KAMILA TOMOKO YUYAMA

CONDIÇÕES FISIOLÓGICAS E AMBIENTAIS QUE FAVORECEM A PRODUÇÃO DE CAROTENÓIDES POR *Rhodotorula mucilaginosa*

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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APROVADA: 22 de outubro de 2013.

Antônio Galvão do Nascimento (Coorientador)	Maria Cristina Dantas Vanetti (Coorientadora)
Vanessa Cristina de Castro	Flávia Maria Lopes Passos (Orientadora)

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BIOGRAFIA

KAMILA TOMOKO YUYAMA, filha de Kaoru Yuyama e Lucia Kiyoko Ozaki Yuyama, nasceu no dia 13 de Abril de 1989, em Ribeirão Preto, São Paulo. Graduou-se em Licenciatura em Ciências Biológicas pela Universidade Federal do Amazonas em Março de 2011. Em julho desse mesmo ano, iniciou o curso de mestrado no Programa de Pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa, Viçosa, Minas Gerais.

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RESUMO

YUYAMA, Kamila Tomoko, M.Sc., Universidade Federal de Viçosa, outubro de 2013. **Condições fisiológicas e ambientais que favorecem a produção de carotenoides por *Rhodotorula mucilaginosa.*** Orientadora: Flávia Maria Lopes Passos. Coorientadores: Antônio Galvão do Nascimento e Maria Cristina Dantas Vanetti.

Em micro-organismos produtores, carotenoides são pigmentos, precursores de vitamina A, que os protegem contra o estresse oxidativo. Fatores ambientais extrínsecos tais como temperatura, fontes específicas de carbono e indutores de espécies reativas de oxigênio (ROS) como luz e peróxido de hidrogênio associados a condições fisiológicas (fase de crescimento exponencial máxima, fase estacionária inicial e tardia) influenciam no acúmulo de carotenoides pelas células microbianas. Uma levedura pigmentada foi isolada do horto da Universidade Federal de Viçosa, Minas Gerais, Brasil. Os objetivos desse trabalho foram identificar esta levedura isolada no horto da UFV e analisar a influência dos fatores ambientais (temperatura, intensidade de luz e concentração de arabinose) e das diferentes condições fisiológicas (fase log, estacionária inicial e tardia) na produção de carotenoides e na velocidade de crescimento da levedura. A identificação taxonômica da levedura foi feita pelo *Centraalbureau voor Schimmelcultures* (CBS) por meio das análises das sequências 5,8S e 26S do rRNA e por ferramentas moleculares e bioquímicas, resultando na identificação da espécie como *Rhodotorula mucilaginosa.* A influência das condições ambientais e fisiológicas na produção de carotenoides e na taxa de crescimento foi verificada pela metodologia de superfície de resposta (RSM) por meio de um delineamento composto central (CCD) com três fatores e seis replicatas do ponto central. Os 20 experimentos foram feitos em 91h de batelada e as amostras foram coletadas em diferentes estados fisiológicos da cultura, isto é na fase de crescimento máximo (fase log), fase de desaceleração do crescimento (2h iniciais na entrada da fase estacionária) e fase estacionária (60h após alcançar a massa celular máxima). Demonstrou-se que a formação de carotenoides pela levedura não é associada ao crescimento. O rendimento máximo de carotenoides por

massa celular (µg/g) ocorreu na fase de desaceleração do crescimento. A velocidade específica máxima de crescimento (μ_{max}) e o rendimento em carotenóide total por massa celular (µg/g) foram ajustados ao modelo matemático, com R^2 de 0,96 e 0,91 respectivamente, e o desajuste não foi significante ($p>0,05$). Os níveis de intensidade de luz (µmol $m^{-2}s^{-1}$), arabinose (%p/v) e temperatura (°C) que criaram a resposta máxima de rendimento de carotenóides totais (93,92 µg/g) foram 100 µmol $m^{-2}s^{-1}$; 5%; 18 °C e da velocidade de crescimento (0,31h^{-1}) foram 63,6 µmol $m^{-2}s^{-1}$; 3,7% e 24,6 °C.

ABSTRACT

YUYAMA, Kamila Tomoko, M.Sc., Universidade Federal de Viçosa, October, 2013. **Physiological and environmental conditions that enhance the production of carotenoids by *Rhodotorula mucilaginosa*.** Adviser: Flávia Maria Lopes Passos. Co-advisers: Antônio Galvão do Nascimento and Maria Cristina Dantas Vanetti.

In pigmented microorganisms, carotenoids are pigments, precursors of vitamin A that provide protective effects against oxidative damage. Extrinsic environmental factors such as temperature, specific carbon sources and inducers of reactive oxygen species (ROS) such as hydrogen peroxide and light associated with physiological conditions (maximum exponential growth phase, early and late stationary phase) influence the accumulation of carotenoids in microbial cells. A pigmented yeast was isolated from an orchard in Universidade Federal de Viçosa, Minas Gerais, Brazil. The main aims were to identify the isolated yeast and analyze the influence of environmental (light, temperature and arabinose) and physiological conditions (log phase, early and late stationary phase) on the production of carotenoids and the growth rate of the yeast. The taxonomical identification of the yeast strain was accomplished through analysis of 5.8S and 26S rRNA by the *Centraalbureau voor Schimmelcultures* (CBS) and biochemical and molecular approaches. The yeast was identified as *Rhodotorula mucilaginosa.* In order to analyze the influence of factors on production of carotenoids and growth rate we used a methodology of response surface (RSM), and a central composite factorial design (CCD) with three factors and six replicates of central point was applied. The 20 experiments were done in batch in 91h and samples were collected at different physiological stages of the culture, at maximum growth rate (log phase), at deceleration growth phase (2h initial at the entrance of the stationary phase) and at stationary phase (60h after reaching the maximum cell mass). It implicates that carotenogenesis is not associated with cellular growth. The maximum carotenoid yield per cell mass (μg/g) occurred at deceleration growth phase. The growth rate (μ) and total carotenoid yield (μg/g) calculations well-adjusted to the mathematical model, with R^2 of 0.96 and 0.91, respectively, and the lack of fit was not significant ($p>0.05$). The levels of light

(μmol $m^{-2}s^{-1}$), arabinose (%w/v) and temperature (°C) that created a maximum answer of total carotenoid yield (93.92 μg/g) were 100 μmol $m^{-2}s^{-1}$; 5% and 18 °C and growth rate ($0.31h^{-1}$) were 63.6 μmol $m^{-2}s^{-1}$; 3.7% and 24.6 °C.

