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ARTICLE

SELECTION OF CULTURE MEDIA FOR THE PRODUCTION OF CAROTENOIDS WITH ANTIOXIDANT ACTIVITY BY *RHODOTORULA GLUTINIS*

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Abstract: *Rhodotorula glutinis is an aerobic yeast with particular metabolic characteristic that can produce large amount of carotenoids during the stationary growth phase. Carotenoid is an important natural pigment with antioxidant properties, which is used in food, pharmaceutical and cosmetics industry as additives. The aim of this work is to study the production of carotenoid with antioxidant activity by R.glutinis R12 in different media conditions. The selected strain was cultivated in three different culture media such as YM broth, Basal and MS3 medium at 28^{0}C for 72–120 hours. After fermentation, cells were harvested by centrifugation and freeze-dried. Carotenoid from the biomass was extracted as a mixture of DMSO, acetone and petroleum ether with a ratio of 1:2:2 and cells were ruptured using ultrasonic wave. The carotenoid content in the supernatant was measured by spectrophotometric method. The highest content of carotenoids extracted from R.glutinis R12 was 283.71μg/g dry biomass. The maximum antioxidant activity of carotenoid by DPPH assays were achieved 52.09 ± 0.4% (IC_{50}= 536.02) and at a concentration of 600 μg ml-1. This study revealed that the R.glutinis R12 strain has the ability to produce carotenoid and has shown antioxidant activity in Basal and MS3 medium. For further study, it is necessary to investigate the improvement of carotenoid yield from R.glutinis R12 strains.*

Keywords: *Rhodotorula glutinis; carotenoids; antioxidant activity; culture media;*

INTRODUCTION

Rhodotorula which belongs to Basidiomycota phylum; *Urediniomycetes* class and *Sporidial* order are the main carotenoid producing microorganisms with predominant synthesis of β-carotene, torulene and torularodin [18]. The genus *Rhodotorula* includes three active species; *Rhodotorula glutinis, Rhodotorula minuta and Rhodotorula mucilaginosa* (formerly known as *Rhodotorula* rubra) [14]. *R.glutinis* often called "pink yeast" is a free living, non-fermenting, unicellular yeast found commonly in nature and is

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Vol. 58 No 04 (228) 2018
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particularly important for food industries because of their biotechnological potential and safety implications. Moreover, they are widely known as a good source of proteins, lipids and vitamins [23].

Carotenoid pigments represent the largest and most diverse class of natural products known to mankind. Nowadays, over 700 structures have been reported [5] and they are natural pigments that can be synthesized by various microorganisms, including bacteria, yeasts, filamentous fungi [3] and microalgae [12]. Carotenoids are derived from isoprene, formed by forty carbon chains whose main characteristic is the presence of a long chain polyene (where the presence of double bonds can range from three to fifteen) responsible for the color perceived by the human eye. Carotenoids represent a group of valuable molecules for the pharmaceuticals, medicine, cosmetics, food and feed industries, not only because they can act as vitamin A precursors, but also for their coloring, antioxidant, and possible tumor-inhibiting activity, and also, enhancement of the immune response leading to protection against bacterial and fungal infections [17], [13]. Moreover, Carotenoids may serve as a protection against many chronic diseases such as cancer, age-related Macular Degeneration, and cardiovascular diseases and also act as an excellent antioxidant system within cells [27]. Interest in carotenoids has recently increased due to the growing demand for such compounds in many similar industries [28]. The global market demand for carotenoids grows 2.9% per year, with estimated annual sales of about US$300 million in synthetic carotenoids [11], [29]. The global carotenoids market was estimated to be valued at USD1.24 billion in 2016 and projected to reach USD1.53 Billion by 2021, at a CAGR of 3.78% from 2016 to 2021.

Commercial production of carotenoids from microorganisms competes mainly with synthetic production by chemical procedures. Carotenoids used industrially are mainly obtained chemically or by extraction of plants or algae. However, due to the concern about the use of chemical additives in foods, there is increasing interest in carotenoids obtained naturally through biotechnological processes and the microbial carotenoids have attracted much attention in recent years [25]. Therefore, the yeast stands out as a natural source of carotenoids.

