

Optimal Conditions for Carotenoid Production and Antioxidation Characteristics by *Rhodotorula rubra*

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Abstract—This study aims to screen out and to optimize the major nutrients for maximum carotenoid production and antioxidation characteristics by *Rhodotorula rubra*. It was found that supplementary of 10 g/l glucose as carbon source, 1 g/l ammonium sulfate as nitrogen source and 1 g/l yeast extract as growth factor in the medium provided the better yield of carotenoid content of 30.39 µg/g cell dry weight the amount of antioxidation of *Rhodotorula rubra* by DPPH, ABTS and MDA method were 1.463%, 34.21% and 34.09 µmol/l, respectively.

Keywords—Carotenoid, *Rhodotorula rubra*, Antioxidation, DPPH, ABTS

I. INTRODUCTION

CAROTENOIDS are a group of bioactive compounds and responsible for bright yellow/orange colours of various plants, microorganisms and animals [1]. It has been found that carotenoids can inhibit various types of cancer and guard one from other important “lifestyle-related” diseases, such as cardiovascular disease and age-related macular degeneration due to their antioxidant activity and provitamin A function. [2-6]. It confers a characteristic colouration to some birds, crustaceans and salmon. It has been increasingly used as a feed and food pigment in the aquaculture industry, and has also been regarded as a potential functional food and pharmaceutical supplement, because of its excellent antioxidant activity [7-8]. However, most of the carotenoids sold in the market are derived from chemical synthesis and cannot meet consumers’ desire for natural carotenoids. Thus, researchers shifted attention from chemical synthesis to isolation of carotenoids from biological sources such as *Chlorella zofingiensis* [9] and *Haematococcus pluvialis* [10] for green microalgae study, *Rhodotorula mucilaginosa* [11], *Rhodotorula rubra* [12] and *Phaffia rhodozyma* [13-14] for yeast study, *Gibberella fujikuroi* [15] for filamentous fungi study, and *Rhodospirillum rubrum* [16-17], and *Rhodobacter sphaeroides* for bacteria study. Effective methods for carotenoids extraction from biological sources have been investigated [18-19]. Yeast, *Rhodotorula rubra*, can be the source of carotenoids. It is in Kingdom Fungi, Phylum Basidiomycota, Class Urediniomycetes, Order Sporidiales, Family Sporidiobolaceae, Genus *Rhodotorula* and Species *rubra* [20-22]. *R. rubra* has a rapid growth rate and is mature in four days.

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The colony is usually pink, but can also be orange-red to yellow. Colony on sabouraud agar is yeast-like, soft and smooth. On YM agar at 25 °C for 72 h incubation, the microscopic morphology shows oval or round budding cells and occasionally, a few rudimentary pseudohyphae. No ascospores are present. [23]. The genus *Rhodotorula* includes three active species; *R. glutinis*, *R. minuta*, and *R. mucilaginosa* which is the current name for the species formerly known as *R. rubra*. Since, it has been shown that the type strain of *R. rubra* is actually a strain of *R. glutinis* and the type strain of *R. mucilaginosa* is identical to the type strain for *R. rubra*. [20-21]. In recent years, antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in many diseases.[24]. Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems have drawn on the attention of many experimental works. Since then it has been proven that these mechanisms may be important in the pathogenesis of certain diseases and ageing. Many synthetic antioxidant components have shown toxic and mutagenic effects, which have shifted the attention onto the naturally occurring antioxidants. Their use has mainly centred around prevention, and the maintenance of health.[25]. Antioxidants are emerging as prophylactic and therapeutic agents. These are the agents, which scavenge free radicals otherwise reactive oxygen species and prevent the damage caused by them. Free radicals have been associated with pathogenesis of various disorders like cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders and are implicated in aging. Several antioxidants like SOD, CAT, epigallocatechin-3-*O*-gallate, lycopene, ellagic acid, coenzyme Q10, indole-3-carbinol, genistein, quercetin, vitamin C, vitamin E and carotenoids have been found to be pharmacologically active as prophylactic and therapeutic agents for above mentioned diseases. Antioxidants are part of diet but their bioavailability through dietary supplementation depends on several factors.[24]. Free radicals are highly reactive molecules or chemical species containing unpaired electrons that cause oxidative stress, which is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage” [26]. Antioxidants are substances which counteract free radicals and prevent the damage caused by them. These can greatly reduce the adverse damage due to oxidants by crumbling them before they react with biologic targets, preventing chain reactions or preventing the activation of oxygen to highly reactive products [27]. Although, the *R. rubra* used in this study was originally considered a high-carotenoids producing strain [7], its carotenoids content is still too low for commercial application. Recently a few carotenoids hyper producing yeast strains have been attained through conventional and modern strain development methods, which can accumulate carotenoids at much higher levels and have a greater commercial potential.