ARTICLE: PHYSIOLOGICAL AND ENVIRONMENTAL CONDITIONS THAT ENHANCE THE PRODUCTION OF CAROTENOIDS BY *Rhodotorula mucilaginosa*

PHYSIOLOGICAL AND ENVIRONMENTAL CONDITIONS THAT ENHANCE THE PRODUCTION OF CAROTENOIDS BY *Rhodotorula mucilaginosa*

ABSTRACT

In pigmented microorganisms, carotenoids are pigments, precursors of vitamin A that provide protective effects against oxidative damage. Extrinsic environmental factors such as temperature, specific carbon sources and inducers of reactive oxygen species (ROS) such as hydrogen peroxide and light associated with physiological conditions (maximum exponential growth phase, early and late stationary phase) influence the accumulation of carotenoids in microbial cells. A pigmented yeast was isolated from an orchard in Universidade Federal de Viçosa, Minas Gerais, Brazil. The main aims were to identify the isolated yeast and analyze the influence of environmental (light, temperature and arabinose) and physiological conditions (log phase, early and late stationary phase) on the production of carotenoids and the growth rate of the yeast. The taxonomical identification of the yeast strain was accomplished through analysis of 5.8S and 26S rRNA by the *Centraalbureau voor Schimmelcultures* (CBS) and biochemical and molecular approaches. The yeast was identified as *Rhodotorula mucilaginosa.* In order to analyze the influence of factors on production of carotenoids and growth rate we used a methodology of response surface (RSM), and a central composite factorial design (CCD) with three factors and six replicates of central point was applied. The 20 experiments were done in batch in 91h and samples were collected at different physiological stages of the culture, at maximum growth rate (log phase), at deceleration growth phase (2h initial at the entrance of the stationary phase) and at stationary phase (60h after reaching the maximum cell mass). It implicates that carotenogenesis is not associated with cellular growth. The maximum carotenoid yield per cell mass (μg/g) occurred at deceleration growth phase. The growth rate (μ) and total carotenoid yield (μg/g) calculations well adjusted to the mathematical model, with R^2 of 0.96 and 0.91, respectively, and the lack of fit was not significant ($p>0.05$). The levels of light (μmol $m^{-2}s^{-1}$), arabinose (%w/v) and temperature (°C) that created a maximum answer of total carotenoid yield (93.92 μg/g) were 100 μmol $m^{-2}s^{-1}$; 5% and 18 °C and growth rate ($0.31h^{-1}$) were 63.6 μmol $m^{-2}s^{-1}$; 3.7% and 24.6 °C.

Keywords: *Rhodotorula mucilaginosa*; carotenoids; growth rate, light, surface response.

1. INTRODUCTION

Carotenoids present antioxidant properties and reduce the risk of chronic degenerative diseases in humans, such as cancer, cardiovascular disease, osteoporosis, cataracts and age-related macular degeneration (Rao and Rao, 2007). Over 600 carotenoids are naturally occurring, and 150 have been found in photosynthetic organisms. However, filamentous fungi, bacteria and nonphotosynthetic yeasts, as *Cryptococcus, Rhodotorula, Rhodosporidium, Sporobolomyces* and *Phaffia,* also produce carotenoids (Britton, 1993; Johnson and Lewis, 1979). Commercial production of natural microbial carotenoids is a new more eco-friendly approach than synthetic manufacture by chemical procedures (Rodríguez-Sáiz et al., 2010).

A great variety of microorganisms accumulates intracellularly carotenoids (Nelis and De Leenheer, 1991). A variety of environmental and cultural stimulants enhances volumetric production and cellular accumulation of carotenoids in microorganisms (Bhosale, 2004). Some studies have used genetic engineering, generating spontaneous mutations in order to improve carotenoid production by microbial strains. However, these strains are not always more robust in terms of resistance and adaptation (An, 1997).

Studies concerning cell physiology and environmental conditions that favor microbial carotenoid synthesis are interesting not only to search for the ideal parameters for industrial production but also to elucidate the microbial physiological advantages provided by such pigments. Changes in temperature of incubation, carbon sources and the presence of reactive oxygen species (ROS) inducers, such as light and hydrogen peroxide, promote increased production of carotenoids (Vázquez, 2001; Bhosale, 2004; Aksu and Eren, 2005; Liu and Wu, 2006; Rodríguez-Sáiz et al., 2010). Carotenoids are potent antioxidants that show protective effects on the organisms exposed to oxidative damage (Bhosale, 2004; Schroeder and Johnson, 1995a; Schroeder and Johnson, 1995b).

A yeast strain was isolated from orchard in Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. The strategies for isolation have favored yeasts from genus *Rhodotorula* sp. (Barreto et al., 2008). Several *Rhodotorula* species are able to synthesize different carotenoids (Frengova

et al., 1994), from different carbon sources including agricultural wastes, as pentoses like arabinose widely distributed in lignocelulosic biomass (Izydorczyk and Biliaderi, 1995; Frengova, and Beshkova, 2009; Parajó et al., 1997). Furthermore, factorial approaches can improve the carotenoid production by yeast, through optimization of culture conditions with reduced number of experimental trials needed to evaluate multiple variables and their interactions (Buzzini, 2000; Gharibzahedi et al., 2012). The main aims of this work were to identify the isolated yeast and analyze the influence of environmental (light, temperature and arabinose) and physiological conditions (log phase, early and late stationary phase) on the production of carotenoids and the growth rate of the yeast.

2. MATERIAL AND METHODS

2.1 Microorganism and maintenance

A yeast strain was isolated from an exudate of *Pinus sylvestris* growing at the University orchard (Universidade Federal de Viçosa-UFV campus, Viçosa-MG-Brazil) at autumn season (Barreto et al., 2008). The main criterion for choice of isolation spots was presence of color on the exudates, indicative of production of carotenoids, like β-carotene or astaxanthin. The UFV-isolated strain was maintained in YM medium [1% glucose; 0.5% peptone, 0.3% malt extract and 0.3% yeast extract] with 40% glycerol at -80 °C.

2.2 Identification of microorganism

Centraalbureau voor Schimmelcultures (CBS) performed the identification of the isolate based on molecular assays. Briefly, the strain was cultured on Malt Extract Agar for 3-4 days in the dark at 25 °C. The MoBio UltraClean™ Microbial DNA Isolation Kit was used for DNA isolation, according to the instructions of the manufacturer. Fragments containing the Internal Transcribed Spacer (ITS) 1 and 2 of the 5.8S gene were amplified using the primers LS266 (GCATTCCCAAACAACTCGACTC) and V9G (TTACGTCCCTGCCCTTTGTA, Gerrits van den Ende and de Hoog, 1999).

