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Research Article

Biotechnological production of lipids and carotenoids from *Rhodosporidium toruloides* Y27012

Furat Alakraa[1], Ayşe Saygün[1], Neşe Şahin Yeşilçubuk[1*]

[1] İstanbul Teknik Üniversitesi, Kimya-Metalurji Fakültesi, Gıda Bölümü, İstanbul, Türkiye (ORCID: 0000-0002-1484-9721: 0000-0002-4179-1932)

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Abstract

Microbial oils are lipids formed by various microorganisms. Microorganisms that are able to accumulate lipids more than 20% of their biomass are named as "oleaginous microorganisms". These oleaginous microorganisms such as bacteria, yeasts, moulds, and algae are able to accumulate SCO (Single Cell Oil) during secondary metabolic growth. *Rhodosporidium toruloides* Y27012 is an oleaginous red yeast, which accumulates both lipids and carotenoids by using different carbon and nitrogen sources. The aim of this present study was to investigate the effects of different nitrogen, carbon sources, C/N (carbon to nitrogen) ratios, and some additives on cell growth, lipid accumulation and carotenoids production by *R. toruloides* Y27012. The maximum biomass, lipid accumulation and carotenoids yield were observed with yeast extract and glucose when used as nitrogen and carbon sources, respectively. *R. toruloides* Y27012 gave the highest values of both biomass and lipid content (53.41±0.93 g/L, 49.83±2.53 %) at C/N ratio of 60, whereas higher nitrogen levels such as C/N ratio of 20 enhanced the production of carotenoids (1001.51±17.87 µg/g). Among the tested additives, ethanol at 10 g/L increased the carotenoids yield up to 1732.17±39.45 µg/g comparing with the control sample at 1001.51±17.87 µg/g. Biomass and lipid contents were found to be higher when acetic acid at 5 g/L was added as an activator (41.97±1.02 g/L, 61.27%±1.77 g/L, respectively). Furthermore, optimization studies for lipid and carotenoids production from *R. toruloides* Y27012 could be achieved and also cost of fermentation could be reduced by using agro-industrial wastes as an alternative cheap carbon and nitrogen sources to produce value-added metabolites.

Keywords: *Rhodosporidiumtoruloides*, single cell oil, carotenoids, lipid accumulation, biomass.

Rhodosporidium toruloides Y27012 mayasından lipit ve karotenoidlerin biyoteknolojik yolla üretimi

Öz

Mikrobiyal yağlar, çeşitli mikroorganizmalar tarafından üretilen lipitlerdir. Biyokütlelerinin % 20'sinden daha fazla lipit üretebilme kapasitesine sahip mikroorganizmalara "oleosus mikroorganizmalar" adı verilmektedir. Bakteri, maya, küf ve algleri içerebilen bu oleosus mikroorganizmalar, sekonder metabolik büyüme sırasında Tek Hücre Yağı (THY) depolayabilmektedir. *Rhodosporidium toruloides* Y27012, farklı karbon ve azot kaynaklarını kullanarak hem lipitleri hem de karotenoidleri üretebilen kırmızı renge sahip oleosus bir mayadır. Bu çalışmanın amacı, farklı azot ve karbon kaynakları, farklı Karbon / Azot (K / A) oranları ve bazı katkı maddelerinin *R. toruloides* Y27012 mayasının gelişimi, lipit ve karotenoid üretimi üzerindeki etkilerini araştırmaktır. Maksimum biyokütle, lipit ve karotenoid verimi, azot ve karbon kaynakları olarak sırasıyla maya ekstraktı ve glukoz kullanıldığında elde edilmiştir. En yüksek biyokütle ve lipit değerleri (53.41 ± 0.93 g / L, % 49.83 ± 2.53) K / A oranı 60 olduğunda, maksimum karotenoid miktarı (1001.51 ± 17.87 ug / g) ise yüksek azot oranında (K / A= 20) elde edilmiştir. Test edilen katkı maddeleri arasında, 10 g / L'deki etanol, kontrol numunesi 1001.51 ± 17.87 ug / g olan karotenoid verimini, 1732.17 ± 39.45 ug / g değerine kadar artırmıştır. Aktivatör olarak 5 g / L'deki asetik asit eklendiğinde biyokütle ve lipit içeriğinin (sırasıyla 41.97 ± 1.02 g / L, % 61.27 ± 1.77 g / L) daha yüksek olduğu bulunmuştur. Ayrıca, *R. toruloides* Y27012 mayasından lipit ve karotenoid üretimi için optimizasyon çalışmaları gerçekleştirilebilir ve alternatif ucuz karbon ve azot kaynakları olarak tarımsal ve / veya endüstriyel atıklar kullanılarak katma değeri yüksek metabolit üretimi için fermantasyon maliyeti de azaltılabilir.

