coşkun, 2017). The fermented vegetables are disposed to the environment as a waste of the process.

Black carrot contains several antioxidant molecules such as vitamin, C, vitamin E, carotenoids and phenolic components and the characteristic purple-black color is due to its high anthocyanin content (Wrolstad, 2004). Black carrot also contains fermentable sugars such as sucrose, glucose and fructose (Kammerer et al., 2004). The rich composition of fresh black carrot makes it attractive for researchers in terms of extraction of different components such as anthocyanins (Agcam et al., 2017; Gizir et al., 2008; Nistor et al., 2021), phytoceuticals (Kumar et al., 2019), pectin (Sucheta et al., 2020) and pectin methylesterase (Ünal & Bellur, 2009). On the other hand, it is mainly used for pigment production in industry (Kumar et al., 2019).

Carotenoids are natural isoprenoid pigments that can be synthesized by photosynthetic organisms and some of non-photosynthetic microorganisms (Moise et al., 2014; Rodriguez-Concepcion et al., 2018). Humans and animals are not capable of synthesizing carotenoids, therefore these molecules should be uptaken do their provitamin A activity that is crucial for human health (Maiani et al., 2009). Besides the significant systemic roles of carotenoids in human body, the lack of vitamin A in humans was proved to cause premature death (Britton, 1995). Carotenoids also serve as antioxidants that protect cells from free radicals (Maiani et al., 2009). Additionally, there are many studies that show the lowered risk of many chronic diseases as a result of the consumption of carotenoids in diets (Mayne, 1996). Related with the awareness of the benefits of carotenoids, not only in human health, but also as additives in different industries such as textile, pharmaceutical, and cosmeceutical products (Hernández-Almanza et al., 2016; Mussagy et al., 2022), global market of carotenoids has grown significantly. The annual growth rate of the market was predicted as 5.7% between 2017-2022 with a market value of 2.0 billion dollars (Rapoport et al., 2021).

The abundant sources of carotenoids are colorful fruits and vegetables such as carrot, orange, mango, bell pepper, etc. They contain mainly, β-carotene, β-cryptoxanthin, α-carotene, lycopene, lutein and zeaxanthin (Maiani et al., 2009). Other sources are filamentous fungi and pigmented yeasts, such as *Rhodotorula glutinis.*

Rhodotorula glutinis is an oleaginous red yeast that is capable of producing carotenoids that gives its unique color and is also capable of accumulating considerable amount of lipid inside (Hernández-Almanza et al., 2016). Besides the well-established carotenoid β-carotene, *Rhodotorula glutinis* can also synthesize torularhodin and torulene, which can only be synthesized by yeast and fungi (Kot et al., 2018). These carotenoids have the potential to be used in several different fields such as food and feed, medical and pharmaceutical industries (Kot et al., 2018).

In the literature, there are numerous studies on the varolization of black carrot pomace as waste of marmalade or juice production. The majority of these studies targets the extraction of anthocyanins or bioactive compounds as the ultimate product (Agcam et al., 2017, 2021; Kumar et al., 2019). However, there are no studies on the valorization of the waste of shalgam juice plant (WSJP) that mainly differs from marmalade or juice production in regard of the fermentation step. Our preliminary experiments on WSJP showed that fermented black carrot could still be evaluated due to the bioactive compounds conserved in the waste.

In this study our aim is the simultaneous valorization of WSJP and production of carotenoids by *R. glutinis*. With this aim, the effects of different parameters such as initial medium pH, carbon and nitrogen sources, and illumination were investigated that are known as stimulators of carotenogenesis (Gu et al., 1997; Johnson & Lewis, 1979; Orosa et al., 2005).