Fragments containing the 26S ribosomal RNA gene, Large Subunit D1 and D2 region (LSU) were amplified using the primers LR0R (ACCCGCTGAACTTAAGC) and LR5 (TCCTGAGGGAAACTTCG, Vilgalys and Hester, 1990). The PCR fragments were sequenced with the ABI Prism® Big DyeTM Terminator v. 3.0 Ready Reaction Cycle sequencing Kit. Samples were analyzed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the program SeqMan from the Laser Gene package.

The sequences of ITS (5.8S rRNA) and LSU (26S rRNA) were compared in a large yeast database of CBS-KNAW Fungal Biodiversity Centre with sequences of most type strains. In order to create a phylogenetic tree of *Rhodotorula* species, ITS (5.8S rRNA) sequences were compared with other ITS sequences in GenBank, through MEGA 5 software (www.megasoftware.net) and the neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap repetitions.

We complemented the identification of UFV-isolated strain by a biochemical assay for nitrate and nitrite utilization (Rhodes & Roberts, 1975). *Escherichia coli* DH5α was used as a positive control.

2.3 Experimental design and statistical analysis

In order to predict the surface response of literature-cited main factors affecting carotenoid yield by yeast (Ambati and Ayanna, 2001; Sharma et al., 2007), a response surface methodology (RSM) was designed. A central composite factorial design (CCD) with three factors [arabinose (%w/v), temperature (°C) and light intensity (μmol $m^{-2}s^{-1}$)] and six replicates of central point was applied. This method was performed at five levels (-α, -1, 0, 1, +α) where, $\alpha=2^{n/4}$, n equals the number of variables and 0 corresponds to the central point in 20 experiments of CCD (Table 1). Two experimental responses [carotenoid yield (μg carotenoid/g dry cell mass) and β-carotene yield (μg carotenoids/g dry cell mass)] from each experiment were recorded. The model was represented by the equation:

$$\mathbf{Y = b_o + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{23}x_2x_3 + b_{13}x_1x_3}$$

Where Y is the predicted response: b_o intercept coefficient; b_1, b_2, b_3 the linear coefficients; b_{12}, b_{23}, b_{13} interaction coefficients; b_{11}, b_{22}, b_{33} the quadratic coefficients; and x_1, x_2, x_3, x_{11}, x_{22}, x_{33}, x_{12}, x_{23}, x_{13} the factor levels. The program for statistical Design Expert 8.0 was used to determine the optimal level of factors (response curve) for maximum responses.

2.4 Inoculum preparation and experimental procedure

Pre-culture was prepared by inoculating a loop full of cell mass from -80 °C stock into 250 mL Erlenmeyer flasks containing 50 mL of YM medium with 1% arabinose and incubating at 30 °C under agitation of 200 rpm. After 24h incubation, cell mass were collected, washed twice and suspended in sterile peptonated water (0.01%) and used to inoculate the 20 cultures according to the designed experiments. All of them were initiated with cell mass concentration ranging from 0.20 to 0.217 g/L, and were carried out in 500 mL jacketed glass reactors containing 180 mL of YM medium with different concentrations of arabinose, and exposed under different light intensities and temperature ranges (Table 1). All the cultures were equally magnetically agitated. The intensity of light was set according to a radiometer and the different temperatures were controlled by a cooled temperature water bath (-10 to 100 °C). The 20 experiments were done in batch in 91h and samples were collected at different physiological stages of the culture, at maximum growth rate (log phase), at deceleration growth phase (2h initial at the entrance of the stationary phase) and at stationary phase (60h after reaching the maximum cell mass).

2.5 Determination of the cell mass and specific growth rate

The cell mass dry weight was determined as previously described (Diniz et al., 2012), where a linear regression of the plot of the absorbance (A_{600nm}) versus dry mass (g/L) allowed the determination of the dry mass corresponding to 1 unit of absorbance at 600 nm. One A_{600nm} unit was found to be equivalent to 0.462 g/L of dry mass.

To determine the growth rate (μ), at least five points in the exponential growth phase were collected to make a linear regression of the plot of ln A_{600nm} unit *versus* time (h).

2.6 Analytical methods

To extract the pigments, we adapted a method described by Kaiser et al. (2007). This method was used to extract carotenoid from bacteria and yeast and it was divided in four steps: disintegration of the cell, extraction, separation, and quantitation. We substituted the process disintegration of the cell to a mechanical cell disruption by shaking with a glass beads (70-100 U.S sieve) and DMSO (dimethylsulfoxide) for 5 min.

After extraction, we dissolved the pigment into petroleum ether to quantify the total carotenoid yield (μg/g) by the formula (Rodriguez-Amaya, 2001):

$$\text{Content of carotenoids } (\mu g/g) = \frac{A \times V\ (mL) \times 10^4}{A^{1\%}_{1\ cm} \times W(g)}$$

Where A is the absorbance at 450 nm of the total carotenoid; V is the total extract volume; W is the sample weight and $A^{1\%}_{1\ cm}$ is the β-carotene absorption coefficient in petroleum ether (2592).

3. RESULTS AND DISCUSSION

The results of molecular identification made by comparison of a large yeast database of CBS-KNAW Fungal Biodiversity Centre were LSU sequence differed in six nucleotides and ITS sequence differed in three nucleotides of the type strain of *Rhodotorula dairenensis* (T. Haseg. and I. Banno) Fell, J.P. Gadanho and Sampaio. However, LSU sequence differed in three nucleotides and ITS sequence differed in seven nucleotides of the type strain of *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison. According to Gadanho and Sampaio (2002) the difference between *R. mucilaginosa* and *R. dairenensis* is nitrate and nitrite utilization. The results of biochemical test

suggest that the UFV isolated *Rhodotorula* sp. is *R. mucilaginosa*, because the nitrate reduction did not occur.

In addition, a comparison of D1 and D2 from LSU 26S sequences and ITS sequences of ribosomal RNA (rRNA) from the studied yeast with those of GenBank specimens showed high similarity (99%) with *R. mucilaginosa*. Corroborating to the molecular identification, we made a phylogenetic tree of ITS sequence from the isolated yeast and from the related species of genus *Rhodotorula* obtained from GenBank, with *Rhodosporidium fluviale* Fell, Kurtzman, Tallman & J.D. Buck as outgroup (Figure 1). The phylogenetic tree showed that the isolated specimen remained in the same branch of *R. mucilaginosa* (AF321544) with a bootstrap value of 74.

