Biosynthesis and profiling of single cell carotenoids of *Phaffia rhodozyma* in waste-based cultivation media

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Abstract

In order to investigate the feasibility of low-cost media for producing well-characterized single-cell carotenoid, the study on biosynthesis and profiling carotenoid in the yeast of *Phaffia rhodozyma* was carried out. We have successfully identified the profiles of single-cell carotenoids of *P. rhodozyma*, which was cultivated in glucose-based medium (MG), molasses-based medium (MM), and coconut water-based medium (MC). Cells were separately cultured in those media under aerobic batch culture system to obtain the carotenoid profiles based on high performance liquid chromatography (HPLC) analysis. The results showed that medium composition strongly affects the profiles of *P. rhodozyma* carotenoids represented by ratio of astaxanthin and beta-carotene (ratio A/B). Astaxanthin was highly synthesized in cells cultivated in MG with ratio A/B as much of 20:1. On the other hand, MM and MC produced a lower ratio A/B than MG as much of 0.4:1 and 0.2:1, respectively. In addition, carotenoids profiles were detected more diverse when this yeast species was cultivated in two waste-based media. This study provided a basic physiological knowledge of *P. rhodozyma* cells for carotenoid biosynthesis using potential low-cost cultivation media.

Keywords: β-carotene, astaxanthin, *Phaffia rhodozyma*, single cell carotenoid, waste-based medium

Introduction

Carotenoids are refered as tetraterpenoids, organic pigments commonly synthesized by photosynthetic and nonphotosynthetic organisms. These substances have a commercial interest as coloring agents for pharmaceuticals, food, animal feed, and cosmetics. Some of them were suggested to prevent chronic diseases because of their antioxidant and anticarcinogenic properties (Krinsky et al. 1993, Miller et al. 1996). Several carotenoids was succesfully produced by chemical synthesis for the commercial purposes (Barreiro & Barredo 2018, Bogacz-Radomska & Harasym 2018, Mata-Gómez et al. 2014). Unfortunately, several countries avoid the use of synthetic carotenoids for food or feed supplements. It was

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reported that the United States of America for Food and Drug Administration (FDA) rejected use of synthetic astaxanthin (3,3’- dihydroxy-β, β-carotene- 4,4’-dione) as feed supplement for fish quality improvement (Sinnot 1988).

Mass production of microbial cell carotenoids could fulfill the organic carotenoids requirements, since microbes are easier to be modified under controlled condition (Nelis et al. 1991). Phaffia rhodozyma is promising yeast for astaxanthin production. This species several advantages properties including high yield of astaxanthin, growing at high rate and utilizing various saccharides under both aerobic and anaerobic conditions. Considering those physiological aspects, P. rhodozyma is economically for astaxanthin production in large scale. Fermentation of P. rhodozyma is reliable methods for natural astaxanthin production (Johnson 2003, Hui et al. 2007). Therefore, the optimal fermentation condition is important for astaxanthin production at the industrial scale.

An important issue of commercial astaxanthin production for microbes is the availability of nutrient sources. The important nutrient are carbon and nitrogen to formulate sufficient carbon/nitrogen (C/N) ratio. Natural carbon sources are promising nutrient especially byproduct or waste materials, such as coconut water and molasses. Coconut water is easily found in tropical country and contains about 4 g carbohydrate per litter and become a waste of virgin coconut oil (VCO) production. Meanwhile, molasses is known as byproduct of sugar processing and contain high sugar. Both coconut water and sugar is a potential carbon source candidate for cultivating yeast in order to yield optimum single cell carotenoids. There is limited information about the use of molasses and coconut water as nutrient source in the relation to carotenoid production. The purpose of this study was to investigate the biosynthesis and profiles of single cells carotenoid of P. rhodozyma in waste-based media.

Materials and methods

Strain and maintenance

Phaffia rhodozyma (Xanthophyllomyces dendrorhous) was maintained on YMA slants (10.0 g/L glucose, 3.0 g/L malt extract, bacto-peptone, 5.0 g/L, 3.0 g/L yeast extract, and 20.0 g/L agar). The culture was stored at 4°C until used.