MATERIALS AND METHODS

Materials

Rhodotorula glutinis (70398) was purchased from DSM, Braunschweig, Germany. Yeast extract (70161), malt extract (70167), peptone (70171), agar (05039) and potassium-sodium tartrate (60410) were obtained from Fluka. Glucose (A1349,5000) was purchased from Applichem, sodium sulfide (13471) from Riedel de Haen. Urea (108488), chloroform (1.022445.2500) and hexane (1.04391.2500) were purchased from Merck. The following chemicals were obtained from Sigma-Aldrich: glycerol (G-5516), DNS (dinitrosalicylic acid) (D-0550), sodium hydroxide pellet (06203), Folin & Ciocalteu's phenol reagent (9252), β-carotene (C4582) and methanol (24229). All other chemicals used were at least analytical grade.

Waste of Shalgam Juice Plant

Waste of shalgam juice plant (WSJP) was supplied from Yeni Kavaklıdere Company (Ankara, Turkiye). Solid waste was grinded by blender (Braun MQ9078X). WSJ was extracted by distilled water at 50°C, 700 rpm for 90 min. Reducing sugar content of the WSJP extract was 6.15 g/L and pH value was 3.4. Supernatant was stored at -30°C until further use.

Fermentation of *Rhodotorula glutinis*

Solid growth medium contained 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract and 20 g/L agar in WSJP extract. The cells were inoculated to the solid growth medium and cultivated at 30°C for 24 h and transferred to the 100 mL of liquid fermentation medium. The liquid medium basically consisted of WSJP extract, urea (0-1 g/L) and glycerol (0-60 g/L), and the cultivations were carried out at 150 rpm and 30°C for 72 h. Then, cells were separated by centrifugation at 7200xg for 10 min (Hettich Zentrifugen Rotina 35 R), washed twice, and lyophilized at -50°C and 0.04 mbar (Hetosicc) and used for further analysis. The effect of illumination was investigated using white light (Osram L 15 W/25, Germany).

Dry Cell Weight

An aliquat was withdrawn from the liquid medium, centrifuged at 7200xg for 10 min at +4°C, the cells were washed twice and dried for 3 h at 80°C (Zhichang- ZRD 5110, Forced-Air Drying Oven) and expressed in dry cell weight (dcw).

Reducing Sugar Concentration

Reducing sugar concentration was determined by Dinitrosalicylic acid (DNS) method (Miller, 1959). 750 µL DNS solution (10 g/L DNS, 0.5 g/L sodium sulfite, 10 g/L sodium hydroxide) was added to 750 µL sample, vortexed and incubated at 90°C for 10 min. After addition of 250 µL of potassium-sodium tartrate solution (40 g/L) absorbance was measured 575 nm (UV-VIS spectrophotometer, Shimadzu 1601, Japan) and reducing sugar concentration was calculated in glucose equivalents.

Total Lipid and Total Carotenoid Content

Chloroform (6.25 mL) and methanol (12.5 mL) were added to a flask containing 0.5 g of lyophilized cells and stirred for 1h. Then, NaCl (0.5%, 6.25 mL) and chloroform (6.25 mL) were added to the flask and stirred for an additional 1 h. The phases were separated by centrifugation (7200xg, 10 min) and chloroform phase was separated and evaporated (Heidolph, Hei-VAP Advantage). The residue containing lipid and carotenoids was determined gravimetrically yielding total lipid amount (Bligh & Dyer, 1959). The residue, then was dissolved in hexane and filtered. Total carotenoid amount was measured in equivalents of β-carotene at a wavelength of 450 nm in a UV-VIS spectrophotometer (Shimadzu 1601, Japan).

Carotenoid Composition

Carotenoid composition was determined by HPLC (Waters Alliance) at 450 nm (UV-VIS Dual Absorbance Detector Waters 2487), using C18 column (XTerra RP18, Waters, 150 mm x 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile, methanol, and chloroform (47:47:6) with a flow rate of 1.0 mL/min. Analysis were performed at 30°C with a sample volume of 50 µl. β-carotene was identified based on the retention time of the external standard.

Thin-Layer Chromatography (TLC)

Torularhodin and torulene solutions were obtained by thin-layer chromatography (TLC) (Kot et al., 2017). The spots, which were carotenoids, were identified using the retention factor coefficients according to the method published (Kot et al., 2017). As a result of many runs, the spots corresponding to torularhodin and torulene were scraped separately into hexane and solutions were filtered through a PVDF filter before injection to HPLC.