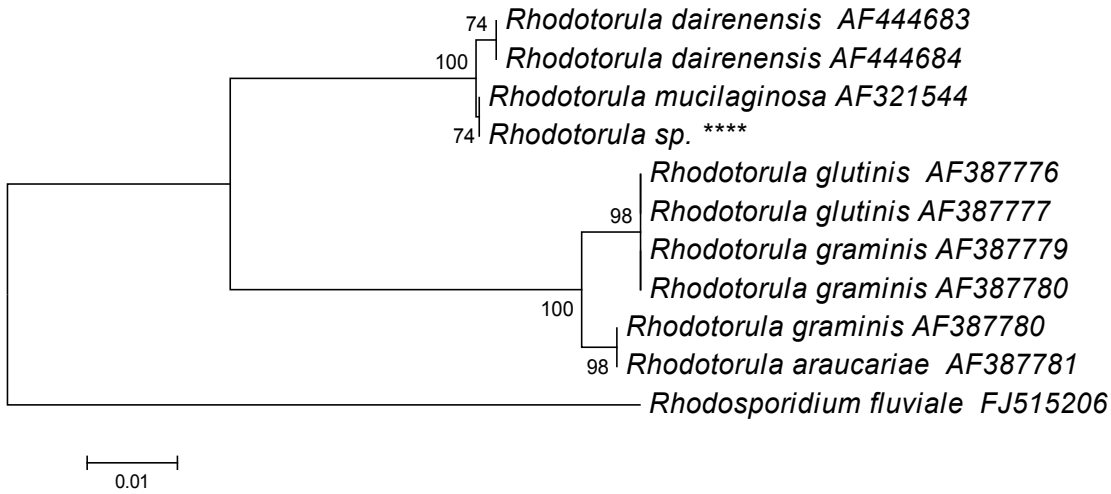

Figure 1. Phylogenetic tree of the UFV- isolated *R. mucilaginosa* and related species of the genus *Rhodotorula*, obtained by neighbor-joining analysis of the ITS region of the 5.8S rRNA, with *R. fluviale* as outgroup. Percentage bootstrap values of 1000 replicates are given at each node. GenBank accession numbers are indicated after strain designation.

The results of biochemical and molecular identification analysis showed that, in fact, the specimen isolated is *R. mucilaginosa.*

R. mucilaginosa is a basidiomycetous yeast, that belongs to *Sporidiobolus* clade (Fell et al., 2000); it has a wide distribution in terrestrial (Butinar et al., 2007; Maldonade et al., 2007), aquatic (Libkind et al., 2003) and marine habitats (Lahav et al., 2002; Nagahama et al., 2001). It can be an endophytic (Bura et al., 2012) and can also be found in human microbiota. Furthermore, the yeast is considered a no virulent saprophyte, even though it

causes serious infections in patients who have immunodeficiency or undergo transient or long-term immunosuppression (Galán-Sánchez et al., 1999).

However, *R. mucilaginosa* presents a high potential to produce diverse and abundant carotenoids, such as β-carotene, but not synthesized astaxanthin (Figure 2). According to Libkind and Brook (2006) the yeast can synthesize beyond β-carotene, also torulene and torularhodin in many proportions. The carotenogenesis is affected by many environmental and physiological conditions, especially temperature (Johnson and Lewis, 1979; Frengova and Beshkova, 2009), carbon sources (Fang and Chen, 1993) and the singlet oxygen and superoxide formed during illumination (Schroeder and Johnson, 1995a; Schroeder and Johnson, 1995b).

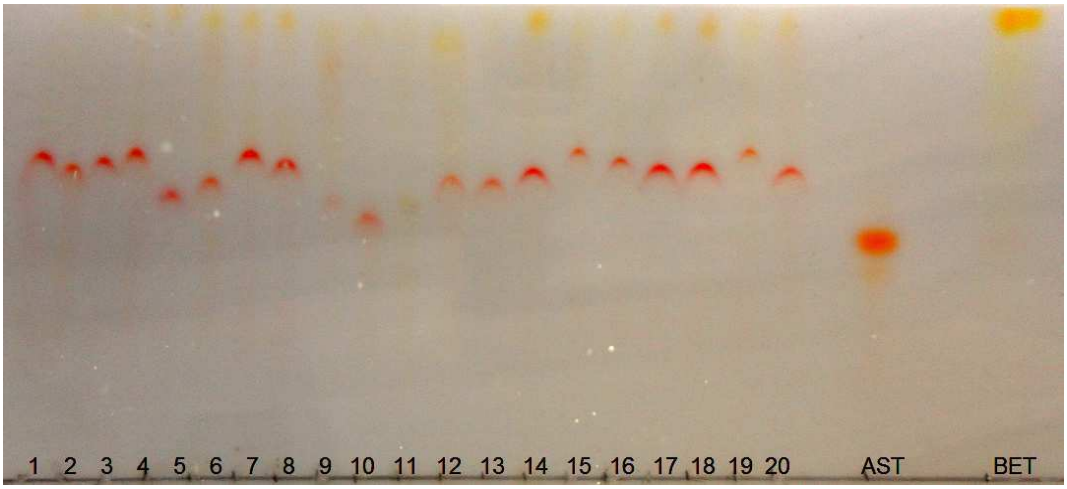

Figure 2. TLC plate showing the separation of the carotenoids synthesized by *R. mucilaginosa* in the 20 experiments of RSM. The mobile phase was methanol-acetone-toluene (5:20:75, v/v/v). AST: astaxanthin standard and BET: β-carotene standard.

The samples were collected at different physiological stages of the culture (Figure 3A). The production of carotenoid was not proportional to the growth rate (Figure 3B), typical of secondary metabolism (Griffin, 1994). Although the amount of carotenoid depends on the amount of cell mass, it was not related to the growth rate of yeast population. It means that the process for carotenoids production should be designed in two stages: cell mass formation in the first stage and carotenoids synthesis in the second. As show in Figure 3B there is a greater accumulation of carotenoids in the beginning of stationary phase (2h) than in logarithmic phase.