Rhodotorula mucilaginosa, Rhodotorula slooffiae and *Rhodotorula glutinis* strains were previously isolated from fruits, soil and insects in Mongolia [2], [22], [25].

But there are not studies and reports on antioxidant activity of carotenoid pigment extracted from Rhodotorula strains isolated from Mongolia. Therefore, we aimed to study carotenoid producing *Rhodotorula glutinis* R12 with antioxidant activity.

MATERIALS AND METHODS

Yeast strain: *Rhodotorula glutinis* R12 strain was supplied from the Culture Collection of the Laboratory of Microbial Synthesis, Institute of General and Experimental Biology, Mongolian Academy of Sciences.

Media: YM broth (10g glucose, 5g peptone, 3g yeast extract and 3g malt extract per liter), Basal medium (20g glucose, 4g yeast extract, 1g KH_2PO_4 and 0.5g $MgSO_4 \times 7H_2O$ per liter) and MS3 medium (30g glucose, 1.5g yeast extract, 5g NH_4NO_3, 1g KH_2PO_4, 0.4g $MgSO_4 \times 7 H_2O$, 0.4g NaCl and 0.4g L-alanine per liter) were used for the cultivation. YM agar, Yeast morphology agar, Yeast Nitrogen base, Yeast Carbon base and Vitamin free medium were used to verify the morphological, physiological and biochemical characteristics

Morphological, physiological and biochemical characterization: Morphological characteristics of the cultures such as shape, cellular dimension and type of cell division

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were examined by microscopic analysis of the strain grown in YM broth and colony characteristics were observed through culture grown in Yeast morphology agar medium at 28°C for 24 hours. After the determination of the morphological characteristics, tests were carried out to verify the principal physiological and biochemical characteristics, according to Barnett et al., 1990.

Inoculum: A single colony from the stock culture on YM agar was transferred to 50 ml of YM broth and incubated in a shaker at 150rpm, at 28°C for an overnight period. The cells in the medium were counted by microscopy in a Fuchs – Rosenthal chamber (Blaubrand, Germany).

Cultivation: The inoculum (10^8 cells/ml) were inoculated with 5% (v/v) in 500ml Erlenmeyer flask containing 100 mL of YM broth, Basal medium and MS3 medium respectively and then incubated in a rotary shaker at 150 rpm, 28°C for 72, 96 and 120 hours respectively.

Determination of dry cell biomass: 100 ml of yeast suspension was centrifuged for 20 minutes at 3000 rpm, washed twice with distilled water, and again centrifuged under same condition. Then the pellet was dried in freeze-dryer (BK – FD18PT, Biobase Biodustry, Shandong, China). Cell biomass was measured by the dry cell weight method [15].

Cell disruption technique: The combination method for cell disruption was used: chemical disruption with DMSO (dimethyl sulfioxide) and ultrasonic wave used as mechanical technique. 1ml DMSO was added in 0.01g dried yeast biomass and this mixture was stood for one hour and 2 ml of acetone added. The mixture was homogenized at 40 kHz for 20 min by using ultrasonic (08895-51 ultrasonic bath, Cole – Parmer Instrument company, Vernon Hills, USA). After the disruption, 2 ml of petroleum ether was added to the mixture [20].

Extraction and determination of pigment: To extract carotenoid from the cells were used a solvent mixture of DMSO, acetone and petroleum ether. 1ml of DMSO and 2ml of acetone and 2ml of petroleum ether was added in 0.01g cell dry biomass separately. The yeast cells were disrupted by ultrasonic wave. Upon standing for 25-30 min, the extracts were centrifuged and the supernatants were filtered through a 0.45µm membrane filter and collected. The extracts were decanted into small separator funnels. Upon addition of 1 mL of 20% NaCl solution, a clear-cut separation of the two phases occurred and the presence of carotenoid pigments was evident as the upper petroleum ether layer was colored yellow, orange, pink or intense red. The absorbance spectra at 474 nm were recorded on a UV-VIS spectrophotometer (UV-1600PC Spectrophotometer, Mapada Instruments Co., Ltd, Shanghai, China) and calculated by using Equation 1, as follows:

$$C = (A \times V \times 10^6)/A_{1cm}^{1\%} * 100 * m$$

Where, C = total concentration of carotenoids (µg/g); A = absorbance; V = volume (mL); m = dry cell mass (g); $A_{1cm}^{1\%}$ = specific absorptivity [8], [9].

