Lipid and β-Carotene Production by *Rhodosporidium diobovatum* Cultured with Different Carbon to Nitrogen Ratios

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HIGHLIGHTS

- Lipids and β-carotene production by *Rhodosporidium diobovatum* yeast was enhanced with increasing carbon/nitrogen ratio.
- Cultures with a carbon/nitrogen ratio of 50 also produced the greatest amount of β-carotene.
- These findings can be used for scale-up production of lipid and β-carotene in food industries by *R. diobovatum*.

ABSTRACT

Background: In food industry, carotenoids are used as food colorants conferring yellow to red color. This research was designed to study on lipid and β-carotene production by *Rhodosporidium diobovatum* cultured with different Carbon to Nitrogen (C/N) ratios.

Methods: *R. diobovatum* was cultured in a medium containing 40 g/l glucose (as the carbon source) and different C/N ratios (20, 50, and 80), which were established by adding different amounts of $(NH_4)_2SO_4$ (3.78, 1.51, and 0.94 g/l) as the source of nitrogen. High performance liquid chromatography, gas chromatography, and microplate reader were used to determine the glucose concentration, lipid production, and β-carotene concentration, respectively. Data were analyzed using IBM SPSS statistics (v. 24).

Results: Cultures with a C/N ratio of 50 produced the greatest amount of lipids during 120 h pi. However, lipid synthesis in the first 48 h pi was very low for all three C/N ratios. Analyses of the lipid composition revealed that oleic acid and linoleic acid were the dominant (60%) fatty acids. Cultures with a C/N ratio of 50 also produced the greatest amount of β-carotene.

Conclusion: *R. diobovatum* in the C/N of 50 culture medium resulted in greater concentrations of lipid and β-carotene. Defining the optimum C/N ratio will enable development of optimized bioprocess engineering parameters for scale-up production of lipid and β-carotene in food industries by this yeast species.

Introduction

In the food industry, carotenoids are used as food colorants conferring yellow to red color. They also have important application in animal feed, cosmetics, nutritional supplements, and medical researches. Carotenoid can be produced by plants and microorganisms, such as bacteria, fungi, and algae (Cazzonelli, 2011; Cheng, 2006; Jaswir et al., 2011). Many carotenoids have antioxidant and antitumor properties and also can reduce the risk of car-

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diovascular disease (Ciccone et al., 2013; Maldonade et al., 2008; Skibsted, 2012). Among natural carotenoids, β-carotene, lycopene, astaxanthin, canthaxanthin, and lutein have very wide applications as food supplements in human and animal diets (Berman et al., 2015; Jaswir et al., 2011).

The international market for carotenoids has been met mainly by chemically synthesized (synthetic) carotenoids that have the similar structure to the natural carotenoids. However, the growing economic value of carotenoids, as well as the potential toxicity caused by synthetic colors, increases the interest in developing new sources of natural carotenoids (Maldonade et al., 2008; Saini and Keum, 2017). Therefore, over the past decade, the use of lipids Triacylglycerides (TAGs) derived from microorganisms, such as microalgae and oleaginous yeasts, have been increasingly explored in the food industry (Liang and Jiang, 2013; Vieira et al., 2014). Comparing to oil seed plants and microalgae, oleaginous yeasts grow much faster, accumulate cell mass to much higher densities, and are less affected by season or weather conditions (Sestric et al., 2014).

Rhodosporidium diobovatum, named red yeast, can produce high levels of carotenoids and TAGs (Buzzini et al., 2007; Munch et al., 2015). It could use low-cost substrates, such as waste glycerol derived from biodiesel production as a carbon source. Nitrogen concentration greatly influences the growth of yeast cells (Sitepu et al., 2014). So, this research was designed to study on lipid and β-carotene production by *R. diobovatum* cultured with different Carbon to Nitrogen (C/N) ratios.