Anahtar Kelimeler: *Rhodosporidium toruloides*, tek hücre yağı, karotenoid, lipit birikimi, biyokütle

[*] Sorumlu Yazar: İstanbul Teknik Üniversitesi, Kimya-Metalurji Fakültesi, Gıda Bölümü, İstanbul, Türkiye, ORCID: 0000-0002-4179-1932, sahinnes@itu.edu.tr

1. Introduction

Microbial oils are oils that are formed using microorganisms such as bacteria, yeasts, fungi, and microalgae. Although all microorganisms have to synthesize minimum amount of lipid for their membranes and other structures, only small number of microorganisms can accumulate lipid more than 20% of their biomass. Therefore, they are named as "oleaginous microorganisms" (RATLEDGE, 2002). These oleaginous microorganisms are able to accumulate SCO (Single Cell Oil) during secondary metabolic growth in the media that contains excess amounts of carbon sources and limited amounts of nitrogen sources.

Examples of oleaginous species are yeasts, such as *Rhodotorula* sp., *Yarrowia lipolytica*, *Cryptococcus* sp., *Lipomyces* sp., *Rhodosporidium* sp. and *Trichosporon* sp. (RATLEDGE, 2002). Among these microorganisms, *R. toruloides* is able to accumulate lipids over 72%, which make it an excellent storage lipid producer (RATLEDGE, 2002; LI et al., 2007; WU et al., 2010). It can grow on many substrates such as glucose, xylose, also lignocellulosic hydrolysate, and excess sludge hydrolysate as well (LI et al., 2007; WANG et al., 2012).

The fatty acid profile of *R. toruloides* is very similar to that of vegetable oils like soybean oil. Such feature makes it attractive for the edible food and biodiesel industry (LI et al., 2007; LIU and ZHAO, 2007; ZHAO et al., 2011). Furthermore, SCOs are now attracting interest since they might include polyunsaturated fatty acids having nutritional, medical and dietetical importance (LI et al., 2007; PAPANIKOLAOU and AGGELIS, 2011; ECONOMOU et al., 2011).

In addition to lipids, *R. toruloides* can also produce carotenoids and biotechnologically important enzymes, such as cephalosporin esterase and epoxide hydrolase (BUZZINI et al., 2007; POLITINO et al., 1997). *R. toruloides* can produce carotenoids including predominantly torulene, torularhodin γ carotene, and β carotene (BERTACCHI et al., 2020; BUZZINI et al., 2007). Carotenoids are yellow to orange-red, lipid-soluble pigments that are ubiquitous in nature (WAITES et al., 2001). They act as membrane protective antioxidants that efficiently scavenge O_2 and peroxyl radicals. Their antioxidative efficiency is apparently related to their structure (GOODWIN and BRITTON et al., 1988). Carotenoids consist of carotenes (are strictly hydrocarbons) and xanthophylls (are oxygenated derivatives) (ARMSTRONG, 1994). Carotenoids can be used for a wide range of commercial applications because of their pharmaceutical, biotechnological and nutraceutical properties (FRENGOVA and BESHKOVA, 2009). There are several papers investigating carotenoid production from different strains. In a study conducted by Aksu and Eren (2005), different glucose concentrations were tested for carotenoids production by the yeast *Rhodotorula glutinis* (AKSU and EREN, 2005). And furthermore, Fontana et al. (1996) studied the production of carotenoids from *Phaffia rhodozyma*. They compared the use of depolymerized bagasse and raw sugarcane juice as a medium for astaxanthin production (FONTANA et al., 1996). PHAM et al. (2020) investigated the effect of light on carotenoid and lipid production by *R. toruloides* (PHAM et al., 2020). According to our literature research, this is the first study on the production of carotenoids from *R. toruloides* Y27012 strain.

