EFFECTS OF SOME FACTORS ON CAROTENOID BIOSYNTHESIS BY *RHODOTORULA MUCLAGINOSA*

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ABSTRACT

Carotenoid compounds are the popular natural antioxidants which are often isolated from plants. There have been more and more researches on carotenoid biosynthesis towards lowering product prices. In this study, in order to produce carotenoid, *Rhodotorula mucilaginosa* was grown on aqueous media composed of carbon source (glucose, glycerol), nitrogen source (yeast extract, $(NH_4)_2SO_4$). The optimum nutrient concentration was 10g/L glucose, 10g/L glycerol, the ratio of yeast extract and $(NH_4)_2SO_4$ (3:7). The fermentation time for obtaining the highest carotenoid yield was 10 days in our research condition. Additionally, some oxidative stress environment for *Rhodotorula mucilaginosa* was be studied. The result has shown that the low level of Cu^{2+} (4.5mM) or 1% H_2O_2 solution (% v/v) in the fermentation media could increase the carotenoid biosynthesis.

Keywords: Arotenoid; Biosynthesis; Fermentation; Rhodotorula mucilaginosa.

1. Introduction

Carotenoid compounds are tetraterpenoid, consisting of highly unsaturated isoprene derivatives. These compounds are the class of natural pigments, displaying yellow, orange or red color in plants. In addition to the popular use as food colorants, carotenoids were also famous for their pro-vitamin and antioxidant activity.

Not only plants but also microorganisms can synthesize carotenoids to protect their cell from radicals. More and more researches on single cell carotenoid have been done in recent years. Red yeast *Rhodotorula* is one of the most popular genus used to produce carotenoids. Most of the researches' purpose was to find out the optimum mediums for carotenoid biosynthesis especially nutrient concentration.

In order to evaluate the effect of supplementation, Bonadio et al. (2018) incubated yeast *Rhodotorula rubra* L02 in mediums with different concentration of nitrogen, phosphorus, zinc and magnesium. The dry biomass and carotenoid yield were 2g/L and 0.003mg/L, respectively. In another report, carbon and nitrogen ratio was changed in the fermentation medium and the result showed that the increase of C/N ratio from 70 to 120 leaded to an increased carotenoid synthesis.

Naghavi et al. (2012) utilized *Rhodotorula slooffiae* and *Rhodotorula mucilaginosa* isolated from leather tanning wastewater as culture to produce carotenoid in the synthetic medium including glucose, yeast extract, $NH_4(SO_4)_2$,… The strain of *Rhodotorula mucilaginosa* had more potential for carotenoid biosynthesis.

Apart from synthetic fermentation medium, some affordable complex medium like inexpensive agricultural product or byproducts were utilized for the yeast growth. Petrik et al. (2014) tested the carotenoid production by four red yeast strains with spent coffee ground as substrate. In 2017, Besarad et al. used beer wort as substrate to biosynthesize carotenoids by some *Rhodotorula* strains. The highest carotenoid yields (over 80µg/g dry biomass) were recorded for the strain *Rhodotorula glutinis* BIM Y-158 and BIM Y-253.

The aim of this study was to evaluate the impact of nutrients (carbon sources, nitrogen sources), oxidant stress factors ($CuSO_4$, H_2O_2) and fermentation time on the biomass and carotenoid yield.

2. Materials and methods

2.1. Microorganism

Rhodotorula mucilaginosa was purchased from Institute of Microorganism and Biotechnology, Vietnam National University, Ha Noi and maintained for further use on YDP agar (20g/L of pepton, 10g/L of yeast extract, 20g/L of glucose and 20g/L of agar) at 4^{o}C.

YDP liquid medium was used to prepare inoculum. Cultivation was carried out in 250mL Erlenmeyer containing 100mL of the medium at 30^{o}C for 24h with shaking at 200rpm. Then the yeast cells were seeded at a density of 10^6 cells/mL in 150mL experimental medium.

2.2. Experiments

Firstly, nutrient concentration was changed in 1L medium: the ratios of glycerol and glucose (10:10, 7:13, 5:15, 4:16 g/g) and the ratios of organic nitrogen (from yeast extract) and inorganic nitrogen (from $NH_4(SO_4)_2$) (10:0, 9:1, 7:3, 5:5, 3:7).