Determination of antioxidant activity: The free radical scavenging activity was measured by the 2-2-diphenyl-1-picrylhydrazyl (DPPH) method as described by Loo et al. (2008). Different dilutions of the carotenoid extracts (1ml) were added to 2 ml of DPPH (5.9 mg/100 ml methanol) and allowed to react at room temperature for 30 min. The absorbance of the mixtures was measured at 517 nm, after 30 min of incubation in the dark. A control was prepared without sample or standard and measured immediately at 0 min – the lower the absorbance of the reaction mixture, the higher free radical scavenging activity. The percentage of the DPPH scavenging effect was calculated by using Equation 2, as follows:

Vol. 58 No 04 (228) 2018
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DPPH scavenging effect (%) = (Acontrol −Asample/Acontrol) × 100

Where Acontrol is the absorbance of the control reaction containing all reagents except the compound tested. Asample is the absorbance of the test compound. The concentration of extract needed to scavenge 50% of DPPH free radicals was calculated from the graph-plotting inhibition percentage against extract concentration [7].

RESULTS AND DISCUSSION

The stock culture of R.glutinis R12 was kept at -80^{0}C and it was inoculated in YM broth medium for the investigation of morphology, physiology and biochemical characteristics. By a microscopic analyses of the yeast culture after 24 h, the production of the spores and pseudohyphae were not observed. The reproduction is budding. The colonies with pink-red color, round and smooth were grown in the YM agar medium (Figure 1).

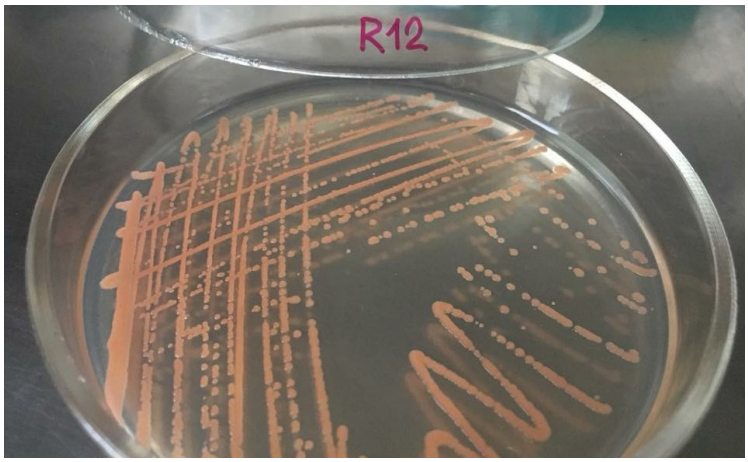

Figure 1. The colonies of R.glutinis R12 were grown in the YM agar.

Carbon and nitrogen assimilation is an important criteria in the taxonomy and identification of yeasts, which depends on organic carbon sources for their energy supply and growth, the carbohydrates being the sources of greater importance. *R.glutinis* R12 was assimilated mono- and disaccharides such as glucose, fructose, maltose mannose, respectively, also glycerol and ethyl alcohol and assimilated all nitrogen sources used in this study. But other sugars and sugar alcohols were not assimilated, expect ethanol (Table 1). It was previously reported that Rhodotorula glutinis did not assimilate carbon source such as melibiose, lactose, erythritol, starch, myo-inositol and methanol and creatine as carbon source, and did not grow at 42^{0}C [1].