Production of single cell carotenoids

Three cultivation media including glucose medium and two waste-based media, i.e. molasses medium containing coconut water with 4% green bean sprout (tauge) extract and 2% molasses (MM); and coconut water medium which consists of coconut water and 4 % sucrose (MC) were used in this study. Glucose medium in this study refers to Yeast Malt broth (3 g/L yeast extract; 3 g/L malt extract; 5 g/L bacto-peptone and 10 g/L glucose) standard medium for carotenoid productionPhaffia rhodozyma grown at 22°C in 500 mL flasks with 250 ml of Glucose Medium (MG), Molasses Medium (MM) and Coconut water Medium (MC) broth, and shake on a rotary shaker at 150 rpm for 168 h. Sample collection was conducted in every 24 h.

Biomass measurements

Measurement of the cell growth was conducted based on the dry cell weight according to method of Schroeder & Johnson (1993). In the corning tube, 5 mL culture from the flask was centrifuged at 12,000 x g for 5 minutes and washed two times with distilled water. Pellet was collected and weighed after drying in the oven at 80°C for 36 h. The growth kinetic of P. rhodozyma cellswas measured by the following formula:
\[ \mu = \frac{\ln(N1-N0)}{\ln(T1-T0)} \]

\[ \mu \], specific growth rate (h⁻¹); N0, biomass at initial growth (g); N1, biomass at final growth (g); T0, time at initial growth (h); T1, time at final growth (h)

**Extraction of single cell carotenoids**

Carotenoids were extracted according the method of Sulistyo et al. (2016), One milliliter of cell suspension was centrifuged at 10,000 x g, 4°C for 10 min and washed twice with distilled water. Dried pellet was added with 2 mL dimethyl sulfoxide (DMSO) and 1 mL 1 M phosphate buffer (pH 7.0), and the homogenized in the 15 mL corning tube containing glass bead (5 mm in diameter). After centrifugation, 5 ml petroleum ether (Mallinckrodt Baker Inc., Phillipsburg, USA) was added to cell pellet in the reaction tube and homogenized for 10 min. After centrifugation, carotenoid in petroleum ether phase was removed to clean reaction tubes and dried. In the final step, 2 ml methanol (Merck KGaA, Darmstadt, Germany) was added and tube was wrapped for carotenoids analysis

**Analysis of total carotenoids**

Total carotenoid analysis (astaxanthin and β-carotene) were performed by high-performance liquid chromatography (HPLC) according to the method of Sulistyo et al. (2016). HPLC (LC-20AB) (Shimadzu Co. Inc., Tokyo, Japan) was equipped with a Spherisorb ODS2 4.0µm by 250 x 4.6 mm C18 column (Luna 5u Silica (2) 100 Å) using a 15-min gradient of ethyl acetate (0 to 100%) in hexane-acetone (86:14, v/v) at a flow rate of 2 mL/min in 20°C. Individual peaks of absorption spectra were obtained with a photodiode array detector with the typical retention times at λ 474 nm. Standard of 10 ppm β-carotene (Sigma, Germany) and astaxanthin (Sigma, Germany) were in the identification

**Results**

**Microbial cell growth**

We investigated cells growth throughout 168 h incubation which was monitored in every 12 h. The yeast grew in all tested media and generally obtained the highest biomass and carotenoid at 96 h incubation. Coconut water (MC) and glucose (MG) media had higher growth rate than that of molasses (MM) at 2.3 and 1.6 fold, respectively (Table 1). The highest growth rate was detected in the cells cultivated on coconut water medium (MC).

| Cultivation medium | Growth rate (h⁻¹) |
|--------------------|------------------|
| MG                 | 0.34             |
| MM                 | 0.24             |
| MC                 | 0.55             |

