FACTORS ENHANCING THE ACCUMULATION OF β-CAROTENE IN RHODOTORULA TAIWANENSIS CT1

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Abstract. β-carotene known as an antioxidant, immunostimulant, provitamin A and natural colour is a popular additive used in food, cosmetics, and animal feed. Microbial β-carotene currently attracts attention thanks to its shorter production time and controllable process in comparison to β-carotene from plant source. This study investigates the factors that enhance the accumulation of β-carotene in the yeast strain Rhodotorula taiwanensis CT1. The addition of stressors including ethanol (0.2%), hydrogen peroxide (10 mM), and NaCl (1 M) to the 48 h CT1 cultures improved the intracellular β-carotene yield by 2.2; 2.05 and 1.35 folds, respectively. The volumetric productivity of CT1 correspondingly increased 2.1, 2.0 and 1.1 folds. As a metabolism regulator, citric acid (1%) added into 48 h CT1 culture had a positive effect on intracellular β-carotene production, resulting in its 1.78-fold increase. The highest β-carotene yield in CT1 was obtained when 1% Tween 80 was introduced into the culture. The intracellular β-carotene content and the volumetric productivity increased 2.84-folds and 2.64-folds reaching 337.56 ± 1.30 µg/g dry biomass and 15.38 ± 0.30 mg/L, respectively.

Keywords: β-carotene, Rhodotorula taiwanensis, factors enhancing.

Classification numbers: 1.1.5, 1.3.2, 1.4.8.

1. INTRODUCTION

β-carotene, a popular carotenoid, known as a provitamin A, an antioxidant, and an immunostimulant is widely used in foodstuff, functional foods, cosmetics, supplements, and animal feed. Currently, microbial production of β-carotene parks interests since it has a higher process efficiency and a much shorter accumulation time compared to the production of β-carotene by plant [1, 2]. In general, β-carotene can be produced by all groups of microorganisms, but β-carotene from yeasts highly interests both scientists and technologists because of its promising features including short fermentation time; safe, effective and controllable process, easily scaling up and automation and its environmentally friendly nature [3, 4].

Production of β-carotene was reported mainly for the genus Rhodospiridium, Sporobolomyces and in particular for Rhodotorula, of which R. glutinis, R. mucilaginosa are
seen as representative producers of β-carotene with yields of 2.18-135.2 mg/L (by HPLC analysis) [3].

To improve the β-carotene production capacity of the yeasts, different approaches were discussed including: 1) Strain engineering [5-7]; 2) Factors control the expression of the genes in the β-carotene pathway [8-10]; 3) Precursors/regulators or stressors for enhancing β-carotene production [11-14]. In addition, since the accumulation of β-carotene starts at the late logarithmic phase, optimizing the fermentation for biomass and production thus may also improve the product accumulated [15, 16].

It had been observed in various carotene-producing microorganisms that carotene is accumulated during exposure to biotic or abiotic stressors, probably because carotene protects microorganisms against oxidative and biological stresses [13, 14, 17]. Precursors, metabolic regulators or surfactants can affect the biosynthesis by either regulating the metabolic pathway or altering membrane permeability. This in turn, leads to enhancing biosynthesis and affecting the metabolite accumulation [13, 16, 18]. Hence, the use of these factors in culture media could be a viable option that improve β-carotene production by yeasts.

During the search for β-carotene producing yeast strains, we found CT1 strain, isolated from eggplant fruit, identified as Rhodotorula taiwanensis, capable of producing β-carotene. R. taiwanensis is considered an industrial yeast for the production of lipid-based bioproducts, reported mostly for carotenoids [1]. There have been very few reports on β-carotene from R. taiwanensis to date, with information on the potential to improve its accumulation. In this paper, we will present the results of our study on the β-carotene production capacity of R. taiwanensis CT1, mainly investigating the effects of various factors (stressor, regulator or precursor) that help increase β-carotene accumulation during fermentation of CT1.