In recent years, carotenoid production has become one of the most interesting applications in biotechnology. The usage of carotenoids that obtained from biological sources especially in the food industry is accelerating. Natural carotenoids attracts more attention against chemically synthesized ones. Therefore, microbial carotenoid production finds important applications in commercial area, the reason for which consumer preferences, cost effectiveness and safety issues are becoming an attractive matter. Also microbial production systems need to be improved for their industrial applications to become more competitive and economically viable because of some difficulties about biotechnological applications (MACHADO et al., 2019; BELLOU et al., 2014).

The objectives of this research were to investigate the effects of various nitrogen, carbon sources, different ratios of C/N, and additives on cell growth, lipid accumulation and carotenoids yield by *R. toruloides* Y27012. Furthermore, ultrasonic assisted and HCl assisted methods were carried out in carotenoids extraction from *R. toruloides* Y27012 and compared with a control method.

2. Material and Method

2.1. *Microorganism and culture conditions*

*R. toruloides*Y27012 strain was provided from the NRRL (Northern Regional Research Laboratory) Culture Collection, IL, USA. The microorganism was kept on the medium of Potato Dextrose Agar (PDA) (Fluka) at +4°C. The culture was sub-cultured twice a month (Hu et al., 2009). During the experiments the strain was cultivated in 250 mL erlenmeyers containing 50 mL of sterile growth medium at 30°C and 200 rpm. The composition of the medium was: 2 % glucose, 0.1 % KH_2PO_4, 0.05 % yeast extract and 0.02 % $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany) (BUZZINI et al. 2007; ZHAO et al., 2011).

The total biomass, lipid accumulation and carotenoids production by *R. toruloides* Y27012 were studied in a cultured medium as described above having glucose (40 g/L) as carbon source and different nitrogenous compounds such as yeast extract (3.72 g/L), peptone (3.38 g/L), and ammonium sulfate (1.73 g/L) as nitrogen sources at C/N ratio of 40:1. Then different carbon sources such as glycerol, and xylose were evaluated with yeast extract (3.72 g/L) as the sole nitrogen source at C/N ratio of 40:1. Besides, the growth conditions for *R. toruloides* Y27012were investigated for growth, lipid accumulation, and carotenoids yields with different C/N ratios of 60:1 and 20:1 by using different glucose concentrations and constant concentration of yeast extract (HU et al., 2009; LATHA et al., 2005). Additives were included at different concentrations to glucose and yeast extract containing growth medium at C/N ratio of 20:1 to

examine the effects of activators on the growth, lipid accumulation, and carotenoids production by *R. toruloides* Y27012 (AKSU and EREN, 2005; TAOKA et al., 2011).

Cultures were cultivated in 250 mL flasks containing 100 mL of the given medium. Each flask was inoculated with 2 mL (2% inoculum volume fraction) of 24 h exponential pre-culture (~ 3 x 10^8 cfu/mL) and incubated in a shaker (New Brunswick Scientific Co., Inc., USA) operating at 200 rpm and 30 °C. The incubation period was five days, since the optimal period for lipid and carotenoids production by *R. toruloides* strains was reported in literature (BUZZINI et al., 2007; LIU and ZHAO, 2007). The pH of the medium was not adjusted during fermentation.

2.1.1. Determination of Biomass

Biomass content was expressed as dry matter per liter of fermentation broth. Cells in 10 mL culture broth was washed twice with distilled water, and then dried at 80°C until obtaining constant weight (ECONOMOU et al., 2011; HU et al., 2009). All measurements were performed in triplicate.

2.1.2. Lipid Extraction

Total lipid that produced in the cells was extracted with chloroform:methanol (2:1; v/v) solvents and then solvents were removed by evaporation with a rotary evaporator (BuchiRotavapor RII, Switzerland) at 40 °C (FOLCH et al., 1957).

2.1.3. Carotenoids Extraction

Two methods of ultrasound assisted and HCl-assisted extraction were compared with the control extraction method, in which acetone was used for extraction, in terms of efficiency (GU et al., 2008). Glucose as carbon source (40 g/L) and yeast extract as nitrogen source (3.72 g/L) were used in the medium of the batch fermentation to have C/N ratio of 40:1. The extraction process of each method is described as follows:

(1) Ultrasound-assisted extraction procedure: The dried biomass of *R. toruloides*Y27012 was put into 250 mL Erlenmeyer and acetone was added at 40 mL/g (liquid /solid) ratio. Ultrasonic treatment procedure was done for 10 min. in ultrasonic crusher (VWR, 75 D, Radnor, PA, USA) at 0°C, the flask was kept in water bath at 100 rpm for 30 min at 28 °C in order to break the cells of yeast. The sample was centrifuged at 3305 g for 20 min to obtain the supernatant containing carotenoids (GU et al., 2008).