**Production and determination of carotenoids**

The measurement of carotenoid profiles was conducted at 96 h incubation time according to the highest biomass content. Two major carotenoids in *P. rhodozyma*, astaxanthin and β-carotene, were determined by HPLC. Two main types of carotenoids (astaxanthin and beta carotene) were detected from cell extracted of *P. rhodozyma* which was cultered in MG medium. However, there were other peaks beside astaxanthin and beta carotene on the electropherogram which indicated the assortments of carotenoid in MM and
MC (Figure 1). The total astaxanthin that was synthetized by the yeast of *P. rhodozyma* in MG reached up to 459.08 µg/g dry cell. This concentration was much higher compared to concentration of total astaxanthin produced cultured in MM and MC medium. Astaxanthin concentration from *P. rhodozyma* cultured in each MM and MC medium was quite similar (3.44 µg/g and 2.38 µg/g dry cell respectively). On the other hand, beta carotene concentration was found highest while cultivated in MM medium (47.86 µg/g) or about 2.6 fold higher than in MC and 2.1 fold than in MG medium (Table 1).

![Figure 1: Profiles of *P. rhodozyma* carotenoids in glucose-based medium (MG), molase-based medium (MM), and coconut water-based medium (MC) detected by HPLC](image)

**Figure 1.** Profiles of *P. rhodozyma* carotenoids in glucose-based medium (MG), molase-based medium (MM), and coconut water-based medium (MC) detected by HPLC

**Ratio astaxanthin and beta-carotene**

The ratio between astaxanthin and β-carotene were calculated based on carotenoid yield from three different media. The result showed that the ratio of astaxanthin and β-carotene (A/B ratio) in MG medium was approximately 20:1. This ratio A/B was higher than those of MM and MC as much of 0.4:1 and 0.2:1, respectively (Figure 2). This study confirmed that medium composition also strongly affects the carotenoids profile in cells of *P. rhodozyma*. 
Table 2. Total astaxanthin and β-carotene of *P. rhodozyma* cells cultivated in three cultivating media

| Medium                          | Biomass (g/L) | Total carotenoid (μg/g [dry weight]) | astaxanthin | β-carotene |
|---------------------------------|---------------|-------------------------------------|-------------|------------|
| Laboratorial cultivating medium |               |                                     |             |            |
| MG                             | 3.00          | 459.08                              | 22.85       |            |
| Waste-based cultivating medium  |               |                                     |             |            |
| MM                             | 5.80          | 3.44                                | 47.86       |            |
| MC                             | 10.75         | 2.38                                | 18.40       |            |

**Figure 2.** Ratio A/B of *P. rhodozyma* carotenoids in glucose-based medium (MG), molase-based medium (MM), and coconut water-based medium (MC) detected by HPLC

**Discussion**

The highest growth of *P. rhodozyma* is in MC medium indicated the effective ability of substrate usage and/or other component of the medium to yield about 12 g/L cell biomass. It can be explained that MC medium are relatively complect medium consist of carbon sources (Sucrose, glucose, fructose), protein and important minerals which important for the growth of this yeast. It well-known that coconut water containing important nutrient for the yeast culture medium (Prades *et al.* 2012). Carbon source gave influence on the yield and carotenoid compositions. previous publication show that carbon source up to 2% is sufficient for carotenoid production (Sumerta *et al.* 2019). Recently, research of the influence of the various carbon sources treatment on the yield and carotenoid of *P. rhodozyma* are still conducted.

We observed that the increased biomass growth and carotenoid production (astaxanthin and beta-carotene) could not always be achieved in the same cultivation condition. Previous study suggested that C/N ratio accelerated the biomass and astaxanthin production. Yamane *et al.* (1997) reported that initial high carbon/nitrogen ratio (C/N ratio)
in the medium increase astaxanthin production. However, high glucose concentration inhibit the cell growth.