2. MATERIALS AND METHODS

2.1. Materials

Rhodotorula taiwanensis CT1 provided by The Yeasts Collection of School of Biotechnology and Food technology – HUST was used in this study.

Basic medium for yeast preculture (g/L): Glucose: 40; (NH₄)₂SO₄: 5; KH₂SO₄: 5; MgSO₄: 0.34; yeast extract: 7 [19].

Media for yeast fermentation (LS10) (g/L): Glucose: 150; MgSO₄·7 H₂O: 1.5; KH₂PO₄: 0.4 ; CaCl₂: 0.167 ; Urea: 0.47 ; Yeast extract: 8.05; ZnSO₄: 1.19.10⁻⁴ mM; MnCl₂: 1.22.10⁻⁴ mM; CuSO₄: 10⁻⁴ mM [20].

2.2. Methods

Yeast cultivation: Yeast was activated in basic medium at 25 °C, shaking 120 rpm for 48 h then 5 mL culture was transferred to 50 mL LS10 medium in 250 mL baffled flasks and was incubated in the same condition.

Influence of the factors on β-carotene accumulation in CT1: CT1 cultures on LS10 at 48 h were added with various compounds: lipids (olive oil at 0.5 % and 1 %; Tween 80 at 1 % and 1.5 %), stressors (0.2 % ethanol; 0.75 M and 1 M NaCl; 10 mM H₂O₂); and a regulator (1 % citric acid). The optical density (OD₆₆₀nm) of the culture was measured every 24 hours to monitor the yeast growth (Amersham Biosciences, Novaspec III). Yeast biomass was collected by
Factors enhancing the accumulation of β-carotene in Rhodotorula taiwanensis CT1

centrifugation (8,000 g, 5 °C for 10 min) at maximal OD_{660nm}, then dried up at 50 °C, used for β-carotene extraction and measurement. The LS10 culture without stressor addition was used as the control. All experiment was duplicate.

Reducing sugar: Reducing sugar in the culture was measured by DNS method [21].

Determination of β-carotene: β-carotene was extracted from dried yeast biomass following the method of Lopez [5] with modification, briefly: 0.1 g dried biomass (DW) was added with 1 mL 4 M HCl, incubated at 65 °C for 1 hour. Centrifugate at 12,000 rpm for 10 min to discard the supernatant. Add 1.5 mL acetone, thoroughly vortex and centrifugate, then collect the top layer which contains β-carotene. β-carotene extract was measured by spectrophotometry at OD_{450nm} (Amersham Biociences, Novaspec III) using standard curve of β-carotene ranging from 1-10 µg/mL [5].

Volumetric productivity of β-carotene (Pv) of the culture was calculated as follows:

Pv (mg/L) = concentration of intracellular β-carotene (µg/g DW) * Biomass (g/L)/1000.

3. RESULTS AND DISCUSSION

R. taiwanensis CT1 grew on PDA medium, giving deep red colonies (Fig. 1). In 7-day culture on LS10 at 25 °C, R. taiwanensis CT1 accumulated β-carotene of 71.54 µg/g DW (analyzed by HPLC, data not shown). This result confirmed that R. taiwanensis CT1 can produce β-carotene but not at high rate [3]. In an attempt to improve the β-carotene capacity of CT1, the influence of stressor-factors on β-carotene accumulation in CT1 was examined.

![Figure 1. Colony of R. taiwanensis CT1 on PDA medium.](image)

3.1. Effect of stressors on β-carotene accumulation in R. taiwanensis CT1

It was reported that the factors causing stress to yeast such as ethanol, NaCl, H₂O₂, light, irrigation, etc., affected the growth and metabolism of the yeasts, influencing the production of yeast carotenoid [14, 18, 19]. To investigate whether those stressors also influence the β-carotene accumulation of CT1, in this experiment, stressors such as ethanol (0.2 %), NaCl (1 M) and H₂O₂ (10 mM) were added to the CT1 cultures at the early logarithm phase. Growth of the yeast and β-carotene content were evaluated at regular intervals and at the end of the culture cycle.