Materials and methods

Yeast strain

The yeast, *R. diobovatum* 08-225, was obtained from the Phaff Yeast Culture Collection, at the University of California at Davis (UCDFST). *R. diobovatum* was grown on Yeast Peptone Dextrose (YPD; Difco™, Sparks, Maryland, USA) agar containing 20 g/l dextrose, 10 g/l yeast extract, 20 g/l peptone, and 15 g/l agar at 30 °C. Grown cells were stored at 4 °C for a few days (Munch et al., 2015).

Culture conditions and analysis of cell growth

R. diobovatum was grown on YPD agar plate at least for 2 days and re-streaked twice. Single colonies were picked and inoculated into 100 ml of nitrogen-limiting medium of Glucose Magnesium sulfate Yeast (GMY) broth containing 3 g/l yeast extract, 8 g/l KH₂PO₄, 0.5 g/l MgSO₄.H₂O, and 40 g/l glucose (Fisher chemicals, New Jersey, USA). Pre-cultures were grown aerobically at

30 °C, 150 rpm for 24 h in a rotary shaker (Series 25, New Brunswick Scientific Co, Inc., Edison, New Jersey, USA) using 500 ml baffled Erlenmeyer flasks (Munch et al., 2015). The optical density of cells was measured using a Spectrophotometer (CAT335905, Thermo Scientific Inc, Wilmington, Delaware, USA) at the wavelength of 600 nm (OD_{600}). All experiments were conducted in triplicate.

Samples (4 ml) for cell growth, Dry Cell Weight (DCW), glucose and glycerol consumption, and ammonium ion (NH_4^+) concentration were taken regularly every 6 h for the initial 30 h. Then, samples were taken at the time-points of 48, 72, 96, and 120 h post-inoculation (h pi). Monitoring of cell growth was carried out at OD_{600}, and cell biomass was harvested by the centrifugation of the samples at 2500 xg for 10 min using a Sorvall RC6 Plus centrifuge with a Sorvall SH-3000BK rotor (Thermo Scientific, USA). Cells were washed with deionized water and centrifuged; then the water was decanted. All the cell pellets were dried at 60 °C for 24 h and weighed to determine the DCW. All the supernatants were stored at -20 °C until required for substrate and ammonium ion concentration analysis. Ten ml of samples were taken every 24 h pi for lipid (5 ml) and carotenoid (5 ml) extraction and characterization.

Experiments using different C/N ratios by glucose and ammonium sulfate

The first treatment was GMY medium containing a C/N ratio of 50, which contained 3 g/l yeast extract, 8 g/l KH₂PO₄, 0.5 g/l MgSO₄.H₂O, 40 g/l glucose, and 1.51 g/l ammonium sulfate (Fisher chemicals, New Jersey, USA). Other C/N ratios were tested by varying the ammonium sulfate content of the medium as shown in Table 1. All other properties of the media were similar. For the calculations of the C/N ratio, the carbon content (mol%) in glucose was assumed to be 40% and the nitrogen content (mol%) in ammonium sulfate was assumed to be 21.205%. The basic GMY medium containing glucose was used. Cultures were inoculated from a fresh overnight culture, and cultivated in 1 L baffled Erlenmeyer flasks for 120 h in a rotary incubator at 130 rpm and 30 °C.

Analytical methods

Ammonium ion concentrations were quantified by using a Lachat Quick Chem 8500 instrument (Lachat instruments, Canada) according to Munch et al. (2015). The supernatants were diluted two times with deionized water and data was processed using Omnion 3.0 software (Lachat instrument, USA). Glucose concentrations at each time-point were quantified using a Waters Breeze High Performance Liquid Chromatography (HPLC), equipped with a 300 mm×7.8 mm HPX-87H Ion Exclu-

sion Column (Aminex, USA), maintained at 45 °C with 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.6 ml/min. All liquid samples were diluted 1:10 or 1:20 with deionized water to prevent overloading of the column and to produce clear peaks. Data were processed by Breeze 2 software (Waters, Breeze™ 2 HPLC, USA).