Basically, carotenoid biosynthesis requires carbon sources in the C formation. It is a complex pathway, which use some enzymes. The most important enzymes are GGPP synthase, phytouene synthase, phytoene desaturase, carotene desaturase, lycopene, β-cyclase, β-carotene hydroxylase, β-carotene oxygenase, zeaxanthin glucosylase, zeaxanthin epoxidase, violaxanthin deepoxidase, capsorubin synthase, β-carotene desaturase, hydroxyneurosporene synthase, methoxyneurosporene desaturase, hydroxyneurosporene-O-methyltransferase, spheroidene monoxygenase, Lycopene elongase and heterodimeric decaprenoxanthin synthase (Amstrong 1997, Lee & Danner 2002). Some specific genes regulate carotenoid synthesis. The research of genes had been conducted for increasing astaxanthin production. For example, based on previous report, after the gene of atxS1 and atxS2 activated, yield of carotenoid had increased up to 8 and 4.6 fold respectively from wild type of P. rhodozyma (Lodato et al. 2004). Based on previously report from Ramirez et al. (2001), Carotenoid production, especially astaxanthin, from the yeast of P. rhodozyma wild type cultivated in YMB is variously with range about 300 – 600 μg/g dry cell.

Until now, it isn’t know whether saccharide have the effect on genes expression in astaxanthin production. Even though, we have been doing experiment to know the carotenoid profile by the yeast of P. rhodozyma cultivated in various monosaccharide and disaccharide as carbon sources. Many research for increasing the yield of astaxanthin were conducted through genetic engineering approach, but the exploration of low cost medium still as requirement to study. P. rhodozyma grew well in MC medium, but the total caroteoid embedded in cell membrane remained to low. It seems, cell growth was accelerated with MG to produced biomass rather than synthesis of astaxanthin and β-carotene.

Although we have not clarified the other peak appearing in HPLC eletropherogram under detection of 474 nm in wave length with other standards of carotenoid, by considering high intensity in detection region, we suggested that more diverse carotenoids was produced by cells grow in two waste-based media (MM and MC) (Figure 1). Production of other carotenoids in those MM and MC may relate to low content of astaxanthin comparing to MG indicating that MG is the best of three medium which used for carotenoid production, because of nutrient content’s. MC and MM medium contain higher carbon compare to MG medium and had no any effects to increase carotenoid production. it probably due to C/N ratio, where in MC and MM have higher C/N ratio. Previous publication reported that an appropriate C/N ratio is very important for carotenoid production (Braunwald et al. 2020). Among the nitrogen sources, peptone, nutrient broth, and yeast extract were utilized effectively for carotenoid production. Peptone was the most effective nitrogen sources for carotenoid production (Fang & Chan 1993, Sun et al. 2004). However, glucose and peptone were the more suitable carbon and nitrogen sources, respectively, when large-scale fermentation is considered.

Cultivation condition influences the expression of genes responsible to astaxanthin production in complex carotenoid metabolisms. In previous studies, several genes in related to astaxanthin production were described (Armstrong 1997). By understanding correlation between the gene expression and environmental factors, improvement of astaxanthin production can be achieved. Although modification of genes have been conducted by mutation to increase astaxanthin yields (Lewis et al. 1990, Sun et al. 2004), studies for applying those genetic modified astaxanthin-producing organisms on low-cost cultivating media are still needed to be conducted in further.

Although in waste-based medium astaxanthin concentration was lower than in MG medium, it does not mean that the properties were lower. Even though the previous research reported that astaxanthin had antimutagen capacity higher than β-carotene, but in the
separated research, it was found out that carotenoid from \textit{P. rhodozyma} synthesized in MC medium showed inhibition ability on carcinogenesis of mice’s lung organ (manuscript will publish elsewhere). This result opened an opportunity to a research on the effect of each carotenoid (astaxanthin and β-carotene) on the reduction of carcinogenic.

**Conclusions**

Medium composition strongly affects not only the total astaxanthin and beta-carotene synthesized in cells of \textit{P. rhodozyma}, but also the ratio A/B and carotenoid profiles. Although high content of astaxanthin in waste-based medium could not be achieved under tested condition, this study provided basic knowledge aspects of caroteoids biosynthesis in cells of \textit{P. rhodozyma} under low-cost cultivating media.

**Conflict of interest**

The authors state no conflict of interest from this manuscript.

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**Author contributions**

All authors have reviewed the final version of the manuscript and approved it for publication. AN, RH, EK and AD designed the study, performed research and collected the data, analyzed the data, wrote and reviewed the paper. IP, MK, NS and M prepared material for research and collected data. All authors are the main contributor of this manuscript.

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