(2) HCl-assisted extraction procedure: The dried biomass of *R.toruloides*Y27012 was soaked into 3 M HCl solution at 28°C, shaking at 100 rpm for 30 min, and then centrifuged at 3305 g for 20 min. The supernatant was cast off, and then acetone was added to the broken cells at 40 mL/g (v/w). Thereafter, extraction of carotenoids was carried out at 100 rpm and 30°C for 30 min, and then centrifuged at 3305 g for 20 min (GU et al., 2008; DEMING et al., 2006; WANG et al., 2008).

2.1.4. Total Carotenoids Content

Total carotenoids content (measured as β-carotene) was determined by measuring the optical density at 480 nm with UV–Vis spectrophotometer (UV-1700, Shimadzu, Japan). Following equation was used to determine the carotenoids yield (GU et al., 2008).

$$\text{Carotenoids yield } x = \frac{1000\text{ADV}}{0.16*\text{W}} \text{ (µg/g dry biomass)}$$

Whereas; A is the absorption at 480 nm, D is the dilution ratio, V is the volume of acetone, 0.16 is the extinction coefficient of carotenoids, W is the weight of dry cell (g)

Statistical analysis

Data were statistically analyzed by ANOVA, General Linear Model, confidence level 95.0 Tukey test with MINITAB program.

3. Results & Discussion

3.1. Carotenoids Extraction Methods

In this work two different extraction methods including ultrasound (US)-assisted and HCl-assisted methods were employed and compared with the control method. Effects of extraction methods on the carotenoids yield are given in Figure 1.

It can be seen from Figure 1 that, HCl-assisted extraction of carotenoids was the most effective method with a carotenoids yield of 977±43.25 µg/g, whereas lower yields of carotenoids (394.71±73.95 µg/g and 544.98±32.10 µg/g) was obtained by ultrasound-assisted and control methods, respectively. Although, duration and intensity of US-assisted extraction procedure were sufficient, yield was the lowest in this method.

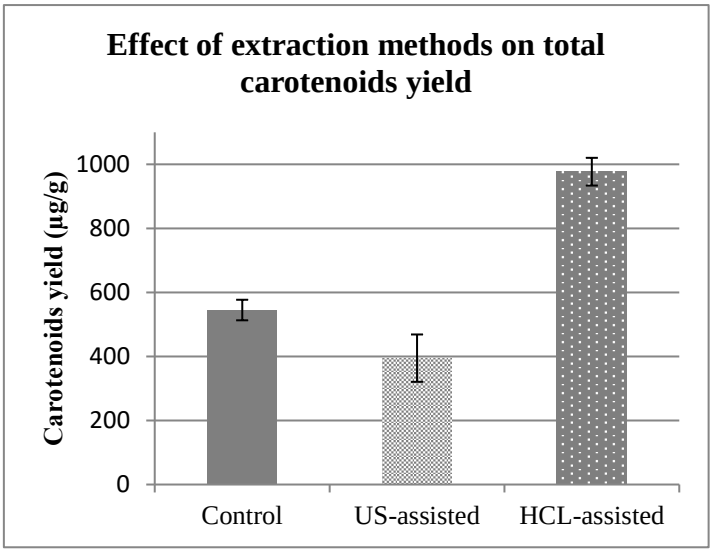

Figure 1. Effect of extraction methods on total carotenoids yield

Studies have been reported on extraction of carotenoids from different microorganisms by various cell disruption techniques (GU et al., 2008; MICHELON et al., 2012). In our study, HCl-assisted extraction revealed HCI-assisted extraction as a promising technique. In contrary, US-assisted extraction revealed lower efficiency for carotenoids. This might be due to the ineffective disruption of cell walls which prevented solubilization of carotenoids from *R. toruloides* cells getting into the acetone solution.