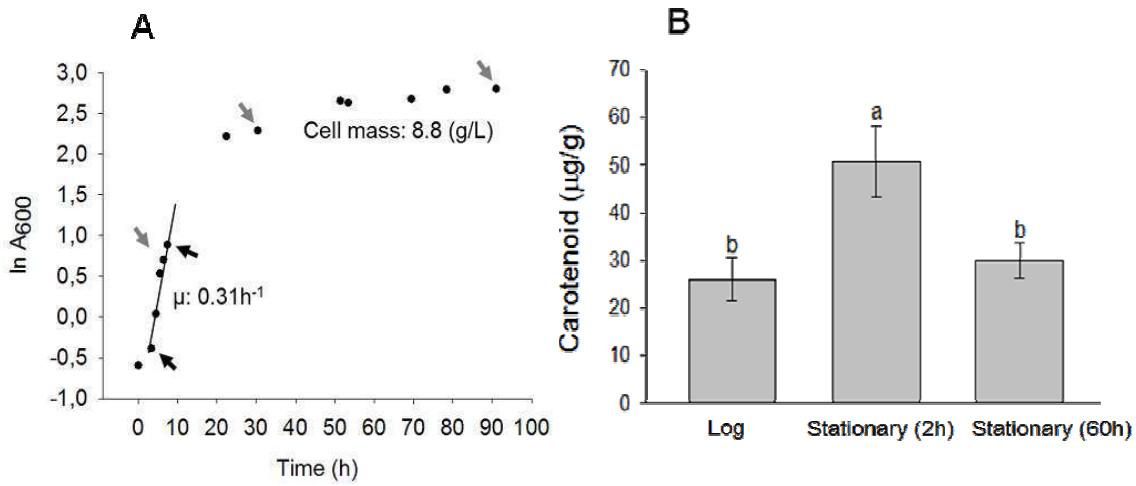

Figure 3. (A) Growth of *R. mucilaginosa* at 23 °C, 60 μmol $m^{-2}s^{-1}$ of light and 3.25% arabinose, gray arrows indicate points where samples were collected to analyze the carotenoid yield (μg/g) and black arrows the points considered to calculate the linear regression from what the slope value was taken as growth rate (B). The carotenoid yield in exponential growth phase and in stationary phase (2h and 60h after reaching maximum cell mass) evaluated by Tukey Test with probability of 5% and six repetitions.

The carotenogenesis of UFV-isolated *R. mucilaginosa* is related to the cellular maintenance and not with cellular growth. According to Goodwin (1972), carotenoid accumulation in most yeast occurs in the late logarithmic phase and continues in the stationary phase. The carotenoid yield was reduced in the stationary phase (60h), perhaps because at this stage a large amount of cells are unviable and growth rate approximate to zero. In that condition the stress is higher than the cell can endure.

The results of central of composite design to determine the optimal levels of light (μmol $m^{-2}s^{-1}$), arabinose (%w/v) and temperature (°C) for the carotenoid yield and growth rate by *R. mucilaginosa* are shown in Tables 1 and 2.

Table 1. Experimental central composite design (CCD) runs and corresponding carotenoid yield (µg/g) after 60 h in stationary phase.

Run order	Light (µmol $m^{-2}s^{-1}$)	Arabinose (%w/v)	Temperature (°C)	Carotenoid (µg/g)
1	20.0	1.50	18.0	62.6
2	100	1.50	18.0	84.6
3	20.0	5.00	18.0	41.4
4	100	5.00	18.0	90.2
5	20.0	1.50	28.0	47.9
6	100	1.50	28.0	53.6
7	20.0	5.00	28.0	50.8
8	100	5.00	28.0	71.5
9	-7.27	3.25	23.0	58.6
10	127	3.25	23.0	101
11	60.0	0.31	23.0	44.8
12	60.0	6.19	23.0	63.6
13	60.0	3.25	14.6	81.3
14	60.0	3.25	31.4	58.2
15	60.0	3.25	23.0	26.9
16	60.0	3.25	23.0	28.2
17	60.0	3.25	23.0	33.1
18	60.0	3.25	23.0	36.1
19	60.0	3.25	23.0	26.9
20	60.0	3.25	23.0	28.0

Table 2. Experimental central composite design (CCD) runs and corresponding cell mass (mg/g) and μ (h^{-1}).

Run order	Light (μmol $m^{-2}s^{-1}$)	Arabinose (%w/v)	Temperature (°C)	Cell mass* (mg/mL)	μ (h^{-1})
1	20.0	1.50	18.0	4.70	0.10
2	100	1.50	18.0	3.60	0.17
3	20.0	5.00	18.0	7.65	0.10
4	100	5.00	18.0	5.50	0.12
5	20.0	1.50	28.0	5.10	0.21
6	100	1.50	28.0	5.00	0.20
7	20.0	5.00	28.0	12.82	0.21
8	100	5.00	28.0	7.60	0.28
9	-7.27	3.25	23.0	3.71	0.12
10	127	3.25	23.0	3.50	0.12
11	60.0	0.31	23.0	4.25	0.23
12	60.0	6.19	23.0	8.00	0.25
13	60.0	3.25	14.6	4.41	0.09
14	60.0	3.25	31.4	8.24	0.15
15	60.0	3.25	23.0	8.80	0.31
16	60.0	3.25	23.0	9.00	0.29
17	60.0	3.25	23.0	8.80	0.29
18	60.0	3.25	23.0	8.37	0.29
19	60.0	3.25	23.0	8.90	0.33
20	60.0	3.25	23.0	9.27	0.35

*Cell mass accumulated after 60h in stationary phase.

The responses that provided total carotenoid yield (μg/g) and specific maximum growth rate (h^{-1}) of *R. mucilaginosa* were evaluated by analysis of variance (Table 3 and 4). After the removal of terms relating to non-significant variables ($p>0.05$), the parameters could be obtain by the functions:

Total carotenoid = 379.14129 - 0.50369 Light - 24.46374 Temperature + 0.074678 Light Arabinose - 0.027770 Light Temperature + 0.52121 Arabinose Temperature + 0.010072 $Light^2$ + 2.29807 $Arabinose^2$ + 0.50135 $Temperature^2$

μ = 1.30657 + 0.11835 Temperature -3.93259 x 10^{-5} $Light^2$ - 6.69223 x 10^{-3} $Arabinose^2$ - 2.51685 x 10^{-3} $Temperature^2$

Table 3. Analysis of variance (ANOVA) results for the quadratic equation developed for total carotenoid yield (µg/g) by *R. mucilaginosa.*

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P* > *F*
Light					<0.0001
Arabinose					0.1245
Temperature					0.0016
Light ^{2}					<0.0001
Arabinose ^{2}					0.0011
Temperature ^{2}					<0.0001
Light x Arabinose					0.0313
Light x Temperature					0.0240
Arabinose x Temperature					0.0500
Model	9232.88	9	1025.88	29.38	<0.0001
Lack-of-Fit	275.38	5	55.08	3.73	0.0874
Error	73.81	5	14.76		
Total	9582.07	19			