Statistics

All cultivations were repeated at least two times and all analyses were repeated at least three times and their average values were presented. The error bars were prepared using Excel 2010.

RESULTS AND DISCUSSION

Effect of Initial Medium pH

The initial pH value of WSJP extract was measured as 3.4 due to the acidic nature of the shalgam juice production process. In order to investigate the effect of the initial medium pH on the growth of *R. glutinis*, and additionally to increase the relatively low pH to ambient values, initial pH was adjusted to 4.4 and 5.4 using 1 M NaOH. The change in pH, biomass and reducing sugar concentrations were measured with time. The results showed that medium pH increased within 24 h of fermentation for all media tested and remained almost constant till 48 h and decreased at 72 h (Figure 1). The pH values of the two media, pH 5.4 and 4.4, were almost equalized after 24 h and showed almost the same profile. On the other hand, the variation of the medium of initial pH 3.4 medium remained lower than the others for 72h.

The change in the biomass with time at different initial pH values are presented at Figure 2. The highest biomass was obtained at pH 5.4 as 1.87±0.055 g/L, followed by pH 4.4 as 1.73±0.06 g/L. The lowest cell concentration was obtained at pH 3.4, as 1.46±0.055 g/L.

Relatively low amount of sugar in the WSJP extract (6.15 g/L) is considered to be the major reason of the low cell concentrations. When the change in reducing sugar concentrations are evaluated (Figure 3), they were found to be compatible with the growth of the cells. Sugar was

consumed higher at the initial pH values of 4.4 and 5.4 than pH 3.4. Considering the decrease in reducing sugar concentration with time, fermentation media was ended after 72 h, in order to maintain the cells at the stationary phase, to produce the target antioxidant components (Mata-Gómez et al., 2014).

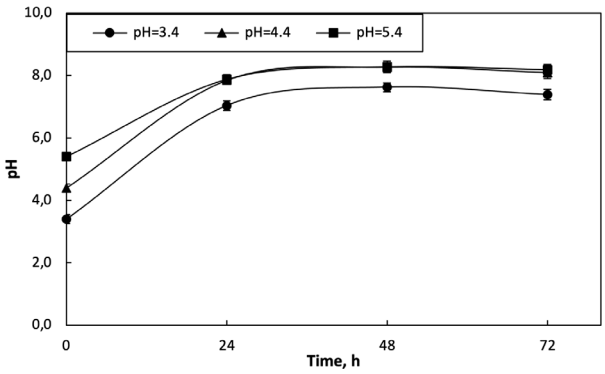

Figure 1. The variation of pH with time at different initial pH values (WSJP extract, 150 rpm, 30°C, 100 mL)

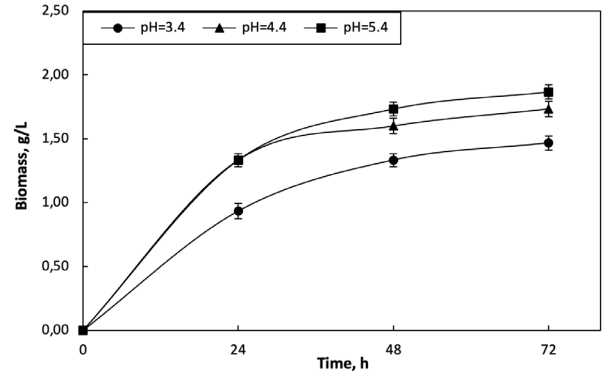

Figure 2. The variation of biomass with time at different initial pH values (WSJP extract, 150 rpm, 30°C, 100 mL)