Gu et al. (2008) compared three methods such as grinding, US and HCl assisted extraction on carotenoids extraction yield from *Rhodobactersphaeroides* and as a result HCl-assisted method gave the highest yield of carotenoids such as 4650 µg/g, whereas US assisted extraction gave 664 µg/g carotenoids yield similar to our study (GU et al., 2008). In another study conducted by Michelonet et al. (2012), different techniques of cell disruption for extracting carotenoids produced by *Phaffia rhodozyma* NRRL Y-17268 were compared (MICHELON et al., 2012). Hurdle technologies of freezing biomass maceration with using diatomaceous earth and enzymatic lysis at pH 4.5 resulted with highest carotenoids concentration (190.35 µg/g) and carotenoid extractability (122.25%). More research involving different mechanical, chemical and enzymatic techniques is required to enhance extraction of carotenoids from *R.toruloides*Y27012 which contains high amounts of carotenoids (MICHELON et al., 2012).

3.2. Effect of nitrogen sources

Nitrogen sources promote growth of microorganisms; high concentrations of nitrogen sources also suppress the biosynthesis of lipids and other secondary metabolites. A number of researchers have tested the effects of nitrogen sources on biomass, lipid content, and carotenoids yield of various microorganisms (WANG et al., 2012; AKSU and EREN, 2005; SAENGE et al., 2011; BHOSALE and GADRE, 2001). In this study, the medium was supplemented with various nitrogen sources such as peptone and ammonium sulfate. The effects of the nitrogen sources on biomass, lipid content, and carotenoids from *R.toruloides* Y27012 are shown in Table 1. Organic or inorganic nitrogen sources were found to increase the lipid content, biomass and carotenoids production. The highest values for biomass, lipid content, and carotenoids yield were obtained with yeast extract when used as nitrogen source (35.41±1.28 g/L, 44.78±5.42 %, and 977.12±43.25 µg/g, respectively), followed by peptone (34.39±0.61 g/L, 27.46±0.59 %, and 626.08±63.21 µg/g, respectively).

Table 1. Effect of nitrogen sources on biomass, lipid content and carotenoids yield from R.toruloidesY27012

Index	Biomass (g/L)	Lipid content (%)	Carotenoids yield (µg/g)
Yeast extract	35.41±1.28[a]	44.78±5.42[a]	977.12±43.25[a]
Peptone	34.39±0.61[a]	27.46±0.59[b]	626.08±63.21[b]
Ammonium sulfate	34.29±1.94[a]	22.30±1.28[b]	334.22±57.76[c]

Results were expressed as mean ± SD deviations of triplicate measurements

C/N ratio=40, 40 g/l glucose, 3.72 g/l yeast extract, 3.38 g/l peptone, 1.73 g/l ammonium sulfate.

T= 30ºC, 200 rpm for 5 days

For the evaluation of nitrogen sources for biomass, lipid content and carotenoids yield, yeast extract was found to be the best source followed by peptone. Furthermore, biomass obtained with ammonium sulfate was comparable to those with yeast extract and peptone. This suggested that certain essential amino acids could be synthesized from inorganic nitrogen sources by *R. toruloides* Y27012. When the effect of yeast extract, ammonium sulfate, peptone and other nitrogen sources were investigated on the carotenoids and lipid production by *Rhodotorula glutinis*, yeast extract gave the highest biomass, lipid content and carotenoids concentrations as 6.33 g/L, 32.63%, and 129.94 mg/L, respectively (SAENGE et al., 2011).

Aksu and Eren (2005) used ammonium sulfate as the single nitrogen source for *Rhodotorulamucilaginosa*growth, and it had been noticed that total carotenoids production and organism growth rates increased with increasing initial ammonium sulfate concentration up to 2 g/L. Carotenoids concentration and yield were 63.0 mg/L and 4.2 mg/g, respectively AKSU and EREN, 2005).

3.3. Effect of Carbon Sources

Yeast metabolism depends on the type of carbon source present in the growth medium. Yeasts in general are able to utilize different carbon sources for the production of biomass, lipids and carotenoids. In this study, the effects of different carbon sources such as xylose and glycerol on the cell growth, lipid accumulation and carotenoids production of *R. toruloides* Y27012 were examined. Table 2 indicates the variations in dry biomass, lipid content and carotenoids yield with the change in carbon sources.

Table 2. Effect of carbon sources on biomass, lipid content and carotenoids production from R.toruloidesY27012

Index	Biomass (g/L)	Lipid content (%)	Carotenoids yield (µg/g)
Glucose	35.41±1.28[a]	44.78±5.42[a]	977.12±43.28[a]
Xylose	34.62±1.23[a]	41.77±7.92[a]	732.89±45.83[a]
Glycerol	34.93±1.39[a]	44.32±4.59[a]	692.73±95.53[a]

Results were expressed as mean ± SD deviations of triplicate measurements

C/N ratio=40, 3.72 g/L yeast extract, T= 30ºC, 200 rpm for 5 days

R. toruloides is known as biogenic yeast and the results presented here confirm its ability to accumulate over 40% of its biomass as lipid. However, lipid accumulation was strongly affected by the nature of the carbon source provided. It was noticed from this research that there was no clear difference between lipid content among glucose, xylose and glycerol sources (Table 2).

