# Effect of Temperature and pH on the Growth Kinetics and Carotenoid Production by *Sporobolomyces ruberrimus* H110 Using Technical Glycerol as Carbon Source

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**ABSTRACT:** *A new isolated strain of Sporobolomyces ruberrimus H110 was cultivated on technical glycerol as carbon source and effect of different temperatures and pH on the growth and pigmentation was studied. The maximum concentration of total carotenoid was 3.84 $mgg^{-1}$ including torularhodin (3.70 $mgg^{-1}$) and β-carotene (0.14 $mgg^{-1}$) using 19ºC at pH 6, but the highest amount of the maximum specific growth rate was obtained ($\mu_{max}$ = 0.094 $h^{-1}$) at 27ºC. The growth yield ($Y_{x/s}$) was equal to 0.52 ± 0.01 g $g^{-1}$ with a correlation coefficient value of 0.98.*

**KEY WORDS:** *Torularhodin, Carotenoids, Technical glycerol, Sporobolmyces ruberrimus H110, Kinetics, β-carotene.*

## INTRODUCTION

Carotenoids are currently produced for use as food colorants, nutritional supplements, cosmetics or health purposes [1]. In addition to their pigmenting abilities, carotenoids may function as antioxidants by quenching photosentizers, interacting with singlet oxygen, and scavenging peroxy radicals [2-4]. The species of the various taxonomic group-bacteria, fungi and yeasts are efficient natural producers of carotenoids [5,6]. Facing the growing economic significance of carotenoids, much interest has been devoted to new supplies of this type of pigment [7]. The fermentation conditions, such as cultivation temperature [3], lightening [8], induced substances [9,10], and inhibitors [11,12] play important roles in the carotenoid-forming activity of microorganisms as well as composition ratio of carotenoids. *Kusdiyantini et al.* showed that pure glycerol can be used as source of carbon for carotenoid production by *P. rhodozyma* using a rich medium containing peptone and yeast extract [13]. To our knowledge, only few microorganisms are able to grow on technical glycerol. The salts released from the

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*1021-9986/06/3/59 6/$/2.60*

oil transesterification, diluted in technical glycerol, exert significant inhibitory effects on many microorganisms. Technical glycerol is a by-product and can become an important feed stock when bio-diesel is applied on a large commercial scale.

The aim of this study was to examine the potential of technical glycerol as substrate and effect of different temperatures and pH on the growth kinetics and production of carotenoids by a wild strain of *Sporobolomyces ruberrimus* H110.

## EXPERIMENTALS

### *Microorganism and growth medium*

*Sporobolomyces ruberrimus* H110 used in this study was isolated in laboratory of Génie des Procédés Biotechnologiques et Alimentaires (L.S.G.C., France). The first preculture was done in a 300 ml baffled Erlenmeyer flask containing 50 ml of medium composed of the following ( per liter): 10 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract on a rotary shaker at 210 rpm, for 24 h at 27 ºC. pH was adjusted at 6 before sterilization. 10 ml of this preculture was used to inoculate a second preculture phase in a 1 l baffled Erlenmeyer flask containing 200 ml, with the following composition (per liter): 1.0 g yeast extract, 0.5 g peptone, 3 g $(NH_4)_2SO_4$, 32.5 g technical glycerol, 2 g $Na_2HPO_4$, 1.5 g $MgSO_4.7H_20$, 4 g $KH_2PO_4$ on a rotary shaker at 210 rpm, for 24 h at 27 ºC and pH adjusted to 6. 150 ml of the second preculture was used as inoculum in a bioreactor. The composition of technical glycerol was 67 % pure glycerol, 2 % fatty acids, 3 % salts and 28 % water.

In order to investigate the effect of the temperature, batch cultures were performed in a 3L fermentor (Applikon, ADI 1030, Holland) containing 1.5 L of the following medium (per liter): 20 g $(NH_4)_2SO_4$, 67 g glycerol (from technical glycerol), 0.5 g peptone, 1 g yeast extract, 2 g $Na_2HPO_4$, 1.5 g ($MgSO_4.7H_20$), 4g $KH_2PO_4$. The initial pH was adjusted with KOH 2M or $H_3PO_4$ (42.5%) solutions before sterilization (121ºC for 20 min). After inoculation, temperature, pH and agitation were maintained at (19, 23, 27, 31 ºC), 6 and 300 rpm respectively. Dissolved oxygen partial pressure was maintained at 50 % of air saturation by controlling air flow-rate and agitation speed (300-900 rpm). Biospumex 153 (Biosoph, Peronne, France) was used as antifoam agent.