Nevertheless, the results derived from the *R. rubra* in the study will still be useful for efficient carotenoids production with the other yeast strains. Ultimately, the study is to attain better understanding of the physiological conditions and an efficient process strategy for carotenoid production and antioxidation characteristic of carotenoids by *R. rubra* production. This study aims to screen out and to optimize the major nutrients (Carbon and nitrogen sources) and growth factor (Yeast extract and Peptone) simultaneously for maximum carotenoid production and antioxidation characteristics (DPPH, ABTS and MDA method) by *Rhodotorula rubra*.

II. MATERIALS AND METHODS

A. Microorganism and Culture Conditions

The *Rhodotorula rubra* was used in this study, which was kindly provided from Maejo University. It was later identified, to be *Rhodotorula rubra* by the Faculty of Associated Medical Sciences, Chiangmai University. The basal medium for routine liquid culture contained 10.0 g glucose, 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 2.0 g KH_2PO_4 , 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.0 g yeast extract (YE) (per litre), with the pH adjusted to 5.5 (buffered by 0.1 M potassium hydrogen phosphate).

For stock culture, the yeast was sub cultured on agar slop (basal medium + 15 g/l agar) and incubated at 30 °C for 72 h. It was kept in the refrigerator. The continuous sub culture was done in every 3 months.

For starter culture, one loop of the yeast was transferred from the culture slop into 100 ml of basal medium contained in 250 ml Erlenmeyer. The flask was rotated on the rotary shaker at 250 rpm, and 30 °C for 72 h.

To investigate carbon, nitrogen and growth factor sources, the liquid culture was done in 250 ml Erlenmeyer shake flasks, each was filled up with 100 ml medium and incubated on the rotary shaker at 250 rpm, 30 °C. Each of the flasks was inoculated with 5 per cent starter culture. Three replication experiments were carried out.

B. Effect of Carbon, Nitrogen Sources and Growth Factor on Growth of *Rhodotorula rubra* and Carotenoid Production

Glucose and sucrose as carbon source, ammonium sulfate and chloride as nitrogen source, and yeast extract and peptone as growth factor were employed in this investigation. The carbon source concentration was 10 g/l, the nitrogen source concentration was 1 g/l, and the growth factor source concentration was 1 g/l. Culture media, 100 ml in 250 ml flask, were incubated on the rotary shaker at 250 rpm, 30 °C for 72 h [28]. The cells of *Rhodotorula rubra* were collected, and analyzed for carotenoid contents and antioxidation characteristics of its carotenoids as previously mentioned. Triplicate experiments were carried out.

1. Measurement of carotenoid content

Yeast cells were separated from the liquid medium by centrifugation at 5,000 rpm. 10 min, and rinsed twice with deionized water, and then freeze dried. The carotenoid content was extracted from the yeast and determined for carotenoid content by Foss method. [29].

2. Measurement of Antioxidation characteristic of *Rhodotorula rubra*

The carotenoids were extracted from the yeast and determined for antioxidation characteristics were as follows :

- 2,2-Diphenyl-1-picrylhydrazyl (DPPH free radical scavenging assay), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS radical cation decolorization assay) and Malondialdehyde (MDA assay) were used to determine antioxidant activity of carotenoids produced by *Rhodotorula rubra* that is modified by Foti *et al.*, Re *et al.*, and Satos *et al.* [30-32].

3. Statistical Analysis

Experimental data were subjected to analysis of variance using the Completely Randomized Design (CRD). Duncan 's New Multiple Range Test was used to identify significant differences among mean of treatments.