Lipid extraction and fatty acid composition

Lipids were extracted from lyophilized biomass by boiling 10-20 mg dried biomass in 2 ml of 15% concentrated H_2SO_4, methanol, and 2 ml of a solution of 0.5 mg/ml methylheptadecanoic acid (C17) as internal standard (Nu-Chek Prep Inc, USA), in chloroform (Fisher chemicals, New Jersey, USA), for 5 h. Then, we added 1 ml deionized water and separated the organic layer after an overnight. A 1 ml aliquot of Fatty Acid Methyl Esters (FAMEs) in chloroform was extracted and transferred into glass vials for FAMEs analysis. FAMEs in chloroform were analyzed using an Agilent 7890A Gas Chromatograph (GC) with a flame ionization detector with a split ratio of 1:10 and a split injector (Agilent Technologies, Inc. Wilmington, USA). Separation of target molecules was achieved using a DB-23 capillary column (30 m×320 µm×0.25 µm; Agilent, California, USA). The carrier gas was Helium, at a flow rate of 1.8 ml/min. The initial oven temperature was set at 75 °C for 4 min and was then increased from 75 to 250 °C with incremental increases of 20 °C/min, with a final hold at 250 °C for 4 min. We used the Supelco 37 component FAME mix (Sigma-Aldrich, Wilmington, USA) as a standard for peak identification and calculation different fatty acids.

Carotenoid extraction and quantification

For carotenoid analysis, 5 ml aliquots of cultured media were harvested at different time points by centrifugation (4500 xg, 10 min), and then washed with physiological water (3 times) and resuspended in 10 ml of distilled deionized water. Thereafter, the suspension was broken with glass beads (0.4 mm, 10%, w/v) for 10 min, with cooling (-18 °C) in a bead-beater (Vibrogen-Zellmuhle, Edmund Bubler, Tubingen, France). The bead-cell mixture was harvested by centrifugation (4500 xg, 10 min). After washing and decanting, the yeast cells were resuspended in 5 ml of ethanol and vortexed for 1 min. This procedure was repeated until the cells became colorless. The final samples were stored at -80 °C under vacuum in the dark by using an anaerobic incubator supplied with vacuum tank.

A standard curve of β-carotene was generated by analyzing a dilution series of β-carotene (Sigma Chemicals, USA) in ethanol, ranging from 0.01 to 10 mg/l. The maximum absorption wavelength of the β-carotene dilutions

was determined at 450 nm, using the scanning spectro-photometer function (from 422 nm to 501 nm) of a UV–vis Synergy HT microplate reader (Winooski, USA). The specific rates of production and concentrations of carotenoids synthesized by *R. diobovatum* were determined at different times post-inoculation by using a line of best fit to the standard curve.

Statistical analysis

All data presented in this work are reported as the means of three independently replicated experiments (biological replicates). Data were subjected to statistical analysis by Analysis of Variance (ANOVA) using IBM SPSS statistics software (version 24).

Results

R. diobovatum cultured in GMY media containing C/N ratios of 20 and 50 grew rapidly in the first 24 h pi, producing a cell mass of approximately 1.3 g/l (Figure 1). These cultures then grew at a slower rate up to 120 h pi. Cells growing in media containing a C/N=20 reached a final DCW of 2.74±0.12 g/l, whereas cells growing in media containing a C/N=50 reached a final DCW of 2.49±0.11 g/l. *R. diobovatum* cultured in GMY media containing a C/N ratio of 80 grew rapidly in the first 18 h pi producing a cell mass of approximately 0.87±0.01 g/l, followed by a slower rate of growth up to 120 h pi, where they reached a final DCW of 2.10±0.13 g/l.

The rate of substrate consumption varied between the three cultures condition (Figure 2). Cells growing in media containing a C/N=20 consumed glucose at a steady rate up to 100 h pi. At the end of the rapid growth phase at 24 h pi, the concentration of glucose in C/N=20 cultures was 35.27±0.24 g/l, and at 100 h pi, the concentration of glucose in these cultures was 28.83±0.24 g/l. The substrate was consumed at a much faster rate after 100 h pi. The final concentrations of glucose at 120 h pi was 16.09±0.29 g/l. Glucose was consumed rapidly in the C/N=50 and C/N=80 cultures up to 24 h pi, followed by a slower rate of consumption up to 120 h pi. The final concentrations of glucose were 18.4 g/l in the C/N=50 and C/N=80 cultures at 120 h pi.