Secondly, the fermentation time was evaluated. The red yeast was grown and two parameters (dry biomass yield and carotenoid concentration) were determined each day so as to identify the best time for cell harvest.

Last, some oxidant stress factors were put into the cultivation medium to evaluate the effectiveness of them on carotenoid biosynthesis. These factors were: $CuSO_4$ concentration (0.0; 0.5; 2.5; 4.5; 6.5mM) and the volume of 1% H_2O_2 solution in 100mL culture (0.0, 1.0; 2.5 and 5.0mL).

2.3. Dry biomass yield determination

After incubation, the specified volume of culture was centrifuged for 15min at 3000rpm and rinsed with distilled water. The wet-cell biomass was dried at 80^{o}C to the constant weight. The dry biomass yield unit has been g/L.

2.4. Carotenoid extraction and determination

The wet-cell biomass was also ground with glass powder (1:1 w/w) within 20 min to break the yeast wall. Subsequently, 15mL acetone was added to extract carotenoid from ground cell. The extraction was implemented again with the same acetone volume. The total acetone extraction was used to quantitatively determine of carotenoid biosynthesis by spectrophotometer at 454nm.

2.5. Statistical analysis

The data analysis of dry biomass yield (g/L) and carotenoid yield (µg/L) from the experiments were carried out by Statgraphic plus 3.0 software with ANOVA method.

3. Results and Discussion

3.1. Effect of the ratio glycerol and glucose on biosynthesis

Glucose is often made use of as carbon source for yeast in inoculum and fermentation medium. In many reports on carotenoid biosynthesis, glycerol as substrate was supplemented to the fermentation medium (Cutzu, 2013; Kot, 2016; Kot, 2017).

In this experiment, the various ratios of glycerol and glucose led to the significantly difference of the carotenoid yield but not change the dry biomass yield. The utilization of glycerol or glucose as the only carbon source indicated the lowest carotenoid yield. The medium contained both carbon sources with

the same quantity of 10g/L was the optimum parameter and produced the highest carotenoid yield: 4703.9µg/L (Table 1).

Many different carotenoid yields were indicated in different reports which utilized *Rhodotorula mucilaginosa* yeast. Cheng (2016) incubated *R. mucilaginosa* to produce carotenoid with some food waste and YM medium (consisting of glucose, peptone, yeast extract and malt extract) as the control medium. The carotenoid yields were obtained from 1107.4 to 2337.5µg/L. In another journal, Manimala (2016) evaluated the carotenoid production using cheap complex substrates (rice bran, wheat bran, coconut oil cake, sesame oil cake,…). The carotenoid yield was ranging from 12.0 -12.5 mg/L.

Table 1
Dry biomass and carotenoid yield in mediums with different glycerol / glucose ratio

Glycerol/glucose ratio (g/g in 1L)	Carotenoid yield (µg/ L)	Dry biomass yield (g/L)
20:0	1977.2^{c}	4.006
10:10	**4703.9^{a}**	**4.160**
7:13	2797.2^{b}	4.407
5:15	2797.2^{b}	4.407
3:17	3733.3^{ab}	4.213
0:20	1972.3^{c}	3.823

Note: The different letters (a, b, c) in the same column showed the significant difference of the dry biomass weight and carotenoid yield. The (ns) showed that the data in the column were not statistically different.

3.2. Effect of the ratio yeast extract and ammonium sulfate on biosynthesis

Yeast extract and ammonium sulfate can supply nitrogen for the yeast growth in many researches. The ratio of yeast extract and ammonium sulfate did not make the effect on carotenoid yield. However, the presence of yeast extract raised the dry biomass yield of *Rhodotorula yeast* (Table 2). To get the high yield of the product and decrease the process cost, the ratio of yeast extract and ammonium sulfate chosen for further research was (3:7).

Table 2
Dry biomass and carotenoid yield in mediums with different yeast extract/ammonium sulfate ratio

Yeast extract/ammonium sulfate ratio (g/g in 1L)	Carotenoid yield (µg/L)	Dry biomass yield (g/L)
10:0	1040.10	6.427^{a}
9:1	540.87	4.934^{ab}
7:3	1340.90	5.670^{a}
5:5	887.63	4.832^{ab}
3:7	**927.50**	**4.793^{ab}**
1:9	568.63	3.247^{b}
0:10	597.20	0.477^{c}

Note: The different letters (a, b, c) in the same column showed the significant difference of the dry biomass weight and carotenoid yield.