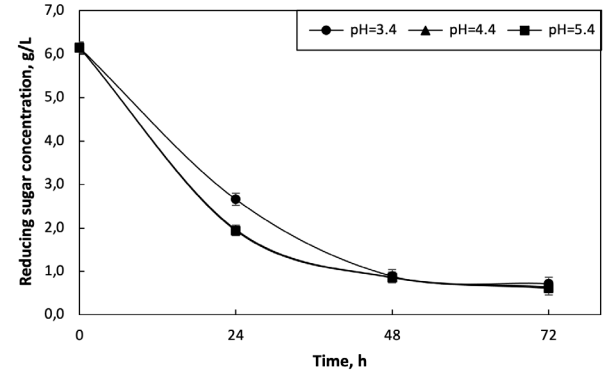

Figure 3. The variation of reducing sugar concentration with time at different initial pH values (WSJP extract, 150 rpm, 30°C, 100 mL)

Carotenoids synthesized by *R. glutinis* were extracted simultaneously with the extraction of lipids and solubilized in lipid phase. According to the results (Table 1), the highest lipid and carotenoid contents were obtained at pH 3.4, as 0.14±0.0007 g/g dcw and 1221±0.68 µg/g dcw, respectively. These results showed that lipid and carotenoid productions were high when the cell growth was low. It is well-known phenomenon that carotenoids are antioxidant molecules that are primarily produced under stress conditions and stress conditions limit the growth of the cells (Goiris et al., 2015; Mata-Gómez et al., 2014; Young & Lowe, 2018).

Table 1. The change in total lipid and total carotenoid contents at different initial pH values

pH	Total lipid content (g/g dcw)	Total carotenoid content (µg/g dcw)
3.4	0.14±0.0007	1221.57±0.68
4.4	0.11±0.0008	448.90±0.65
5.4	0.10±0.0007	587.24±0.72

Torulene and torularhodin are significant carotenoids produced by *R. glutinis*, besides β-carotene. In order to determine the distribution of the three components, HPLC analysis was performed and the results are presented in Figure 4. According to the results, carotenoid profile was found to be the same for all initial pH values and β-carotene was found to be the most abundant component. Evaluating the results of the effect of initial pH value experiments, pH 3.4 was selected as the optimum pH for the production of target carotenoids.

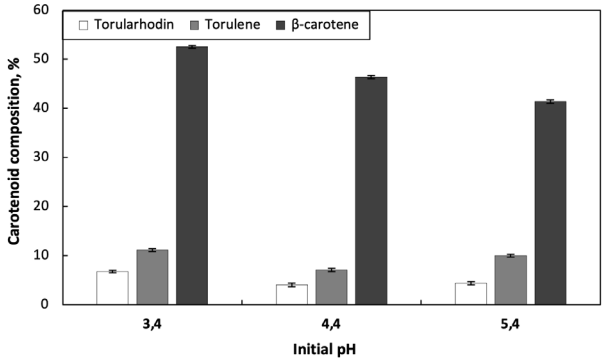

Figure 4. Carotenoid composition of *R. glutinis* cells grown at different initial pH values

Effect of Nitrogen Source

In order to observe the effect of nitrogen source, different amounts of urea were added to the WSJP extract. Addition of urea did not affect the initial medium pH, however, affected the time-course pH values of the fermentation media. As presented in Figure 5, pH values of 0.5 g/L and 1.0 g/L urea containing media were found to be higher

than that of 'no urea' medium. Similarly, higher biomass was obtained when additional nitrogen source was utilized (Figure 5). The highest cell concentration was 2.13±0.05 g/L at 1 g/L urea, followed by 1.73±0.06 g/L at 0.5 g/L urea concentration. The change in the growth of cells were found to be compatible with the decrease in the reducing sugar concentrations (Figure 6). The higher the growth rate, the higher the consumption rate of the reducing sugar was obtained, as expected (Figure 7).