Table 4. Analysis of variance (ANOVA) results for the quadratic equation developed for growth rate (h^{-1}) by *R. mucilaginosa.*

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P* > *F*
Light					0.2677
Arabinose					0.6293
Temperature					0.0025
Light ^{2}					<0.0001
Arabinose ^{2}					0.0482
Temperature ^{2}					<0.0001
Light x Arabinose					0.7653
Light x Temperature					0.7653
Arabinose x Temperature					0.2133
Model	0.13	9	0.014	11.93	0.0003
Lack-of-Fit	8.675×10^{-3}	5	1.735×10^{-3}	2.64	0.1550
Error	3.283×10^{-3}	5	6.567×10^{-4}		
Total	0.14	19			

The regression model presented statistical significance (F test), and the analysis of variance was performed for the response surface of the quadratic model (Tables 3 and 4). In addition, R^2 of the model was 0.96 for total carotenoid yield and 0.91 for growth rate (h^{-1}), demonstrating that the quadratic model was significant and the lack of fit was not significant ($p>0.05$). The high R^2 indicates fitness of the model (Weisberg, 1985).

In Figure 4 and 5 are shown the three-dimensional response surfaces of the final model of total carotenoid yield (µg/g) and growth rate (h^{-1}), produced by *R. mucilaginosa*. The response surfaces were based on the final model, with one constant variable fixed at its optimal level, while the remaining two factors varied within the experimental range.

The levels of light (µmol $m^{-2}s^{-1}$), arabinose (%w/v) and temperature (°C), which created a maximum response of total carotenoid yield (93.92 µg/g) were 100 µmol $m^{-2}s^{-1}$, 5 % and 18 °C; and growth rate (0.31 h^{-1}) were 63.6 µmol $m^{-2}s^{-1}$, 3.7 % and 24.6 °C. Furthermore, carotenoids are intracellular components that cannot be excreted to the medium and the key for biological carotenoid production is the cost of the cell mass production, the concentration of carotenoids and their production inside the cell (Ausich, 1997). Although maximum specific growth rate does not always means maximum total cell mass (Table 2), the productivity depends on response of growth rate, because it can indicate how fast the cell will enter into stationary phase, the phase of higher accumulation of carotenoids (Figure 3).

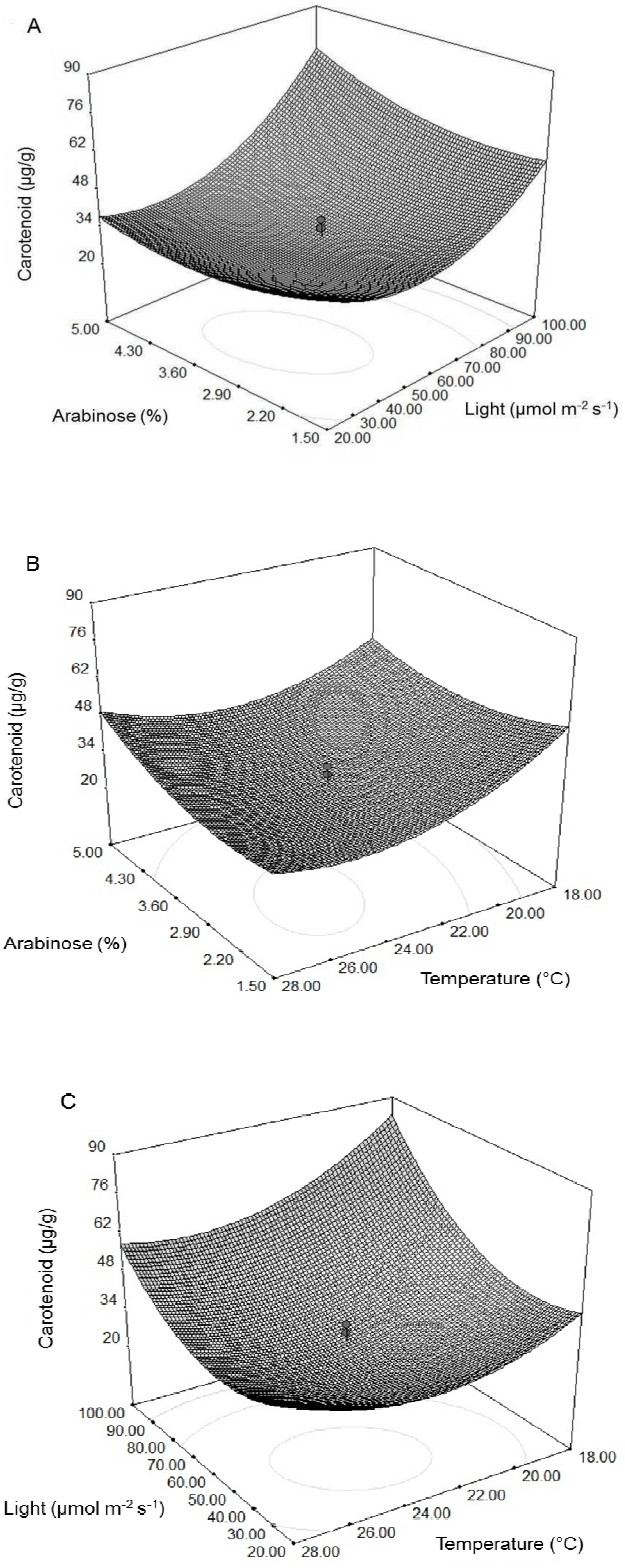

Figure 4. Response surface of total carotenoid yield (µg/g) produced by *R. mucilaginosa*. (A) Arabinose and light; (B) Arabinose and temperature; (C) Light and temperature.