### *Analytical Methods*

Yeast concentration was determined by filtering the samples through a 0.45 µm-pore size polyamide membrane filter, washing it twice with physiological water (NaCl 0.9 %) and drying at 105 ºC for 12h. Biomass was also measured by optical density using a spectrophotometer (LKB-Biochrom, Cambridge, UK) at 600 nm. Glycerol concentration was determinated by HPLC on a polypore column H 250 mm x 7mm using $H_2SO_4$ 0.04 N as eluent at 65ºC.

### *Carotenoid extraction and analysis*

5 ml of medium was harvested by centrifugation (4500 g for 10 min) then washed in physiological water (three times), and resuspended in 10 ml of distilled water. The suspension was broken with glass beads (0.4 mm, 10 % w/v) for 10 min with cooling (-18 ºC) in a bead beater (Vibrogen-Zellmuhle, Bioblock, France). The bead-cell mixture was harvested by centrifugation (4500g for 10 min) and after washing and decanting, the yeast cells were resuspended in 5 ml ethanol and vortexed for 1 min. The pigment contained in the ethanol was recovered. (The procedure was repeated until the cells remained colourless). The samples were filtered through P.T.F.E. (Polytetra Fluoroethylene) membranes and stored at -80 ºC under vacuum in the dark. Carotenoids were analysed by High Performance Liquid Chromatography (HPLC) using a Symmetry analytical C18 column, (150 mm x 4.6 mm, 300 Å) and sphere diameter 3.5µm (waters, USA). The UV detector operated at 420 -500 nm and the column temperature was maintained at 35 ºC. Mobile phase consisted of 1ml $min^{-1}$ of isocratic acetonitrile-methanol-dichloromethane mixture solvent (71:22:7, by vol).

## RESULTS AND DISCUSSION

### *Choice of the carbon source*

In a first step, growth and carotenoid production by *Sporobolomyces ruberrimus* H110 were quantified using different carbone sources such as glucose, pure glycerol and technical glycerol, in Erlenmeyer flask for 50 h. The concentration of the carbon source was 32 g $l^{-1}$ for all of the cultures. As shown in Fig. 1, *SP. ruberrimus* can consume each carbon source, but growth was approximately twice higher using technical glycerol (4.16 g $l^{-1}$). The maximum biomass was formed after 24 h with

***Table 1: Effect of different carbon sources on growth and total carotenoid by SP. ruberrimus H110.***

| Carbon source (32 g $l^{-1}$) | Growth (g $l^{-1}$) | Cellular carotenoid (mg $g^{-1}$) | Volumetric carotenoid (mg $l^{-1}$) |
|---|---|---|---|
| Glucose | 1.79 | 0.99 | 1.77 |
| Pure glycerol | 1.93 | 0.81 | 1.56 |
| Technical glycerol | 4.16 | 1.15 | 4.81 |

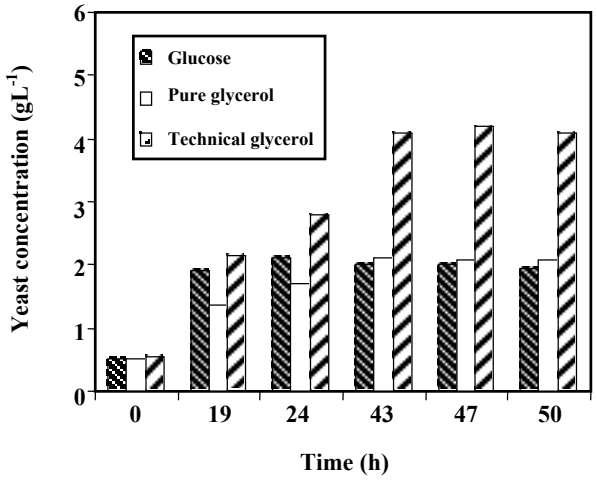


***Fig. 1: Effect of different carbon sources on growth and carotenoid production by Sporobolomyces ruberrimus in Erlenmeyer flask (pH: not controlled, 27 °C).***

glucose, while the same yeast concentration was obtained after 43 h with pure glycerol.

The growth rate was higher when glucose was used but as indicated in table 1, the highest carotenoid concentration was observed when technical glycerol was the carbon source (1.15 mg $g^{-1}$). In the view of a favoured pigment production, these preliminary informations suggest technical glycerol as the effective carbon source.