A minor influence of ethanol and H₂O₂ on CT1 growth was observed (Fig. 2a): CT1 biomass slightly increased after the addition of the stressors (48 h) but it was repressed afterwards (CT1-H₂O₂, 72 h and CT1-ethanol, 96 h). However, all cultures attained the highest OD_{660nm} at the same time, biomass accumulated and remaining reducing sugar contents were
Pham Tuan Anh, Hoang Thi Ngoc Trang, Phung Thi Thuy, To Kim Anh

similar in these cultures (Fig. 2b), confirming the concentrations of ethanol and H$_2$O$_2$ used were thus below the toxic threshold to CT1 growth.

Conversely, ethanol and H$_2$O$_2$ strongly affected the accumulation of β-carotene in CT1 (Fig. 2b): intracellular β-carotene achieved 255 ± 0.11 µg/g and 243.60 ± 2.20 µg/g DW, equivalent to 2.2 and 2.05 folds increased in adding 0.2 % ethanol and 10 mM H$_2$O$_2$, respectively. $P_v$ of the cultures was calculated of 11.9 and 12.1 (mg/L), respectively, higher than that of the control of 2.0 - 2.1 folds.

**Figure 2.** Influence of stressors on growth (a) and β-carotene accumulation (b) in *R. taiwanensis* CT1.

It was reported that once added to the yeast culture, ethanol was subsequently oxidized in the cells by alcohol dehydrogenase followed by aldehyde dehydrogenase. The reaction with aldehyde dehydrogenase formed reducing coenzymes NAD(P)H, known as a negative regulator to TCA cycle, repressing energy metabolism then the growth of the yeast. On the other hand, ethanol activated oxidative metabolism in the cells, forming HMG-CoA reductase, a key enzyme of the isoprenoid pathway, hence improving carotenoid production in the yeasts. Marova *et al.* [22] explained the effect of hydrogen peroxide on yeast in another way. It is a stressor that may activate the yeast to produce anti-stressor compounds. In this case, β-carotene probably is an antioxidant for the cell against the stressor. The obtained result in this study is similar to the report of Gu *et al.* on the influence of ethanol on carotene production in *Phaffia rhodozyma* [14]. Both ethanol and H$_2$O$_2$ thus can be enhancers for β-carotene production by *R. taiwanensis* CT1.

Contrast to the addition of ethanol and H$_2$O$_2$, NaCl acts as an osmotic stressor, depressing CT1 growth: growth rate decreased, time course to attain maximum biomass much longer than that of LS10 culture. Conversely beside the depression of CT1 growth, NaCl in the studied concentration did increase the accumulation of β-carotene in CT1 to 1.35 folds (160.26 ± 1.93 µg/g DW). However due to the lower biomass, $P_v$ of the NaCl culture just slightly improved (increasing 11.2%). The same reaction conformed with reported observation by Li *et al.* [23] and Tamás *et al.* [24], which had proved the presence of NaCl in the culture did enhance the expression of *crtYB* and *crtI*. These are the two key genes in the β-carotene pathway in *Sporidiobolus pararoseus* NGR and *Saccharomyces cerevisiae*, that increase intracellular β-carotene in these yeasts.

The result all showed positive effects of studied stressors on the β-carotene accumulation in *R. taiwanensis* CT1. Nevertheless, in this experiment, the stressors added at the early logarithm
Factors enhancing the accumulation of β-carotene in Rhodotorula taiwanensis CT1

phase of CT1 cultures somehow affected the growth of CT1 resulting in lower Pv than expected. In order to avoid this phenomenon, the addition time of these agents should be further investigated.