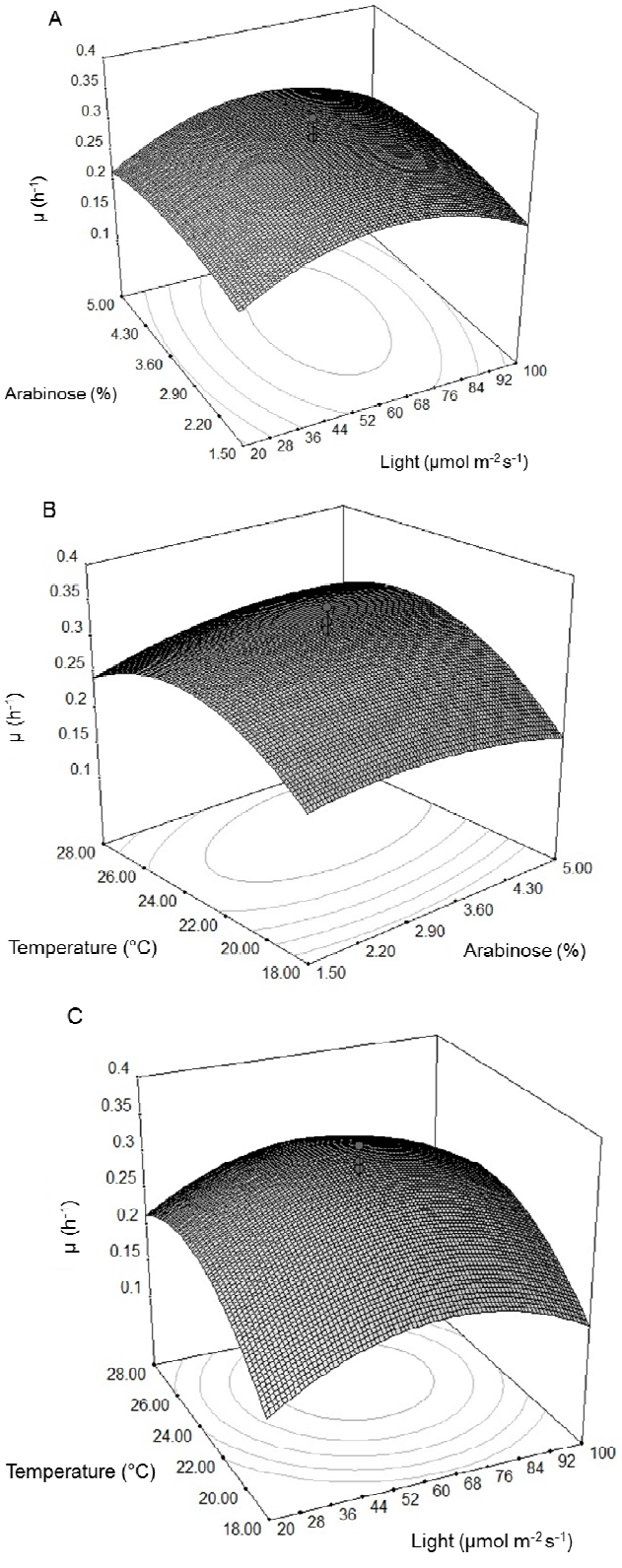

Figure 5. Response surface of the maximum specific growth rate (μ_{max}) from *R. mucilaginosa*. (A) Arabinose and light; (B) Temperature and arabinose; (C) Temperature and light.

The light intensity influenced carotenogenesis in *R. mucilaginosa* (Table 3 and Figure 4), especially in a high intensity of light, such as 100 μmol $m^{-2}s^{-1}$, which was similar to result found in *R. minuta* (Tada and Shiroishi, 1982). There are many reports on light effect in carotenogenesis (Meyer and Du Preez, 1994; An, 1997; Sasaki et al., 2000; Stachowiak and Czarnecki, 2007). According to Schroeder and Johnson (1995a), carotenoids protect cells from photo-oxidative damage by scavenging harmful agents such as singlet and triplet molecular species produced upon illumination, through its structure of nine or more conjugated double bond. Carotenogenesis is an important factor to protect *R. mucilaginosa* against oxidative stress, because Cu/Zn superoxide dismutase genes are silent in this yeast, as consequence of no-functional promoters (Moore et al., 1989; Hernandez-Saavedra, 2003). Furthermore, the color of carotenoid serves to block out certain wavelengths of light that could otherwise damage the cell (Chandi and Grill, 2011).

The interaction of light and temperature in carotenogenesis (Table 3) was significant and our results agree with the reported of Bhosale and Gadre (2002), which have investigated the production of β-carotene by *R. glutinis* in a 1 L fermenter at different temperature and illumination conditions. In their experiments, the optimum temperature for growth and β-carotene production was 30 and 20 °C, respectively. At 30 °C, illuminating the fermenter in late logarithmic phase resulted in a 58% increase of β-carotene production with a concurrent decrease in torulene. However, at 20 °C, no appreciable increase was observed, thus the interaction of light and temperature was essential to carotenogenesis.

Although luminosity has higher influence on carotenogenesis, it also affects growth rate (Table 4 and Figure 5). In this work, moderate intensity of light (63.6 μmol $m^{-2}s^{-1}$) contributed to the maximum growth rate ($0.31h^{-1}$) from *R. mucilaginosa*. According to Yen and Zhang (2011) the light could greatly enhance the cells growth rate and total lipid productivity in *R. glutinis*.

Temperature affects not only the growth kinetics of the microorganism (Table 4) but also the carotenogenesis (Table 3), which can change the biosynthetic pathway of carotenoids (Frengova and Beshkova, 2009;

Johnson & Lewis, 1979). In this work, the interaction of the variables showed that the best temperature for total carotenoid yield (µg/g) was 18 °C, while for the growth rate it was 24.6 °C. Different from earlier report, which mention *R. mucilaginosa* with higher specific growth and carotenoid formation rates at 25 to 30 °C, decreasing sharply above 30 °C (Aksu and Eren, 2005).

We demonstrated that arabinose concentration in the range of 1.5 to 5%w/v has a positive influence in carotenogenesis. Arabinose is one of the most abundant pentose in nature (Izydorczyk and Biliaderi, 1995; Pan et al., 2007). There are few studies on the use of pentose by *R. mucilaginosa* and the genes of this metabolic pathway (Xu et al., 2011; Bura et al., 2012). However, *R. mucilaginosa* can grow at both pentose and hexose as carbon source (Bura et al., 2012). In this work, glycerol also showed higher cell mass yield (data not showed) which suggest that it could be an alternative substrate for carotenoid production from biodiesel, similar to the results reported by Taccari et al. (2012).

The interaction of temperature and carbon concentration on growth rate of *R. mucilaginosa* (Figure 5) also has been related in others studies of pigmented yeast (Rossi et al., 2009 and Aksu and Eren, 2005).

Hence, this study established the light intensity, temperature and arabinose concentration for higher growth rate ($0.31h^{-1}$) and carotenoid production (93.92 µg/g) by *R. mucilaginosa*. The potential of this yeast to synthesize carotenoids, in stressful conditions, such as simulated by low growth rate is a promising condition for industrial production.

4. CONCLUSION

The pigmented yeast isolated from UFV campus orchard was identified as *Rhodotorula mucilaginosa.* There is a greater accumulation of carotenoids in the earlier stationary phase than in maximum exponential growth phase. The light intensity, temperature and arabinose concentration for higher growth rate ($0.31h^{-1}$) and carotenoid production (93.92 µg/g) by *R. mucilaginosa*. This yeast was able to grow and synthesize carotenoids even

in stressful conditions, and is considered as a candidate for future industrial applications.