### *Effect of pH*

For evaluating the effect of pH on the pigmentation and growth of strain, *SP. ruberrimus* was cultivated at 28 °C for 72 h. The pH was adjusted to 3.5, 4, 4.4 5, 5.5, 6.0, 6.6, 7.0, 7, 6 and 8.2 respectively. The pH had a pronounced effect on the pigment and biomass. Minimum carotenoid and biomass were observed when cells were grown at pH 3.5 (growth: 6.81 g $l^{-1}$, cellular carotenoid: 2.02 mg $g^{-1}$). The carotenogenesis and growth were induced by increasing of pH (pH>6). The results showed (Table 2) that the optimum pH for cell growth and total pigmentation formation by this strain was 6.0.

### *Effect of temperature*

To study the effect of temperature on yeast growth and carotenoid production, *Sporobolomyces ruberrimus* was grown on technical glycerol under pH controlled at four temperature values 19, 23, 27 and 31 ºC , in a batch reactor. The initial glycerol and ammonium sulfate concentrations were respectively 67 g $l^{-1}$ and 20 g $l^{-1}$ for all experiments. As shown in Fig. 1, carotenoid production occurred whenever the temperature was kept between 19 ºC and 27 ºC, but at 31 ºC the cells remained colourless and no pigment production was seen. The overall growth yield was always approximately the same and, when glycerol was exhausted, biomass and pigment accumulation stopped. The most rapid production of carotenoids occurred during the exponential growth phase under all temperature values, except 31 ºC. The production of carotenoids approximately paralleled the amount of yeast growth in each experiment. It was also determined that the maximum pigment production was usually observed at the end of the exponential growth phase.

As indicated in Figs. 2 and 3, temperature has a high effect on the yeast growth rate and carotenoids production. The maximum production of torularhodin occurred at 19 ºC and the minimum at 27 ºC. On the contrary, the process time course at 27 ºC was short.

Torularhodin was synthesized in much higher amounts than β-carotene. The torularhodin content was 70.4, 91.1, 120 mg $l^{-1}$ at 27, 23, and 19 ºC repectively. The highest concentration of β-carotene was seen at 19 ºC (Table 3). Although the production of total carotenoid at 19 ºC was higher than those at 23 ºC and 27 ºC, the above results show that the process time course became strongly lengthened.

The yield was always equal to 0.52 ± 0.01 g $g^{-1}$ with a correlation coefficient value of 0.98, but it decreased to 0.33 g $g^{-1}$ when temperature was 31 ºC. The correlation between torularhodin syntheseis and glycerol consumption is determined from the plot of torularhodin formation ($T_t$-T) versus consumed glycerol ($S_0$-S). It was shown that the yield of torularhodin production increased in all cases in a similar way with the growth. (Fig. 4).

***Table 2: Effect of different pH on the growth and pigmentation by SP. Ruberrimus.***

| *p*H | Growth ($g\ l^{-1}$) | Cellular carotenoid ($mg\ g^{-1}$) | Volumetric carotenoid ($mg\ l^{-1}$) | Torularhodin ($mg\ l^{-1}$) | β-carotene ($mg\ l^{-1}$) | Average carotenoid production rate ($mg\ l^{-1}$) |
|---|---|---|---|---|---|---|
| 3.5 | 6.81 | 2.02 | 13.75 | 9.87 | 3.88 | 4.58 |
| 4 | 8.12 | 3.0 | 24.36 | 21.2 | 3.16 | 8.12 |
| 4.4 | 9.20 | 3.22 | 29.62 | 26.3 | 3.3 | 9.87 |
| 5 | 9.19 | 3.18 | 28.93 | 26.64 | 2.29 | 9.64 |
| 5.5 | 10.5 | 3.34 | 35.07 | 29.87 | 5.2 | 11.69 |
| 6 | 11.3 | 3.43 | 38.75 | 31.54 | 7.21 | 12.91 |
| 6.6 | 11.0 | 3.15 | 34.65 | 28.74 | 5.91 | 11.55 |
| 7 | 10.8 | 3.18 | 34.34 | 29.22 | 5.12 | 11.44 |
| 7.6 | 10.2 | 3.17 | 32.33 | 28.53 | 3.8 | 10.77 |
| 8.2 | 9.70 | 3.02 | 29.29 | 26.3 | 2.99 | 9.76 |