III. RESULTS AND DISCUSSION

A. Effect of Carbon Source on growth of *Rhodotorula rubra* and Carotenoid Production

Glucose and sucrose were used as supplementary carbon for carotenoid production. The concentration of 10 g/l was employed. The results were shown in table 1

TABLE I
PRODUCTION OF CAROTENOIDS BY *RHODOTORULA RUBRA* IN BASAL MEDIUM WITH GLUCOSE OR SUCROSE SUPPLEMENTATION AND THEIR ANTIOXIDATION CHARACTERISTICS

Carbon sources	Carotenoid Content (µg/g cell dry weight)	Antioxidation Characteristics		
		DPPH (%)	ABTS (%)	MDA (µmol/l)
Glucose	15.63 ^a	0.778 ^a	3.605 ^a	22.57 ^a
Sucrose	12.39 ^b	0.352 ^b	1.484 ^b	21.40 ^b

From Table I, it was found that glucose was more efficient on carotenoid production. Maximum carotenoid production of 15.63 µg/g cell dry weight was obtained when 10 g/l glucose was supplemented. *Rhodotorula rubra* grew well in yeast malt extract medium (Glucose 10 g/l) and produced 30.679 µg / g (cell dry weight) of carotenoids. [29]. The optimal ratio of molasses to water for carotenoid production by *Rhodotorula rubra* in molasses medium, was 1 to 20. The supplementation of 5 per cent (w/v) sucrose in the medium provided the better yield of carotenoid content of 164.54 µg / g cell dry weight [11]. In general, increase in sugar concentration in the growth medium increased the growth of yeast and carotenoid formation. In 20 g/ l molasses sucrose-containing medium, the yeast showed the maximum growth (4.2 g/l) and produced 89.0 mg/l carotenoids with a 21.2 g/l carotenoid production yield while the maximum growth and carotenoid formation parameters were obtained as 2.4 g/l dry biomass concentration, 70.0 mg/l carotenoids with a 29.2 carotenoid production yield in 6.6 g/ l whey lactose-containing medium. The ability of *R. mucilaginosus* yeast for growing on a variety of carbon sources, such as glucose, sucrose, and lactose is a remarkable advantage. When compared with the results obtained with other yeasts in the literature, the high carotenoid productivity of the yeast also

suggests a feasible process [22,33]. Thus, the yeast *R. mucilaginosa* will be one of the most promising microorganisms for the commercial production of carotenoids by the use of agricultural wastes as a cheap carbon source. The highest carotenoid concentration (125.0 mg total carotenoids per liter of fermentation broth) was obtained when 20 g/l molasses sucrose was used as the carbon source while the highest product yield based on the maximum cell concentration (35.5 mg total carotenoids per gram of dry cells) was achieved when 13.2 g/l whey lactose was the carbon source in the broth.[11]. Maximum antioxidation characteristics of this produced carotenoids determined by DPPH, ABTS and MDA method (0.778%, 3.605% and 22.57 $\mu\text{mol/l}$) was obtained when 10 g/l glucose was supplemented.

B. Effect of Ammonium Source on Growth of *Rhodotorula rubra* and Carotenoid Production

Ammonium sulfate and ammonium chloride were used as supplementary nitrogen source for carotenoid production. The supplemented concentration of 1 g/l was employed. The results were shown in table II

TABLE II
PRODUCTION OF CAROTENOIDS BY RHODOTORULA RUBRA IN BASAL MEDIUM WITH AMMONIUM SULFATE OR AMMONIUM CHLORIDE SUPPLEMENTATION AND THEIR ANTIOXIDATION CHARACTERISTICS

Nitrogen sources	Carotenoid Content ($\mu\text{g/g}$ cell dry weight)	Antioxidation Characteristics		
		DPPH (%)	ABTS (%)	MDA ($\mu\text{mol/l}$)
Ammonium sulfate	28.31 ^a	1.168 ^a	14.43 ^a	27.43 ^a
Ammonium chloride	21.69 ^b	0.722 ^b	10.35 ^b	23.80 ^b

Table II, it was found that ammonium sulfate was more efficient on carotenoid production than that of ammonium chloride. Maximum carotenoid production (28.31 $\mu\text{g/g}$ cell dry weight) was obtained when 1 g/l ammonium sulfate was supplemented. An initial ammonium sulphate concentration of 2 g/l gave the maximum carotenoid production by *Rhodotorula mucilaginosa* (89.0 mg total carotenoids per liter of fermentation broth).[34]. Maximum antioxidation characteristics of the produced carotenoid determined by DPPH, ABTS and MDA method (1.168%, 14.43% and 27.43 $\mu\text{mol/l}$) was obtained when 1 g/l ammonium sulfate was supplemented.