Utilization of different sugars as carbon source by *R. toruloides* Y27012 for its pigmentation shows that highest carotenoids yield was 977.12±43.28 µg/g when glucose was used as carbon source at a C/N ratio of 40:1.

In case of carbon sources, glycerol, xylose and glucose had similar results on lipid content. Wiebe et al. (2012) compared the lipid content from *R. toruloides* CBS14 between two carbon sources; glucose and xylose. They showed that glucose increased biomass and lipid content (9 g/L, 56%), whereas when xylose was used as carbon source biomass and lipid content was 8 g/L and 45%, respectively (WIEBE et al., 2012).

To our knowledge, this is the first study on production of carotenoids from *R. toruloides* Y27012, so comparing the results with *Rhodotorula glutinis* at different glucose concentrations showed that carotenoids formation enhanced with the increase in glucose concentration. The highest carotenoids production yield of 13.8 mg/g was observed by *R. glutinis* at 20 g/L glucose content (Aksu and Eren, 2005). Varying the carbon sources in the culture medium affected carotenoids production by *R. glutinis* mutant 32. Glucose yielded a proportionally higher production of carotenoids comparing with glycerol (9.8 mg/L, 2.9 mg/g) and xylose (23.9 mg/L, 2.2 mg/g) (BHOSALE and GADRE, 2001).

3.4. Effect of C/N Ratios

Generally, microbial lipid production by yeasts requires growth medium with high amount of carbon source and limited amount of nitrogen source (RATLEDGE, 1988). Thus, microbial lipid production capacity is significantly affected by the C/N ratio of the culture. At a high C/N ratio, excess C source is used to produce microbial lipids (SAENGE et al., 2011). In this study, the highest biomass and lipid contents were obtained at a C/N ratio of 60 and a nitrogen concentration of 3.72 g/L (53.41±0.93 g/L biomass, 49.83 ±2.53 % lipids of the dry biomass). At lower C/N ratio of 20, biomass content was 24.76±1.78 g/L and lipid content was 40.28 ±2.44 % as seen in Table 3.

Table 3. Effect of C/N ratios on biomass, lipid content and carotenoids yield from R.toruloidesY27012

Index	Biomass (g/L)	Lipid content (%)	Carotenoids yield (µg/g)
C/N=20	24.76±1.78[a]	40.28±2.44[a]	1001±17.87[a]
C/N=40	35.41±1.28[b]	44.78±5.42[a]	977.12±43.28[a]
C/N=60	53.41±0.93[c]	49.83±2.53[a]	445.38±40.88[b]

Results were expressed as mean ± SD deviations of triplicate measurements

Glucose as carbon source, 3.72 g/l yeast extract, T= 30ºC, 200 rpm for 5 days

The influence of C/N ratio on carotenoids production has remained elusive until now. In this study it was observed that increasing C/N ratios caused decrease in carotenoid yield. At C/N ratio of 20, carotenoids yield (1001±17.87 µg/g) was higher than carotenoids yield (445.38±40.88 µg/g) at C/N ratio of 60. C/N ratio of 20 was statistically different from 40 and 60 ratios by means of carotenoids yield.

Since carotenoids and lipid production share acetyl CoA as common precursor, it cannot be excluded that there are certain interactions between both production pathways at certain C/N ratios. Park (2005) indicated that the best carotenoid production occurred at a C/N ratio of approximately 44.5 by *Rhodotorulaglutinis* whereas C/N ratio of 31.6 gave the highest biomass (PARK et al., 2005). Somashekar and Joseph (2000) found that a medium with a high C/N ratio tended to produce lipids rather than carotenoids (SOMASHEKAR and JOSEPH, 2000). Also, the C/N ratio of 44.5 observed in the study of Park (2005) for maximum carotenoids production differed from the results of Somashekar and Joseph (2000). They investigated carotenoids production from semi-defined minimal salts media with three different C/N ratios by the yeast *R. gracilis*, and found that a C/N ratio of 10 favored maximum carotenoids production. This result might be due to the different types of medium and yeast strain used (SOMASHEKAR and JOSEPH, 2000).