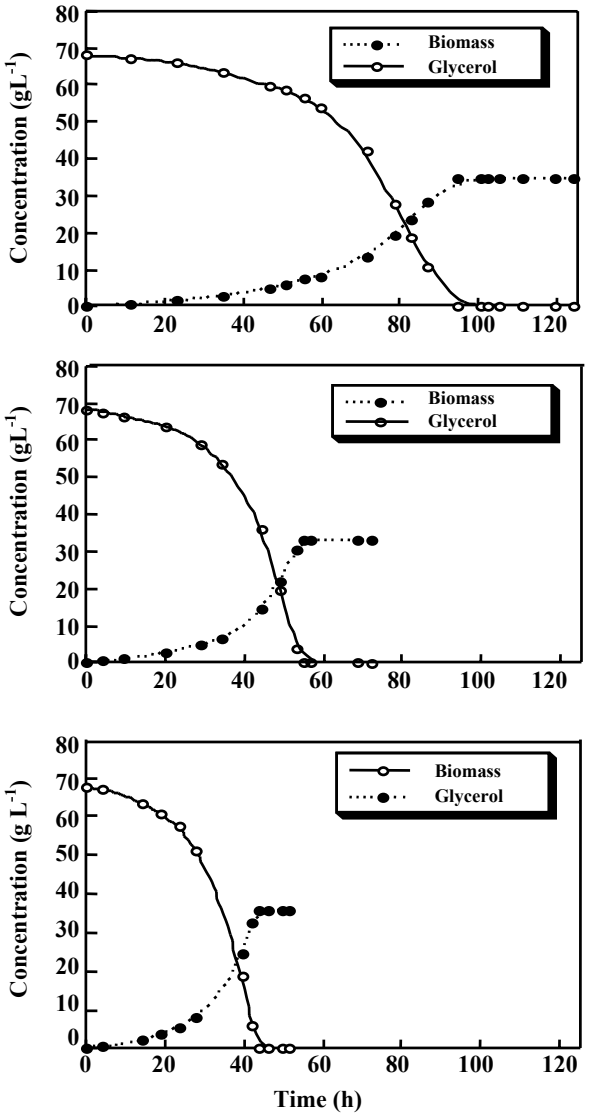


***Fig. 2: Effect of temperature on growth by Sporobolomyces ruberrimus H110 in a 3 l fermentor with technical glycerol ($67\ g\ l^{-1}$) , pH controlled at 6.0.***

***Table 3: Concentration of final invidual carotenoids synthesized by SP. ruberrimus, at various temperature values.***

| Temperature (°C) | $\mu_{max}$ ($h^{-1}$) | Torularhodin ($mg\ g^{-1}$) | β-carotene ($mg\ g^{-1}$) |
|---|---|---|---|
| 19 | 0.042 | 3.70 | 0.14 |
| 23 | 0.072 | 2.70 | 0.10 |
| 27 | 0.094 | 2.00 | 0.11 |
| 31 | 0.083 | N.D.[a] | N.D. |

*a) N.D.: not detected*

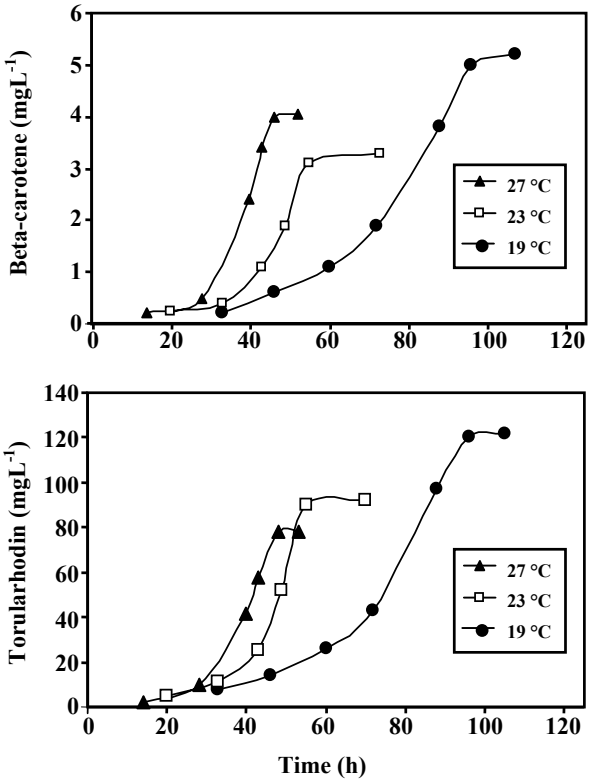


***Fig. 3: Effect of temperature on production of torularhodin and β-carotene by Sporobolomyces ruberrimus H110 in a 3 l fermentor with technical glycerol ($67\ g\ l^{-1}$) pH controlled at 6.0.***