C. Effect of Growth Factor on Growth of *Rhodotorula rubra* and Carotenoid Production

Yeast extract and peptone were used as supplementary growth factor for carotenoid production. The supplemented concentration of 1 g/l was employed. The results were shown in table III.

TABLE III
PRODUCTION OF CAROTENOIDS BY RHODOTORULA RUBRA IN BASAL MEDIUM WITH YEAST EXTRACT OR PEPTONE SUPPLEMENTATION AND THEIR ANTIOXIDATION CHARACTERISTICS

Growth factor	Carotenoid content ($\mu\text{g/g}$ cell dry)	Antioxidation Characteristics		
		DPPH (%)	ABTS (%)	MDA ($\mu\text{mol/l}$)

	weight)			
Yeast extract	30.39 ^a	1.463 ^a	34.21 ^a	34.09 ^a
Peptone	26.32 ^b	1.018 ^b	18.12 ^b	27.25 ^b

From Table III, it was found that yeast extract was more efficient on carotenoid production in basal medium than that of peptone. Maximum carotenoid production (30.39 $\mu\text{g/g}$ cell dry weight) was obtained when 1 g/l yeast extract was supplemented. Maximum antioxidation characteristics of carotenoids by DPPH, ABTS and MDA method (1.463%, 34.21% and 34.09 $\mu\text{mol/l}$) were achieved when 1 g/l yeast extract was supplemented. It was reported elsewhere that many yeast fermentation companies in Korea used ammonium sulfate and urea as nitrogen source.[35]. Yeast extract has been successful in culture media for microorganism studies in milk and other dairy products. [36] Several media containing Yeast extract have been recommended for cell culture applications. Yeast extract provides vitamins, nitrogen, amino acids, and carbon in microbiological and cell culture media. [37]

Glucose, ammonium sulfate and yeast extract have been screened out as the significant factors affecting on carotenoid biosynthesis for *R. rubra* in shake-flask cultures. The optimal levels of glucose and ammonium sulfate for cell growth are very different from those for carotenoid biosynthesis. The cell growth required relatively high concentrations of carbon and nitrogen sources for carotenoid production, while carotenoid biosynthesis required much lower concentrations of glucose and ammonium sulfate. A possible explanation for the low optimal nutrient concentrations required for carotenoid biosynthesis is that carotenoids as the secondary metabolites of *R. rubra* are mainly synthesized when the cells are under stress (such as nutrient limitation) and the cell growth (primary metabolism) is suppressed. [38]. This point is in accordance with the timing of significant carotenoid biosynthesis after the rapid growth and during the stationary growth period. [38].

Sugars such as glucose in the culture medium provides both the major energy source for cell metabolism and the carbon element for biosynthesis of biomolecules. However, excessive glucose has been found to repress the carotenoid synthesis due to the so-called Crabtree effect [38,39]. For example, an initial glucose concentration 50 g/L or higher led to a marked decrease in the astaxanthin synthesis and increase in the accumulation of ethanol and organic acids (*Phaffia rhodozyma*). [40]. Nitrogen source, such as ammonium sulfate, is another major nutrient which has been shown to affect the growth and carotenoid production of several *Xanthophyllomyces dendrorhous* mutant strains [41]. The marked decrease in cell growth was concomitant with an increase in the astaxanthin content of cell as the C/N ratio of medium was increased in *Phaffia rhodozyma* cultures, and rapid biomass growth was coupled with lower activities of the key enzymes involved in astaxanthin synthesis. [42].