In another study conducted by Libkind and Brook (2006), low C/N ratio enhanced biomass yield while different C/N ratios did not have any effect on carotenoids yield from *Rhodotorulamucilaginosa* CRUB 0138 (LIBKIND and VAN BROOCK, 2006.

In another research conducted by Libkind et al. (2004), maximum carotenoids yield (2.32 mg/L) were obtained from *Rhodotorulamucilaginosa* CRUB 0138 at C/N of 5 in culture medium containing 10 and 40 g/L glucose, respectively. Different C/N ratios did not influence carotenoid pigment production but low C/N ratio enhanced biomass yield (LIBKIND et al., 2004). The optimized conditions showed that cell growth required low C/N, whereas highest C/N caused increase in lipid content and carotenoids production by *R. glutinis*, at C/N of 170 the lipid content and carotenoids production were 39.74 g/L, 148.77 mg/L, respectively (SAENGE et al., 2011).

It was observed in the medium at different C/N ratios between 10/1 and 70/1, that C/N ratio had no effect on cellular pigment accumulation by *R.glutinis* mutant 32. However, a low ratio resulted in high volumetric production of carotenoids (33 mg/L, 2.90 mg/g) due to high cell mass yield (BHOSALE and GADRE, 2001).

There are many studies that considered C/N factor in lipid production from yeasts. Nitrogen amount should be taken into consideration, because C/N ratio was important for lipid production. C/N ratio of 70 was enough for lipid production, and a higher C/N ratio gave higher lipid content. However, lipid productivity could be decreased when nitrogen sources were extremely scarce (PAPANIKOLAOU and AGGELIS, 2011).

The results have showed that the culture with a C/N molar ratio of 14.3 gave a slightly higher biomass but a lower lipid yield than those with a C/N ratio of 22.3. Both biomass and lipid contents decreased as culture C/N molar ratios went down. Lipid contents almost held constant at around 60% even the C/N molar ratio reduced to 6.1 (WU et al., 2010).

3.4. Effect of Additives

Some agents such as surfactants and detergent additives are known to have ability to increase lipid production (KIM et al., 2006). Although the mechanism is not completely understood, these additives can cause variations in membrane fluidity. In this study, a number of surfactants and solvents including Tween 20 and 80 at 0.1 % and 1 % concentrations, ethanol (10 g/L) and acetic acid (5 g/L) were tested for enhancement of growth, lipid and carotenoids production by *R. toruloides* Y27012 cultivated on glucose and yeast extract.

Effects of Tween 20, Tween 80, ethanol and acetic acid on biomass, total lipid and carotenoids yield are given in Table 4. It can be concluded that, when Tween 20 and Tween 80 were added at a level of 0.1 %, carotenoids yield decreased (606.50±30.16 µg/g, 754.68±33.65 µg/g, respectively) along with a little increase in lipid content (42.38±1.36 %, 41.96±3.01 %, respectively) comparing with the control. It can be noticed that lipid contents reached to 44.92±5.44 % and 41.34±7.94 %, respectively with Tween 80 and 20 at 1 % (v/v). Also an increase in carotenoids yield was observed as 1014.46 ±68.44 µg/g when Tween 80 was used in the medium with glucose, yeast extract, and C/N of 20 as can be seen in Table 4. Ethanol at 10 g/L causes an increase in both lipid content and carotenoids yield (45.21%±0.87, 1732.17±39.45 µg/g, respectively) with lower biomass (13.33±0.05 g/L) comparing with control sample as seen in Table 4. Acetic acid also gave the highest lipid content as 61.27±1.77 % among the additives tested (Table 4).

Table 4. Effect of additives on biomass, lipid content and total carotenoids yield

Index	Biomass(g/L)	Lipid content (%)	Carotenoids yield (µg/g)
Control	24.76±1.78[a]	40.28±2.44[a]	1001.51±17.87[a]
0.1 %Tween 20	35.41±1.30[b]	42.38±1.36[a]	606.50±30.16[b]
0.1% Tween 80	28.60±2.80[c]	41.96±3.01[a]	754.68±33.65[c]
1 %Tween 20	25.00±1.13[a]	41.34±7.94[a]	1002.79±46.87[a]
1% Tween 80	24.00±0.46[a]	44.92±5.43[a]	1014.46±68.44[a]
Ethanol (10 g/L)	13.33±0.05[d]	45.21±0.87[a]	1732.17±39.45[d]
Acetic acid (5 g/L)	41.97±1.02[e]	61.27±1.77[b]	NR