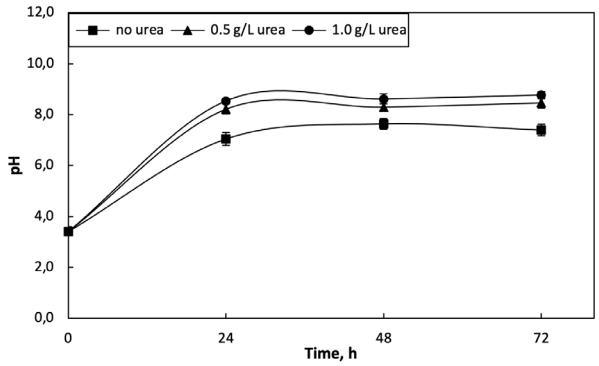

Figure 5. The variation of pH with time at for different urea concentrations (WSJP extract, 150 rpm, 30°C, 100 mL)

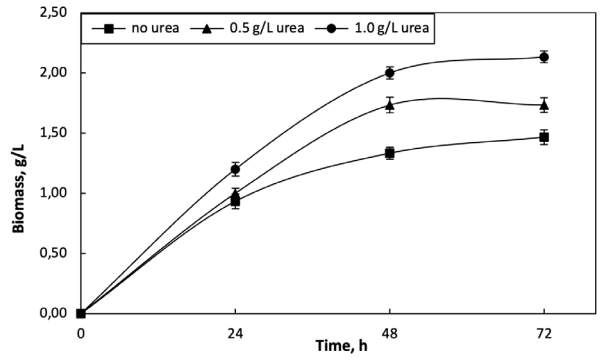

Figure 6. The variation of biomass with time at for different urea concentrations (WSJP extract, 150 rpm, 30°C, 100 mL)

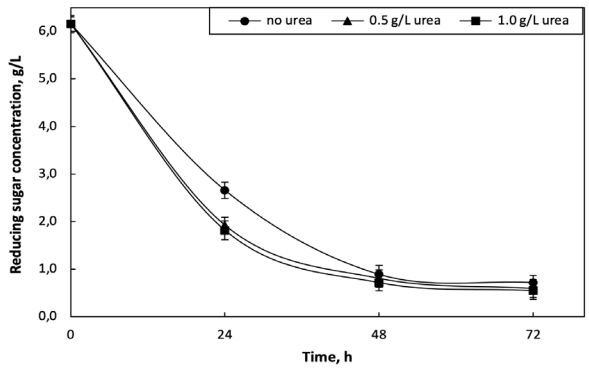

Figure 7. The variation of reducing sugar concentration with time for different urea concentrations (WSJP extract, 150 rpm, 30°C, 100 mL)

The highest total lipid and total carotenoid contents were achieved when no nitrogen source was added to the media, as 0.14±0.0006 g/g dcw and 1221.57±0.63µg/g dcw, respectively (Table 2). When urea-containing media were compared, 1 g/L of urea provided higher carotenoid content (587.24±0.71 µg/g dcw) than 0.5 g/L (448.9±0.58 µg/g dcw). When carotenoid profiles are evaluated, β-carotene was found to be the most abundant component at all media tested (Figure 8). The profiles were found to be similar for 0 and 0.5 g/L urea containing media while they were found to change at 1 g/L urea containing medium. The relative amount of torularhodin exceeded torulene. This showed that nitrogen source had a considerable effect on the composition of carotenoids. This effect of nitrogen source was also presented before in the literature (Mussagy et al., 2022).

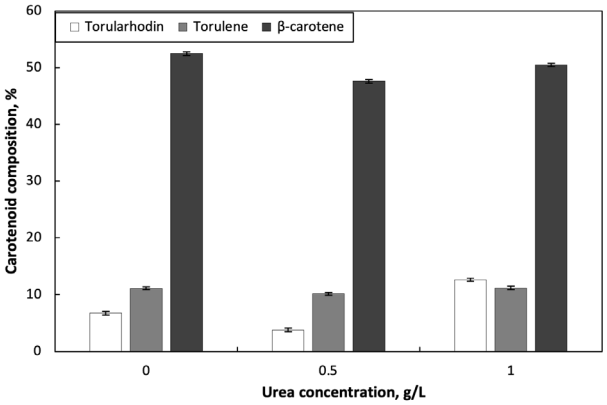

Figure 8. Carotenoid composition of *R. glutinis* cells grown at different urea concentrations (150 rpm, 30°C, 100 mL)

Effect of Carbon Source

To investigate the effect of an additional carbon source on WSJP extract, different concentrations of glycerol were added to the fermentation media. Glycerol was selected as the additional carbon source for being the side-product of biodiesel production process. Therefore, utilization of glycerol also contributed to valorization of another waste. The initial pH values of 'no glycerol' and 30 g/L glycerol containing media remained constant at pH 3.4, while an increase was observed 60 g/L glycerol containing medium. Interestingly, the variation of pH was found to be significantly different for glycerol containing media (Figure 9). pH increased very slowly during the first 24 h. This could be explained by the utilization of glycerol by *R. glutinis*, as the major carbon source in the medium, by releasing catabolism products that alter the pH profile.