3.3. Effect of the fermentation time on biosynthesis

The fermentation time is also an important parameter for harvesting the bio-product. In this experiment, the dry biomass and carotenoid yield were identified once per two days through ten-day incubation. Generally, there was an increase in both the yields due to the rise of the incubation time. The highest dry biomass gained at the eighth day at 7.437g/L but after that the carotenoid yield continue rising until the last day of this experiment. Hence, ten days was the time to harvest the carotenoid of our *Rhodotorula mucilaginosa* and the carotenoid obtained at 809.59µg/L. Compare with some reports, the carotenoid quantity accumulated from other *Rhodotorula mucilaginosa* strains quite different. Petrik (2014) and Naghavi (2012) fermented *R.*

mucilaginosa and carotenoid production gained at 4.69mg/L and 8mg/g dry biomass, respectively.

Table 3
Dry biomass and carotenoid yield in ten-day fermentation

Fermentation time (day)	Carotenoid yield (μg/ L)	Dry biomass yield (g/L)
2	248.77^{c}	3.520^{c}
4	540.88^{b}	5.749^{b}
6	618.03^{b}	6.136^{ab}
8	579.02^{b}	7.437^{a}
10	**809.59^{a}**	**7.200^{a}**

Note: The different letters (a, b, c) in the same column showed the significant difference of the dry biomass weight and carotenoid yield.

3.4. Effect of the oxidant stress factors on biosynthesis

Carotenoids are the secondary metabolic products which protect the yeast cell from oxidant factors. Marova et al. (2012) used some stress factors (high concentration of NaCl and peroxide) to test the carotenoid accumulation of some yeast strains.

Exposure to H_2O_2 or Cu(II) cation would modified the carotenoid content in *R. mucilaginosa* RCL-11, both qualitatively and quantitatively (Irazustaa et al., 2013). Hence, in our research, the solution of H_2O_2/$CuSO_4$ were supplemented to the medium to create an oxidant stress condition in the cell growth.

The addition of H_2O_2 solution made the effect on the carotenoid synthesis clearer than the yeast biomass. Without H_2O_2, the carotenoid yield was significantly lower but too much H_2O_2 concentration (from 5% solution of H_2O_2) could inhibit the yeast growth and carotenoid biosynthesis. The H_2O_2 solution percentage of 1.0% and 2.5% obtained the significantly higher carotenoid yield (1379.1 and 1380.3 μg/L, respectively) (Table 4).

Table 4

Dry biomass and carotenoid yield in mediums with different volume of 1% H_2O_2 solution

The percentage of 1% H_2O_2 solution (% v/v)	Carotenoid yield (μg/L)	Dry biomass yield (g/L)
0.0	442.07^{b}	5.626^{ab}
1.0	1379.1^{a}	6.845^{a}
2.5	1380.3^{a}	6.288^{a}
5.0	310.31^{b}	4.477^{b}

Note: The different letters (a, b, c) in the same column showed the significant difference of the dry biomass weight and carotenoid yield.

The concentration of $CuSO_4$ also made the various carotenoid yields after fermentation. The best biosynthesis was identified with 4.5mM $CuSO_4$ in the medium and gained the carotenoid yield of 1855.0μg/L. The lower than 4.5mM of $CuSO_4$ concentration was decreased the yield but the increase of this parameter to 6.5mM led to the death of the yeast because of stress. (Table 5)

Table 5

Dry biomass and carotenoid yield in mediums with different concentration of $CuSO_4$

Concentration of $CuSO_4$ (mM)	Carotenoid yield (μg/ L)
0.0	764.58^{b}
0.5	573.68^{b}
2.5	918.03^{b}
4.5	1855.00^{a}
6.5	0.00^{a}

Note: The different letters (a, b, c) in the same column showed the significant difference of carotenoid yield.

4. Conclusion

In conclusion, the ratios of carbon sources influenced on the carotenoid yield and the ratios of nitrogen sources mainly affected the dry biomass yield in our fermentation conditions. After eight-day incubation, the highest biomass gained, while the carotenoid production continuously rose until tenth day. Both of the oxidation stress factors ($CuSO_4$ and H_2O_2) could increase the carotenoid accumulation in their limitation. Over the optimum concentration, the oxidant stress inhibited or even stopped the yeast growth■

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