Results were expressed as mean ± SD deviations of triplicate measurements, NR not released

C/N ratio=20, Glucose as carbon source, 3.72 g/L yeast extract, T= 30ºC, 200 rpm for 5 days

The results of effects of Tween 80 on carotenoids production are consistent with the results of Aksu and Eren (2007). In their study, addition of Tween 80 has increased total carotenoids concentration (AKSU and EREN, 2007). Tween 80 and Tween 20 not only act as carbon sources but also act as enhancer of nutrient uptake into the cell bodies, meaning that the permeability of the cell membrane increases (TAOKA et al., 2011).

An increase in both lipid content and carotenoids yield was observed when ethanol was used as an additive. Taking into consideration that the theoretical yield of lipid production per 1 g of ethanol is 0.54 g; it can also be considered as a very proper additive since no residual carbon arises from its uses in fermentation processes (RATLEDGE, 1988). Detailed studies revealed that ethanol activates oxidative metabolism with induction of HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase, which in turn enhances carotenoids production. Similarly, many studies reported increased carotenoids production upon addition of ethanol to cultures of many types of yeasts (KIM et al., 2003; GU et al., 1997). Ethanol has been considered as a potential substrate for the *de novo* lipid production of the oleaginous microorganisms since they have the ability to utilize ethanol. With the presence of alcohol dehydrogenase, yeast converts ethanol to acetaldehyde, and acetaldehyde dehydrogenase enzyme converts acetaldehyde to acetate that will be further converted to acetyl-coA (RATLEDGE, 1988).

Among the additives tested; acetic acid gave the highest lipid content. Acetic acid has been equally considered as substrates for SCO production in many studies. Roux et al. (1995) had used acetic acid as a carbon source and the lipid content observed was 47 % of the biomass (ROUX et al., 1995). The major reasons for this improved lipid production would appear to originate from both acetic acid and ethanol feeding directly into the pool of acetyl-CoA which is necessary for lipogenesis. In glucose-grown cells, the main flux of carbon involves glucose uptake, glycolysis, transport of pyruvate into the mitochondria, conversion of pyruvate into citrate, transport of citrate into the cytosol, and cleavage of citrate by adenosine triphosphate (ATP): citrate lyase to acetyl-CoA (RATLEDGE and WYNN, 2002).

4. Conclusions and Recommendations

The strain of *R. toruloides* Y27012 was cultivated on various carbon and nitrogen sources with different C/N ratios and in the presence of various additives. The effects of nitrogen, carbon sources, different C/N ratios, and some additives on cell growth, lipid accumulation and carotenoids production from *R. toruloides* Y27012 were determined by measuring dry biomass, lipid and carotenoids yield.

According to ANOVA analysis of the results, there was no significant difference between nitrogen sources on biomass production but nitrogen sources had significantly affected the lipid and carotenoid production. Among carbon sources, there was no significant difference on biomass, lipid and carotenoid production. C/N ratio was found to be important on biomass and carotenoid production, in contrast there was not any significant effect on lipid contents. Additives, except 1% Tween 80 and Tween 20, changed the biomass and carotenoid content significantly while only acetic acid was found to be statistically important on lipid content.

The results showed that HCl-assisted extraction method of carotenoids was found to be the most effective method for carotenoids extraction from *R. toruloides* Y27012. Among the nitrogen and carbon sources that were investigated, yeast extract and glucose were found as the best sources for cell growth, lipid accumulation, and carotenoids yield. Highest biomass and lipid production was favoured with high C/N ratios whereas lower C/N ratios caused high carotenoids production. Lipid content and carotenoids yield were increased when Tween 80 and Tween 20 were added at 1 % (v/v) and at C/N ratio of 20. Ethanol (10 g/L) when used as an additive also caused an increase in carotenoids production.

Further studies should focus on the optimization of temperature, light, pH, and aeration for lipid and carotenoids production from *R. toruloides* Y27012 since there is limited research on this subject. On the other hand, agro-industrial wastes and by-products such as

whey, fruit waste extracts, sugar cane juice and sugar beet molasses, corn hydrolysate can be used in the growth medium as low-cost substrates and these substrates should be explored as alternative cheap carbon and nitrogen sources.

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