Table 1. Physiological and Biochemical characteristics of R.glutinis R12

Characteristics	*R.glutinis*	*Characteristics*	*R.glutinis*	*Characteristics*	*R.glutinis*
Fermentation	-	*Inulin*	-	*25^{0}C*	+
Glucose	+	*Cellulose*	-	*30^{0}C*	+
Fructose	+	*Glycerin*	+	*37^{0}C*	+
Lactose	-	*Dulcitol*	-	*42^{0}C*	-

Vol. 58 No 04 (228) 2018
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Maltose	+	*Mannitol*	-	*Vitamin free*	+
Sucrose	+	*Methanol*	-	*Urease*	+
Xylose	-	*Ethanol*	+	*Starch formation*	-
Rhamnose	-	$(NH_4)_2SO_4$	+	*50% - Glucose*	-
Melibiose	-	KNO_3	+	*60% - Glucose*	-
Mannose	+	$NaNO_3$	+	*0.01% cyclohex.*	-
Starch	-	20^0C	+		

(-) Negative test, (+) Positive test

The yeast culture was inoculated in three different media (MS3, YM broth and Basal medium) and cultivated for 5 days (Figure 2). Stationary phase of all the cultures in the different culture media was obtained between 72 to 120 hours. *R.glutinis* has the ability to produce carotenoid pigments during the stationary phase [12]. Therefore, it was not necessary to incubate for more than 120h. The further assays were studied in those times.

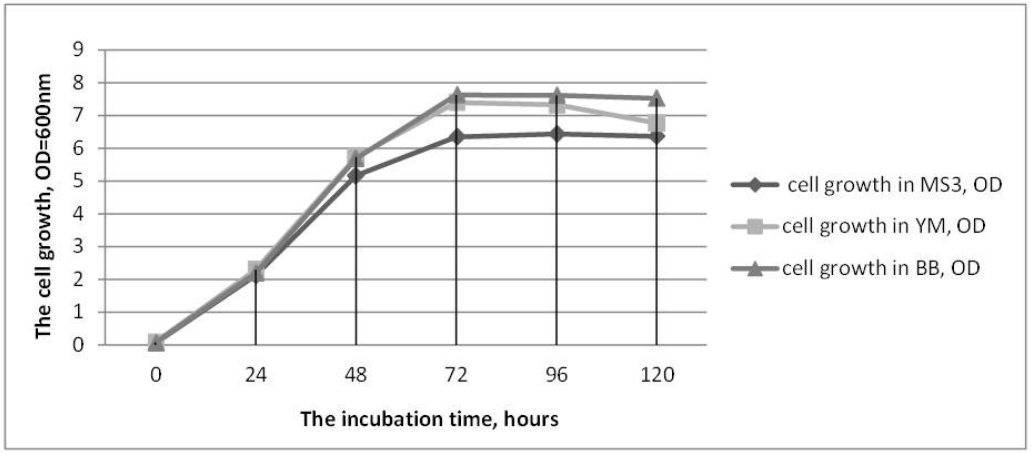

Figure 2. The cell growth of R.glutinis R12 during 5 days fermentation in different media

During the fermentation, the amount of biomass and total carotenoid of the strains were evaluated by dry cell weight and spectrophotometric methods respectively (Figure 3). The dried cell biomass and the carotenoid content in the produced extract fluctuated between 5.4±0.14 – 8.76±03 g/L and 97.46µg/g (0.7 mg/L) - 284.6µg/g (2.49 mg/L) respectively, depending on the culture medium and incubation time. The highest biomass yield (8.76±03 g/L) and the carotenoid content (284.6µg/g) were obtained from Basal medium culture after 120h. Many studies of carotenoids by strains of *R.glutinis* have been reported. Maximum yield (5.95 mg/L of total carotenoids culture fluid, 630 µg/g dry cell weight) was obtained after a batch culture of 120h in a substrate containing concentrated rectified grape must as the sole carbohydrate source [6]. The mutant of *R.glutinis* produced β-carotene by getting up to 2.2 mg of carotenoids/L in 72 h [4]. On the other hand, Turkan et al reported that carotenoid concentration obtained from *R.glutinis* strains was between 0.23 and 1.23 mg/L [26].The results in this study obtained moderate value to compare other studies.