R. diobovatum cultured in GMY media containing a C/N ratio of 50 produced the greatest concentrations of TAGs, whereas the cultures containing C/N ratios of 20 produced the lowest concentrations of TAGs (Figure 3). Interestingly, the C/N=50 and C/N=80 cultures synthesized most of their TAGs between 48 and 120 h pi, whereas the C/N=20 culture synthesized most of its TAGs between 48 and 72 h pi.

Under all three culture conditions, the fatty acid compositions of the TAGs synthesized by *R. diobovatum*

were similar (Table 2). The predominant fatty acids (>30% of the total TAGs production) in the C/N=50 and C/N=80 cultures were oleic acid (18:1) and linoleic acid (18:2). The other fatty acid compositions contained stearic and arachidic acids with <10%. Oleic acid and linoleic acid constituted >40% of the fatty acids synthesized by *R. diobovatum* in the C/N=20 culture while palmitic acid and arachidic acid constituted <6% of the fatty acids in this ratio.

Although β-carotene production by *R. diobovatum*

gradually increased in all three culture conditions between 48 and 96 h pi (Figure 4), cultures containing a C/N ratio of 50 produced significantly greater amounts of β-carotene (0.20±0.00 mg/l at 96 h pi) than the C/N=20 (0.03±0.00 mg/l at 96 h pi) and C/N=80 cultures (0.04±0.01 mg/l at 96 h pi). The β-carotene concentrations in the cells decreased in all culture conditions after 96 h. The decline in the β-carotene concentrations was 85.0%, 45.5%, and 46.05% in the C/N=20, C/N=50, and C/N=80 cultures, respectively.

Table 1: Levels of glucose and ammonium sulfate in *Rhodosporidium diobovatum* cultures, and carbon to nitrogen ratios used in the treatments

Treatment	Glucose (g/l)	Ammonium sulfate (g/l)
C/N 20	40	3.78
C/N 50	40	1.51
C/N 80	40	0.94
Control 1	40	0.00
Control 2	0	1.51

Table 2: Fatty acid composition of TAGs synthesized by *Rhodosporidium diobovatum* cultivated with different carbon to nitrogen ratios

Treatment	Palmitic acid C 16:0 (%)	Stearic acid C 18:0 (%)	Oleic acid C 18:1 (%)	Linoleic acid C 18:2 (%)	Arachidic acid C 20:0 (%)
C/N 20	4.90±0.8 [b]	6.14±0.2 [b]	38.99±0.1 [a]	44.01±0.2 [a]	3.93±0.4 [c]
C/N 50	20.93±0.0 [a]	9.90±0.0 [a]	35.31±0.8 [a]	24.90±0.07 [a]	6.98±0.2 [b]
C/N 80	23.31±0.1 [a]	9.04±0.3 [a]	36.26±0.5 [a]	23.61±1.1 [b]	7.41±0.3 [a]

[a, b, c] Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels of probability
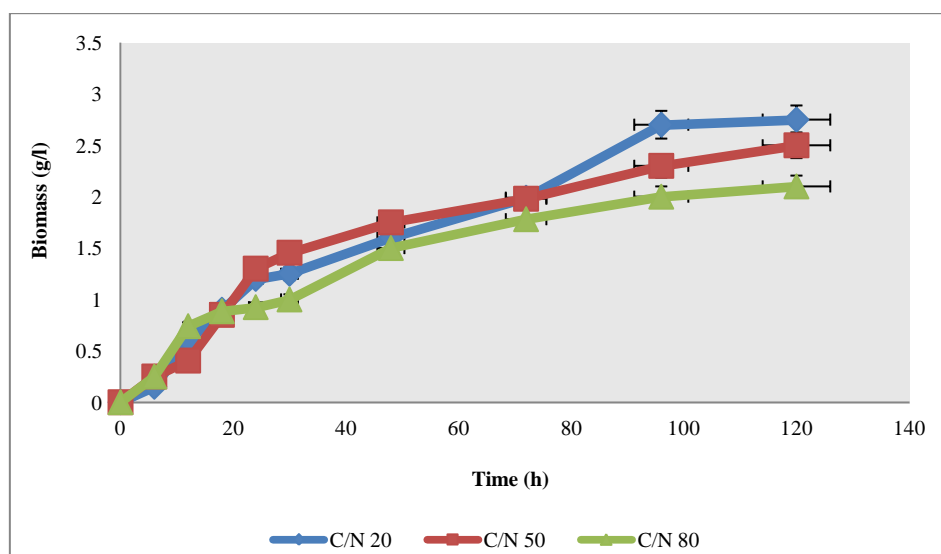