On the other hand, biomass increased significantly in glycerol containing media (Figure 10). The final cell concentration increased to 4.67±0.07 g/L at 60 g/L glycerol, that is almost 3-fold when compared to 'no glycerol' medium.

Table 2. The change in total lipid and total carotenoid contents at different urea concentrations (150 rpm, 30°C, 100 mL)

Urea concentration (g/L)	Total lipid content (g/g dcw)	Total carotenoid content (µg/g dcw)
0	0.14±0.0006	1221.57±0.63
0.5	0.12±0.0006	448.90±0.58
1	0.10±0.0005	587.24±0.71

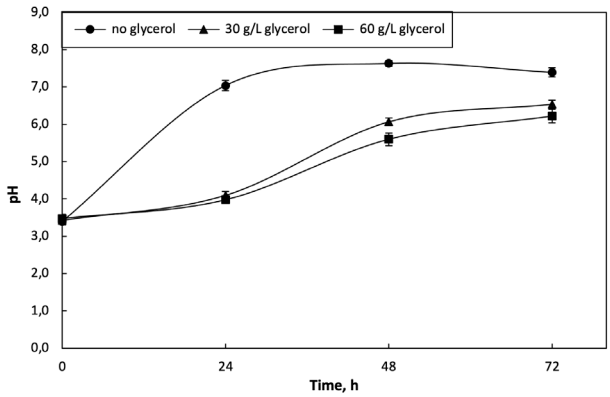

Figure 9. The variation of pH with time at for different glycerol concentrations (150 rpm, 30°C, 100 mL)

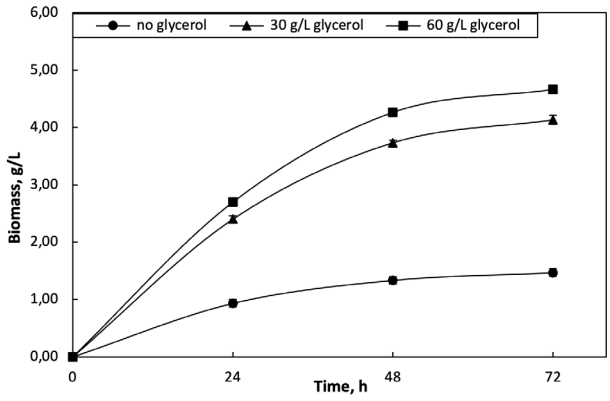

Figure 10. The variation of biomass with time for different glycerol concentrations (150 rpm, 30°C, 100 mL)

Reducing sugar concentrations decreased at the same rate for all three media in the first 24 h and then differed according to the glycerol concentrations (Figure 11). On the other hand, the increase of biomass for glycerol containing media in 24 h (Figure 10) illustrated that *R. glutinis* consumed both glycerol and reducing sugar. Reducing sugar concentrations at 48 h and 72 h (Figure 11) showed that reducing sugar consumption decreased with increasing glycerol concentration.

In contrast to the cell growth, total lipid and total carotenoid contents were found to be the highest without glycerol (Table 3). Total carotenoid content showed almost a 16-fold decrease when 60 g/L of glycerol used. This result supported the fact of induction of the production of antioxidant molecules at stress conditions, ie., insufficient carbon source.