They then suggested that abundant nitrogen in the medium might enhance the cell growth but suppress the enzymes for β -carotene conversion to astaxanthin. The proposed that high initial C/N ratio might decrease the NADPH consumption for primary metabolism such as protein synthesis, so as to leave more NADPH available for astaxanthin biosynthesis. [43].

In mycelia cultures of *Gibberella fujikuroi* fungus, while nitrogen feeding increased the cell growth, nitrogen limitation stimulated carotenoid biosynthesis, perhaps by imposing C/N imbalance and driving most of assimilated carbon to the secondary metabolism pathways [15]. Yeast extract is another significant factor affecting on the carotenoid production in *R. rubra* cultures found in this and many previous studies. Most previous studies have chosen and identified yeast extract 1 g/l for cell growth and carotenoid biosynthesis in the cultures of wild *R. rubra* strains [44,45]. Both the timing of carotenoid accumulation after the rapid cell growth phase and its low nutrient requirement are apt for two-stage production process (or fed-batch process with different nutrient compositions for growth and product formation). An effective fed-batch strategy is to grow the culture first to a high cell density and then switch to conditions favorable for product accumulation [46]. Fed-batch processes have been exercised for astaxanthin production in *Xanthophyllomyces dendrorhous* cultures, however, only selected nutrients were fed to a spent culture medium rather than complete medium replacement in these studies [39, 43]. As productivity is a major index for the efficiency of a bioprocess, a higher productivity means a higher efficiency. A higher volumetric yield or concentration of product in the bioreactor is also most desirable and favorable for a bioprocess as it may decrease the cost for concentration and recovery of product at the downstream.

The results of the antioxidation capacity assessment of the studied *R. rubra* yeast as determined by DPPH, ABTS and MDA assays are shown in Table 3. These differences could be explained by different mechanisms of analytical method. ABTS and DPPH assays are based on the reduction of ABTS [7] and DPPH free radicals [47], but values from DPPH assay might be lower than those from ABTS assay. Wang et al. [48] showed the some compounds which have ABTS scavenging activity may not show DPPH scavenging activity. Arts et al. [49] found that ABTS scavenging reaction may have a higher antioxidant capacity and can further react with remained ABTS radicals.

A newly-isolated strain of *Rhodotorula rubra*, which has been reported to have the ability to synthesize high concentrations of carotenoids, has been adapted and grown in liquid phase using acid extracts of peat as the main substrate source. The growth of the yeast was studied as a function of the medium carbohydrate concentration, pH, time, temperature, and agitation rate. The optimal growth conditions were found to be an initial total carbohydrate concentration of 15 g/l, a pH of 5.5, an incubation time of 3.5 day, a temperature of 22 °C, and an agitation rate of 200 rpm. Under those conditions, the growth parameters were: approximately 4.8 g/l dry biomass, 70 per cent yield and 32 per cent efficiency. Also, a total β -carotene concentration of 1,256 μ g/g dry biomass was produced by the yeast. [50].

In addition, the proximate analysis of *Rhodotorula rubra* was analyzed. It was found that it contained 164.540 μ g/g (cell dry weight) of carotenoids, 39.123 per cent of protein, 3.142 per cent of lipids, and 24.335 per cent of carbohydrates. [29]. *Rhodotorula rubra* NRRL Y-15596 was found to produce the highest amount of carotenoids. The maximal yield of total carotenoids expressed as β -carotene was 1,041 μ g/l or 131 μ g/l yeast dry weight after 48 h of growth at 30 °C in Sauerkraut brine with an initial pH of 5.0. [51].

IV. CONCLUSION

The optimal culture conditions for carotenoid biosynthesis in *R. rubra* cultures, it was found that 10 g/l glucose as carbon source, supplementary of 1 g/l ammonium sulfate as nitrogen source and 1 g/l yeast extract as growth factor in the medium provided the better yield of carotenoid content of 30.39 μ g/g cell dry weight.

The antioxidant capacity of carotenoids obtained from *Rhodotorula rubra* was determined by three different methods : Malondialdehyde (MDA assay), improved ABTS radical cation decolorization assay, and DPPH free radical scavenging assay. It was found that at the optimal condition, it provided the better the antioxidation characteristics by DPPH, ABTS and MDA method were 1.463%, 34.21% and 34.09 μ mol/l, respectively.

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