Figure 1: Biomass production by *Rhodosporidium diobovatum* during cultivation at different carbon to nitrogen ratios, initial glucose, and nitrogen contents. Data points are presented as the means of three independent biological replicate experiments. Vertical bars represent the standard error about the means, while horizontal bars represent the range of values observed
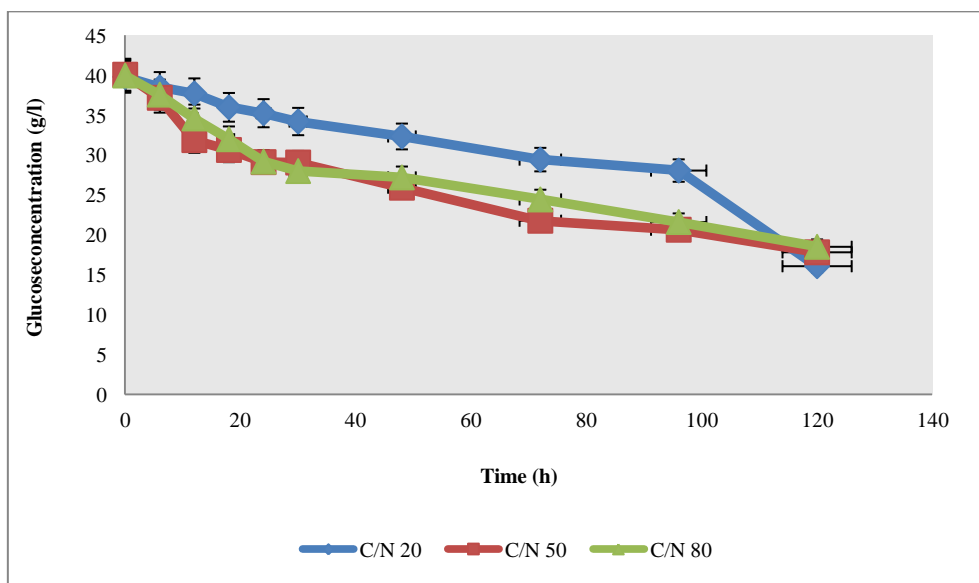
Figure 2: Glucose utilization by *Rhodosporidium diobovatum* during cultivation at different carbon to nitrogen ratios, initial glucose, and nitrogen contents. Data points are presented as the means of three independent biological replicate experiments. Vertical bars represent the standard error about the means, while horizontal bars represent the range of values observed
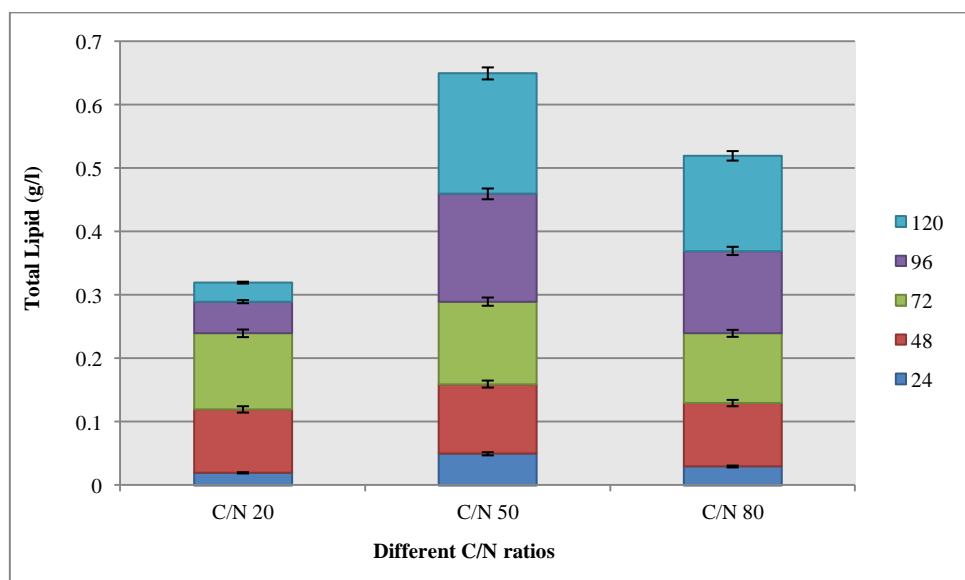