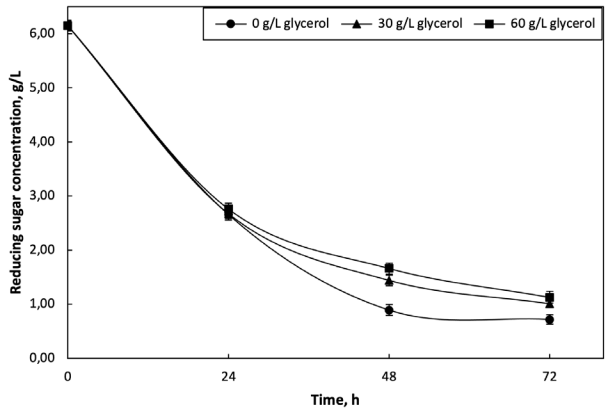

Figure 11. The variation of reducing sugar concentration with time for different glycerol concentrations (150 rpm, 30°C, 100 mL)

When carotenoid profiles are evaluated (Figure 12) the percentage of torularhodin was found to increase significantly, from 8% to 30%, at 60 g/L of glycerol. This showed the considerable effect of glycerol concentration on the carotenoid composition. This result is consistent with the carotenoid profiles obtained for different urea concentrations (Figure 8) and also with the literature. The change in the composition of carotenoids produced by *R. glutinis* with different carbon and nitrogen sources was reported in different studies (Buzzini & Martini, 2000; Peng et al., 2021). Nevertheless, since total carotenoid content was quite low as 75.89±0.71 µg/g dcw when 60 g/L glycerol was used, further experiments were conducted without additional carbon source.

Table 3. The change in total lipid and total carotenoid contents at different glycerol concentrations (150 rpm, 30°C, 100 mL)

Glycerol concentration (g/L)	Total lipid content (g/g dcw)	Total carotenoid content (µg/g dcw)
0	0.14±0.0004	1221.57±0.59
30	0.22±0.0007	209.60±0.65
60	0.12±0.0005	75.89±0.71

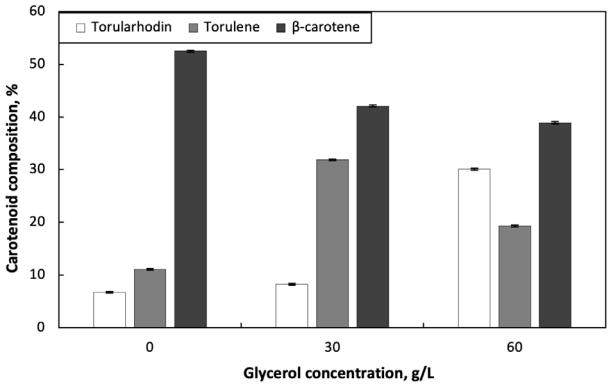

Figure 12. Carotenoid composition of *R. glutinis* cells grown at different glycerol concentrations (150 rpm, 30°C, 100 mL)

Effect of Illumination

Carotenoid production is reported to be enhanced by illumination for different microorganisms. This is associated with the promoted activity of the enzymes that take place in carotenogenesis (Bhosale, 2004). Therefore, to enhance the production of carotenoids, fermentation medium of WSJP extract was subjected to illumination for 72 h. According to the results, illumination decreased the cell growth during fermentation and caused nearly 9% of decrease in final biomass concentration, in accordance with the literature (Bhosale & Gadre, 2002) (Figure 13). The change in reducing sugar concentration with time was found to be similar for illuminated and non-illuminated media (Figure 13). Similarly, the variation of pH values of illuminated and non-illuminated media with time showed almost the same values (Table 4). On the other hand, total lipid content was found to increase from 0.14±0.0004 g/g dcw to 0.24±0.0005 g/g dcw after 72 h of illumination (Table 5), showing the stress effect on *R. glutinis* cells. Despite the minor change in the total carotenoid content (Table 5), carotenoid composition of *R. glutinis* was significantly affected by illumination. Torulene and β-carotene percentages was found to decrease while torularhodin percentage increased from 6.74% to 19.96% (Figure 14).