***Table 4: Comparison of the carotenoid content in SP. ruberrimus H110 to the other yeasts.***

| Yeast | Total Carotenoid (mg $g^{-1}$ dry weight) | Type of Carotenoid | Content % | Culture medium | Reference |
|---|---|---|---|---|---|
| *P. rhodozyma* (mutant (N9) | 2.12 | Astaxanthin | 64 | Grape Juice | Meyer & Preez. 1994 |
| *R. Glutinis 22 P* | 0.268 | Torularhodin | 68 | Whey ultrafiltrate | Frengova et al. 1994 |
| *R. Glutinis (mutant 32)* | 3.8 | β-carotene | 79 | Sugarcane molasses | Bhosale & Gare, 2001 |
| *R. rubra*[a] | 1.25 | β-carotene | 100 | Peat extract | Martin et al. 1993 |
| *SP. ruberrimus H110*[b] | 3.7 | Torularhodin | 96 | Technical glycerol | In this work |

*a) A new isolated strain of Rhodotorula rubra.*

*b) The wild strain was isoltated by authors' laboratory.*

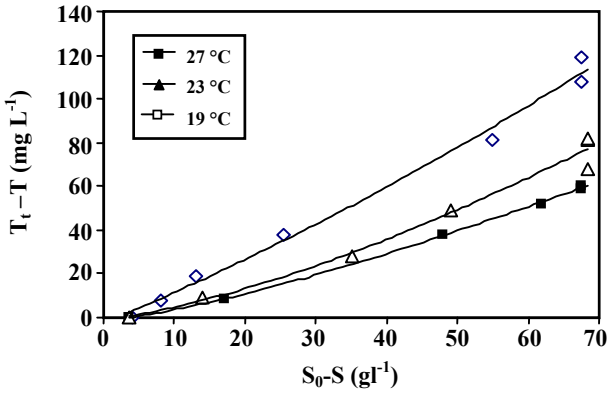


***Fig. 4: Yield of torularhodin as a function of glycerol consumption for different temperature.***

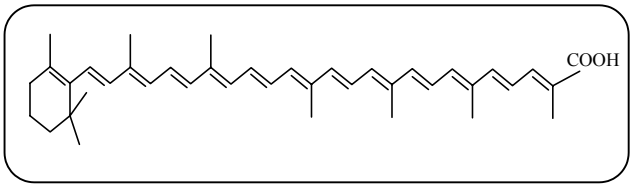


***Fig. 5: Chemical structure of torularhodin.***

ammonium sulfate and pH 6. Table 4 compares the carotenoid contents obtained for *SP. Ruberrimus* in this work with a few previously reported works for other microorganisms. Our values obtained for *SP. Ruberrimus* cultivated in a medium containing technical glycerol are in a high level compared to those obtained with other microorganisms considered as high producers.

However this strain is strongly aerobic and growth can be slowed down by lack of oxygen. Torularhodin (Fig. 5) has a very important antioxidant activity (*Sakaki et al*., 2002 [14]; *Ershov et al*., 1992 [15]; *Eugiena et al*., 1997 [16]) and is reported to be a provitamin A [15,16]. It is abundantly biosynthesized by this strain. In conclusion, *SP. ruberrimus* H110 could be a promising source of carotenoids for industry, using an inexpensive medium containing technical glycerol.

*Received : 29th June 2005 ; Accepted : 26th December 2005*

## CONCLUSIONS

These preliminary experiments demonstrated that technical glycerol has to be an effective carbon source favournig growth and carotenoid production, specially torularhodin, by *Sporobolomyces ruberrimus* H110. This can be due to the presence of different fatty acids (approximately 2%) in technical glycerol compared to pure glycerol. Temperature and pH play important roles in the production of pigments. At a low temperature (19 °C), maximum specific growth rate is minimum ($\mu_{max}$ = 0.042 $h^{-1}$). The cultivation of yeast under quasi-optimal growth conditions produced 3.84 mg of total carotenoid per g of yeast, at 19 °C with 20 g$l^{-1}$

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