Figure 3: Temporal analysis of triacylglyceride yields in *Rhodosporidium diobovatum* cultured with different carbon to nitrogen ratios. Data points are presented as the means of three independent biological replicate experiments. Vertical bars represent the standard error about the means

Figure 4: β-carotene production of *Rhodosporidium diobovatum* cultivated with carbon to nitrogen ratios. Data points are presented as the means of three independent biological replicate experiments. Vertical bars represent the standard error about the means
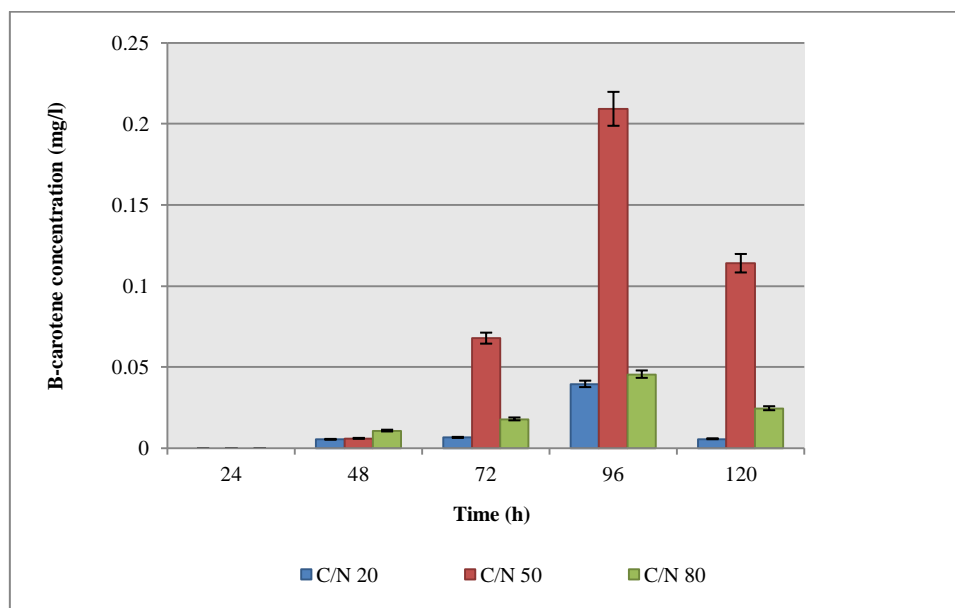
Discussion

In the present study, the production of biomass by the C/N=20 culture was greater than the amount of cell mass produced by the C/N=50 and C/N=80 cultures. Despite a lower rate of glucose consumption between 48 and 120 h pi, the C/N=20 culture displayed a sudden and dramatic increase in cell mass production between 72 to 96 h pi. This may be due to the high amount of ammonium sulfate (3.776 g/l) in the C/N=20 culture, compared to the other two culture conditions.