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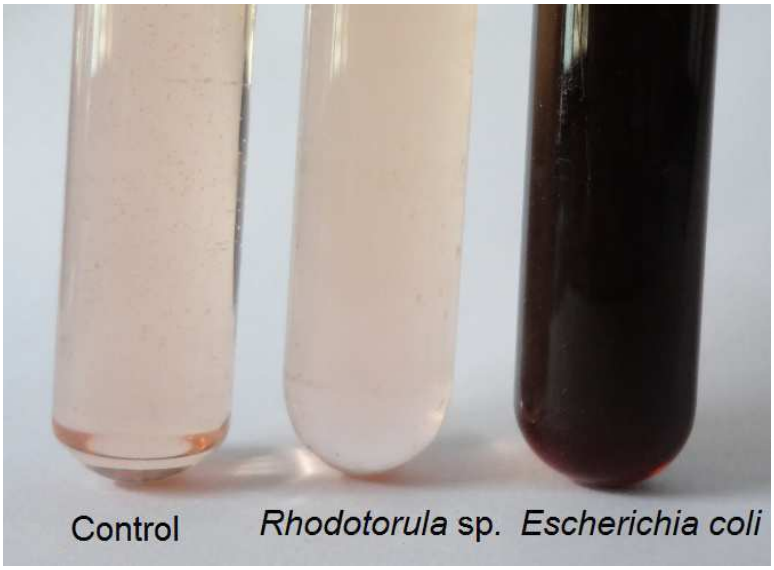
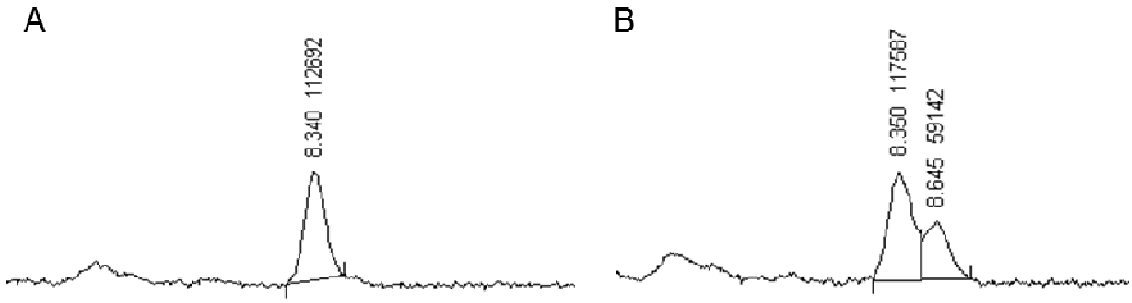
ANEXO

Biochemical test of nitrate reduction



Right tube, positive control (*Escherichia coli*) for nitrate reduction. Left tube, uninoculated negative control. Middle tube, UFV-isolated *Rhodotorula* sp. with no nitrate reduction.

HPLC chromatograms



A: Standard of β-carotene (Sigma Aldrich). B: Carotenoids synthesized by *R. mucilaginosa*, especially β-carotene. HPLC Shimadzu coupled with a UV-visible detector (450nm), reverse phase column C18 Luna (5 µm, 100 Å, 250x 4.6 mm). Isocratic mobile phase, MTBE: methanol: water (65:30:5 v/v/v) at a flow rate of 0.8 mL/min during the running time 20 min.

Experimental central composite design (CCD) runs and corresponding cell mass (mg/mL) and carotenoid yield (µg/g) of *R. mucilaginosa*.

Run order	Malt extract (%w/v)	Glycerol (%v/v)	H_2O_2 (mmoL/L^{-1})	Cell mass* (mg/mL)	Carotenoid yield (µg/g)
1	1.50	1.50	2.00	0.56	1857
2	5.00	1.50	2.00	0.78	1385
3	1.50	5.00	2.00	0.83	619.2
4	5.00	5.00	2.00	0.97	1055
5	1.50	1.50	8.00	0.62	730.5
6	5.00	1.50	8.00	0.75	1206
7	1.50	5.00	8.00	0.85	987.3
8	5.00	5.00	8.00	0.97	652.9
9	0.30	3.25	5.00	0.55	1757
10	6.19	3.25	5.00	0.84	624.9
11	3.25	0.31	5.00	0.61	799.1
12	3.25	6.19	5.00	0.78	838.8
13	3.25	3.25	-0.04	0.94	737.9
14	3.25	3.25	10.0	0.75	979.3
15	3.25	3.25	5.00	0.82	989.4
16	3.25	3.25	5.00	0.82	851.7
17	3.25	3.25	5.00	0.80	1092
18	3.25	3.25	5.00	0.76	883.4
19	3.25	3.25	5.00	0.84	767.0
20	3.25	3.25	5.00	0.81	731.5

*Cell mass accumulated after 12 days at 30 °C and 200 rpm.

Experimental central composite design (CCD) runs and corresponding carotenoid yield (µg/g).

Run order	Light (µmol $m^{-2}s^{-1}$)	Arabinose (%w/v)	Temperature (°C)	Carotenoid (µg/g)		
				Log	S* (2 h)	S* (60 h)
1	20.0	1.50	18.0	16.5	39.3	62.6
2	100	1.50	18.0	64.2	53.8	84.6
3	20.0	5.00	18.0	70.9	59.7	41.4
4	100	5.00	18.0	59.6	54.8	90.2
5	20.0	1.50	28.0	15.4	45.3	47.9
6	100	1.50	28.0	17.2	60.5	53.6
7	20.0	5.00	28.0	20.1	33.3	50.8
8	100	5.00	28.0	36.7	56.6	71.5
9	-7.27	3.25	23.0	44.4	30.0	58.6
10	127	3.25	23.0	45.0	95.3	101
11	60.0	0.31	23.0	17.8	64.6	44.8
12	60.0	6.19	23.0	32.8	40.0	63.6
13	60.0	3.25	14.6	28.5	42.0	81.3
14	60.0	3.25	31.4	36.1	35.0	58.2
15	60.0	3.25	23.0	22.0	46.0	26.9
16	60.0	3.25	23.0	31.4	32.7	28.2
17	60.0	3.25	23.0	27.9	42.4	33.1
18	60.0	3.25	23.0	29.2	42.4	36.1
19	60.0	3.25	23.0	26.0	44.3	26.9
20	60.0	3.25	23.0	19.2	52.8	28.0

*Stationary phase