3.2. Influence of regulator on the β-carotene accumulation in R. taiwanensis CT1

Citric acid is an intermediate metabolite known as a negative regulator of TCA cycle. At a specific concentration in the cell, it becomes a repressor of TCA, led to reducing the oxidative metabolism to energy in the cell, and glucose then can be shifted to another pathway [13]. In this experiment, 1% of citric acid was added to CT1 cultures on LS10 at 48 h and 72 h to examine the influence of citric on CT1 culture.

![Figure 3](image)

**Figure 3.** Effect of citric on growth (a) and accumulation of β-carotene (b) in R. taiwanensis CT1.

With the addition of 1% citric, a slight decrease in CT1 growth rate was observed (Fig. 3a). Both biomass and remaining sugar of citric cultures were slightly lower than that in LS10. It suggested that CT1 did not use citric as a substrate for growth. Once added in the culture, citric repressed the growth of CT1, due to the negative regulation of TCA cycle for energy metabolism, and thus lowering the growth of the yeast.

Intracellular β-carotene of CT1 increased 1.73 - 1.78 folds (204.36 - 211.36 µg/g DW) in comparison to that of LS10 culture. Pv of β-carotene reached 10.04 ± 1.28 mg/L, 1.73-fold higher than that in the control (Fig. 3b). The result aligned with the assumption that citric regulates the TCA cycle and promotes the β-carotene pathway [13, 14]. It was observed that the addition of citric to the culture at earlier logarithm phase at 48 h seemed to have an appropriate effect on both biomass and β-carotene accumulation in CT1.

3.3. Influence of some lipid compounds on β-carotene accumulation

To investigate the influence of some lipid compounds to CT1, olive oil and Tween 80 were added to the LS10 cultures at 48 h incubation.
Different effects of olive and Tween 80 addition on CT1 growth were observed: the growth rate of CT1 was significantly increased in the presence of olive but heavily depressed by Tween 80 (Fig. 4a). Maximum biomass of olive cultures was attained at 144 h of incubation, similar to that of the control but the biomass of olives was higher than that of the control, equivalent to 8.6 % and 13.5 % folds increased respectively (53.25 ± 0.91 and 55.65 ± 2.23 g/L in 0.75 % and 1 % olive culture). On the contrary, the CT1 attained the maximum biomass in the Tween 80 cultures after 168 h of incubation, it was 24 h longer than that of the control. The biomass decreased 6 and 14 % when 1 % and 1.5 % Tween 80 respectively added to the cultures.

Accumulation of intracellular β-carotene of CT1 however impressively increased in all cultures in compared to that’s in the control with the highest value attained in 1% Tween 80 culture, which increased 2.84 folds. P\textsubscript{v} of β-carotene increased in all cultures. P\textsubscript{v} in olive and 1% Tween cultures achieved 15.38 ± 0.30 (mg/L); 2.64-fold (Fig. 4b). This result was promising more than the case of \textit{S. pararoseus} KM281507, when 1 % Tween 80 was added to the culture, P\textsubscript{v} increase of 1.43 folds only [14].

Observing the biomass, remaining sugar, and β-carotene accumulated of the cultures, it can be assumed that olive oil was an additional substrate for biomass and precursor for the product while Tween 80 was not a substrate for CT1 growth but enhanced the β-carotene production. The effect of olive on CT1 may agree with the assumption that lipid provides fatty acids as precursors and promoting the β-carotene production. In addition, Tween 80 may act as a surfactant compound, interferes with the cell membrane permeability, then affecting the production of β-carotene [13, 16].

4. CONCLUSIONS

The accumulation of β-carotene in \textit{R. taiwanensis} CT1 improved in the presence of stressors such as NaCl, hydrogen peroxide, ethanol; regulator such as citric acid; precursor-lipids such as olive oil and surfactant as Tween 80. Within the factors at investigated concentrations, adding 1 % Tween 80 resulted in the highest β-carotene accumulation, attained 337.56 µg/g DW, increased 2.84 folds. This is a promising result which suggests further study on how to enhance β-carotene production in CT1.
Factors enhancing the accumulation of $\beta$-carotene in Rhodotorula taiwanensis CT1

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