We showed that the greatest amounts of TAGs were synthesized by *R. diobovatum* cultured with a C/N ratio of 50, and that lower concentrations of TAGs were synthesized in cultures with both higher (C/N=20) and lower (C/N=80) concentrations of nitrogen. It is well established that C/N ratios can have a positive effect on TAGs synthesis by oleaginous yeasts (Chi et al., 2011; Papanikolaou and Aggelis, 2011). Our data also indicated that C/N ratio influenced the fatty acid composition of the TAGs synthesized by *R. diobovatum*. Oleic (C 18:1) and linoleic (C 18:2) acids were the dominant fatty acids in the TAGs synthesized by *R. diobovatum* under all three culture conditions. However, oleic and linoleic acids accounted for 30% of the fatty acids synthesized in the C/N=50 and C/N=80 cultures, and 40% in the C/N=20 cultures which same results were taken by

Munch et al. (2015). The Oleic (C 18:1) and linoleic (C 18:2) acids with 33% and 20% of total fatty acid compositions were the predominant fatty acids in their experiment. Correspondingly, TAGs synthesized by *R. diobovatum* cultures with high C/N ratios (high carbon/low nitrogen) contained greater amounts of saturated fatty acids.

In spite of presence of sufficient amount of glucose and an increase in the biomass, we obtained low overall lipid production which this finding could be attributed to the presence of nitrogen in the growth medium. Accordingly, not only lipid production process did not progress, but also glucose was used to produce more biomass instead of lipid production which is in agreement with the findings of Braunwald et al. (2013) who got the same results by growing the *Rhodotorula glutinis* in glucose as the carbon source.

Beneficial effects of unsaturated fatty acids on human health are obvious. Oleic acid, as omega-9 fatty acid, is known to reduce heart-related diseases such as arterial sclerosis, and could be act as an anticancer agent. Linoleic acid, as omega-6 fatty acid, has been shown to have positive effects for treatment of the diabetic neuropathy, rheumatoid arthritis, and dermatological disorders, such as psoriasis and eczema (Belury, 2002; Calder, 2015;

Koba and Yanagita, 2014). In the current investigation, the fatty acid profiles of the TAGs synthesized by *R. diobovatum* were similar to those of vegetable oils, which are the very nutritional importance in the food industry.

Similar to microbial lipids, microbial carotenoids are synthesized as secondary metabolites. Both lipid and carotenoid synthesis use acetyl-CoA as a precursor (Tinoi et al., 2005). While high C/N ratios have a positive effect on TAGs synthesis, the effect of C/N ratio on carotenoid production is not well documented in oleaginous yeasts. According to this study, the C/N ratio also had a significant influence on β-carotene synthesis by *R. diobovatum*. The greatest amount of β-carotene produced by *R. diobovatum* was observed in the C/N=50 cultures, whereas the C/N=20 and C/N=80 cultures produced much less β-carotene. A possible explanation for the level of β-carotene synthesis in the C/N=20 cultures, especially significant depletion between 96 to 120 h, may be the higher concentrations of nitrogen in the culture media resulted in more rapid growth, and consequently a lower pH. Another probable reason for the reduction of the β-carotene production in our study may be the high amount of ammonium sulfate which causes a drop in the synthesis of β-carotene's precursor, especially in the late stages of cultivation (Flores-Cotera et al., 2001). Some previous researchers showed that with decreasing nitrogen content in the media to the less than 1.0 g/l, the yeast cells tend to produce fewer carotenoids and higher lipids, because, the yeast desires to produce lipids instead of carotenoid with the available carbon (Aksu and Eren, 2007; Somashekar and Joseph, 2000).

Conclusion

Our work suggests that C/N ratio has a considerable influence on the synthesis of both β-carotene and TAGs by *R. diobovatum*. Moreover, the C/N ratio affected the fatty acid composition of TAGs. These findings may be useful in food industries in order to scale-up production of TAGs and β-carotene by *R. diobovatum*.

Author contributions

M.M. and D.B.L. designed the study and did the experimental works; M.M., S.S.S-D, and D.B.L. analyzed the data and wrote the manuscript. All authors read and confirmed the revised manuscript.

Conflicts of interest

All the authors declared that there was no conflict of interest in this research.

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