Carotenoid pigment has the ability to act as antioxidants and thus protect cells against photooxidation. The ability of carotenoids to quench singlet oxygen is well known and reactions with radical species have also been studied [10]. Antioxidant activities of extracts showed highest carotenoid content were studied by the DPPH radical scavenging method. The results are shown in Table 2.

Vol. 58 No 04 (228) 2018
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https://www.mongoliajol.info/index.php/PMAS

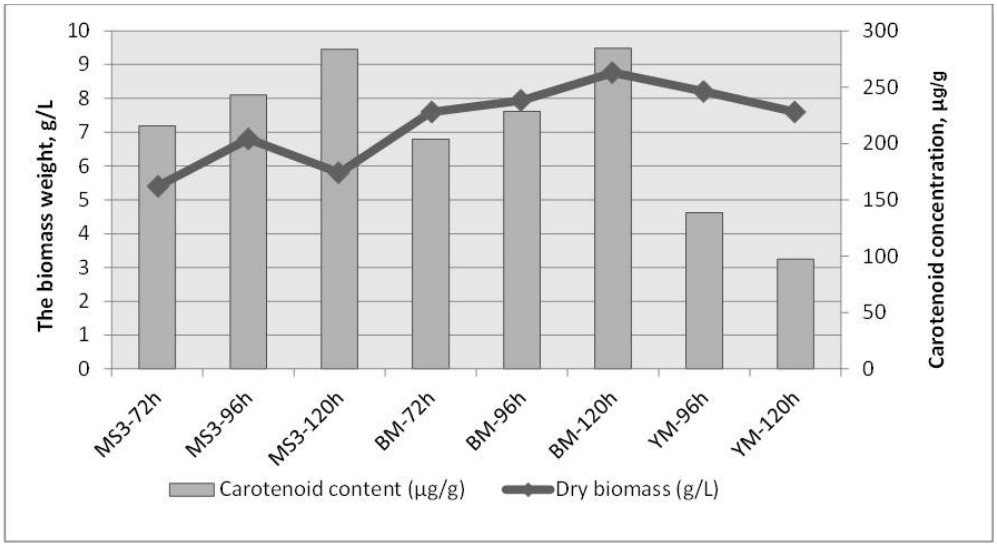

Figure 3. Carotenoid content and biomass yield of R.glutinis R12 in the different culture media and at different time.

Table 2. Antioxidant activity of carotenoid extracts from R.glutinis R12

Cultivated media	Carotenoid extract concentration, µg/ml	DPPH radical scavenging activity, %	IC_{50}, µg/ml
Basal medium	600	52.09 ± 0.4	**536.02**
	300	42.29 ± 0.48	
	150	30.28 ± 1.06	
MS3 medium	600	51.96 ± 0.26	568.75
	300	33.15 ± 0.2	
	150	20.75 ± 0.81	

mean±SD (n=3), IC_{50} sample concentration required scavenging 50% of the DPPH radical, µg/ml

The maximum antioxidant characteristics of carotenoid by DPPH radical scavenging assays were achieved 52.09±0.4%(IC_{50}=536.02) at the concentration of 600 µg/ml by pigmentation of *R.glutinis* R12. Michalowska and Stachowiak reported that highest percentage of DPPH scavenged radicals was recorded in pigment produced from *Phaffia rhodozyma* for an addition of 0.05% carotenoid extract (94.58%) [21]. Pigmentation of *Sporobolomyces sp.* at a concentration of 100 µg ml-1 exhibited 75.04% [18].

Further study needs to be done to improve the strain and it is possible to use the carotenoids extracted from strains of *Rhodotorula glutinis* in feed and foods as natural preservatives as well as colorants.

CONCLUSIONS

This study revealed that *R.glutinis* (R12) is able to produce up to 284.6µg/g carotenoid pigments under 120h cultivation in the Basal medium, and these pigments have a significant antioxidant effect. The maximum antioxidant activity was 52.09 ± 0.4% at a concentration of 600 µg $ml^{-1}$and sample concentration required scavenging 50% of the DPPH radical was 536.02µg ml-1.

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