Table 4. The effect of illumination on the variation of pH with time (WSJP extract, 150 rpm, 30°C, 100 mL)

	pH			
	Incubation time, h			
Illumination	**0**	**24**	**48**	**72**
No illumination	3.40±0.04	7.04±0.07	7.63±0.07	7.39±0.05
72 h illumination	3.40±.0.04	7.08±0.05	7.63±0.06	7.76±0.06

Table 5. The effect of illumination on total lipid and total carotenoid contents (WSJP extract, 150 rpm, 30°C, 100 mL, 72 h)

Illumination	Total lipid content (g/g dcw)	Total carotenoid content (µg/g dcw)
No illumination	0.14±0.0004	1221.57±0.59
72 h illumination	0.24±0.0005	1260.30±0.62

When the effect of initial medium pH, carbon and nitrogen sources on the total carotenoid content are evaluated, the highest total carotenoid amount was obtained at initial pH value of 3.4 in the absence of urea and glycerol, therefore, further experiments were conducted using WSJP extract.

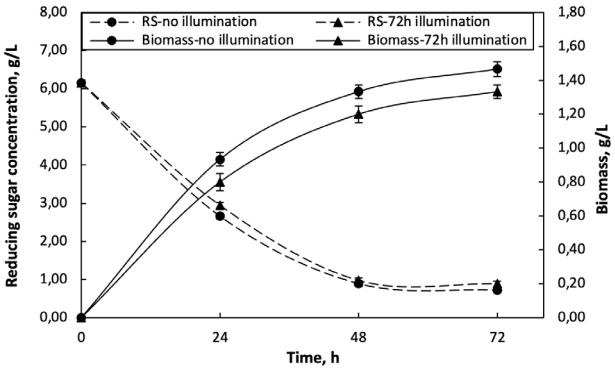

Figure 13. The effect of illumination on the variation of biomass and reducing sugar concentration RS: reducing sugar (WSJP extract, 150 rpm, 30°C, 100 mL)

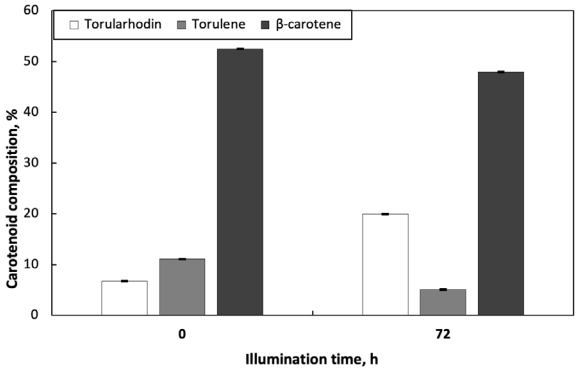

Figure 14. Carotenoid composition of *R. glutinis* cells under different illumination time (WSJP extract, 150 rpm, 30°C, 100 mL)

CONCLUSION

In this study we aimed to evaluate the waste of shalgam juice plant, to produce carotenoids, that are high-value-added components. The results showed that biomass reached to higher values when initial pH was tuned to 5.4 and additional nitrogen and carbon sources were added to the fermentation medium. On the other hand, lipid and carotenoid synthesis was induced when cell growth was hindered. The highest total carotenoid and lipid contents were achieved when crude WSJP extract was used. This is an advantage in terms of the cost of the fermentation medium. The waste can directly be extracted and used as the growth medium. On the other hand, illumination had a positive effect on total lipid and total carotenoid contents and additionally, increased the relative amount of torularhodin significantly. This is an important result, since torularhodin is a rare member of carotenoids that can only be synthesized by yeast and fungi. Nevertheless, even with no light, carotenoids were synthesized by *R. glutinis*. Regarding the promising results of this study, the process may be scaled up using a bioreactor. In addition to the investigated parameters, the effect of dissolved oxygen concentration may be investigated on the production of carotenoids which is a significant stress effect on the microorganisms. Moreover, pH-stat or chemostat operation strategies may be performed and the effect on the total lipid and carotenoid contents, as well as their compositions may be presented.

To our knowledge, this is the fist study in the literature, that presents a process for the valorization of the waste of shalgam juice plant and the simultaneous production of carotenoids as high-value-added components.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that they have no conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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