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ORIGINAL PAPER

From crude glycerol to carotenoids by using a *Rhodotorula glutinis* mutant

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Abstract In this work eighteen red yeasts were screened for carotenoids production on glycerol containing medium. Strain C2.5t1 of *Rhodotorula glutinis,* that showed the highest productivity, was UV mutagenized. Mutant 400A15, that exhibited a 280 % increase in β–carotene production in respect to the parental strain, was selected. A central composite design was applied to 400A15 to optimize carotenoids and biomass productions. Regression analyses of the quadratic polynomial equations obtained ($R^2 = 0.87$ and 0.94, for carotenoids and biomass, respectively) suggest that the models are reliable and significant ($P < 0.0001$) in the prediction of carotenoids and biomass productions on the basis of the concentrations of crude glycerol, yeast extract and peptone. Accordingly, total carotenoids production achieved (14.07 ± 1.45 mg l^{-1}) under optimized growth conditions was not statistically different from the maximal predicted (14.64 ± 1.57 mg l^{-1}) ($P < 0.05$), and it was about 100 % higher than that obtained under un-optimized conditions. Therefore mutant 400A15 may represent a biocatalyst of choice for the bioconversion of crude glycerol into value-added metabolites, and a tool for the valorization of this by-product of the biodiesel industry.

Keywords *Rhodotorula glutinis* · β–carotene · Crude glycerol · Central composite design · Bioconversion

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Introduction

Carotenoids are the most common class of pigments naturally occurring in nature. Besides being responsible for the colour of vegetables, microorganisms and some animals, they are precursor of vitamin A (Johnson and Schroeder 1996), different hormones (Vershinin 1999) and harbour photo-protective (Moliné et al. 2009; Vershinin 1999) and antioxidant properties (Edge et al. 1997; Johnson and Schroeder 1996). Moreover, carotenoids show immunostimulating (Emodi 1978) and putative antitumor activities (Hennekens 1997), and may modulate membrane fluidity also by affecting their permeability to small molecules such as oxygen (Gruszecki and Strzalka 2005). For their important biological properties carotenoids are largely utilized in medicine but also in chemical, pharmaceutical, cosmetic, food and feed industries.

Since carotenoids global market grows yearly of 2.3 %, and in 2018 it is expected to reach 1.4 billion dollars (BCC Research 2011), there is growing interest in developing processes aimed at efficiently producing these pigments. Indeed, carotenoids may be easily chemically synthesized. However, also due to a general concern on the safety of artificial synthetic pigments, the exploitation of carotenoids accumulating microorganisms represents the most interesting approach to their production.

At present, in spite of the number of microbial carotenoids already characterized, just a few are produced at the

industrial scale. Among these, astaxanthin from *Phaffia rhodozyma* and *Haematococcus pluvialis* and β-carotene from *Blakeslea trispora* are already on the market and are utilized as food addictives (Dufossé 2006). However, also other microbial species accumulate carotenoids. In particular, yeasts ascribed to the genera *Rhodosporidium, Rhodotorula, Sporobolomyces* and *Sporidiobolus* are known producers of carotenoids (Frengova and Beshkova 2009).

Biotechnological processes aimed at carotenoids production with these yeasts should be based on the utilization of cheap substrates. Among these crude glycerol seems particularly appealing. Crude glycerol is the main by-product of biodiesel industry which produces about 1 kg of glycerol by transesterification of 10 kg of vegetable oil. Due to the worldwide increase in biodiesel production also the production of crude glycerol is increasing with a consequent decrease of its market price (Nicol et al. 2012). In this context, the objectives of the work were the selection of a yeast strain to be used for carotenoids production on crude glycerol and the optimization of the bioconversion of crude glycerol into value-added metabolites through the application of a central composite design.

Materials and methods

Yeast strains and culture media

The yeast strains utilized are reported in Table 1. Strains C2.5t1 e C71t0 were from Yeast Collection of Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche, while all the other strains were purchased by Centraalbureau voor Schimmelcultures (CBS) and the Industrial Yeast Collection of the University of Perugia (DBVPG). Culture media were: YEPD (yeast extract 1 %, bacto-peptone 2 %, dextrose 2 %); YEPGLY (yeast extract 1 %, bacto-peptone 2 %, pure glycerol 2 %), added with agar 2 %, when required, and YEPCGLY (yeast extract 1 %, bacto-peptone 2 %, crude glycerol as indicated). Crude glycerol, kindly provided by a private company showed the following characteristics: pH 7.10, purity 88–92 % with impurities composed by potassium and sodium salts (4–5 %), non glycerol organic matter 0.5–1 %, water 3–5 %, methanol <0.2. Yeasts were maintained on YEPD at 4 °C for short term conservation and on YEPD added with 20 % glycerol at −80 °C for long term conservation.

Yeast cultivation

Yeast cultures were inoculated in 10 ml of YEPD and incubated at 25 °C, unless otherwise stated, under shacking conditions (150 rpm). After 48 h 5 ml of starter culture were harvested and cells were rinsed in sterile water, pelleted by centrifugation, and re-suspended in 50 ml of culture medium in 250 ml Erlenmeyer flasks with baffles (inoculum 5 % vol vol^{-1}). Flasks were incubated as above indicated unless differently specified. Cell growth was monitored by evaluating OD600 nm (SmartSpecPlus, Biorad) and dry weight of biomass. Briefly: 5 ml of cell culture were centrifuged for 5 min at 3,500*g*, rinsed twice with sterile distilled water to remove any residual glycerol, filtered through 0.45 μ Millipore pre-weight filter and dried at 80° for 48 h.

Residual glycerol and glucose concentrations in the growth medium were evaluated enzymatically (Kit K-GCROL and K-GLUC, Megazyme, Ireland). All trials were carried out at least in triplicate. Specific growth rate (μ, h^{-1}) was evaluated during logarithmic growth phase by using the following equation: $\mu = (\ln x_t - \ln x_o)\, t^{-1}$ where x_t is OD600 nm at time t; x_o OD600 nm at time 0; t is time (h).

Carotenoids extraction

Carotenoids extraction was carried out according to Dominguez-Bocanegra and Torres Muñoz (2004), modified as follows. Cells contained in 1 ml aliquots of yeast cultures were harvested, washed twice in sterile distilled water, re-suspended in 1 ml acetone, centrifuged for 3 min at 3,500*g* and let dry briefly. The cell pellet was re-suspended in 2 ml DMSO pre-heated at 60 °C, added with 0.5 g acid washed glass beads (ø 212–300 μm) (Sigma-Aldrich, Italy) vigorously shaken for 2 min and incubated at 60 °C for 15 min. 2 ml acetone, 2 ml petroleum ether (added with hydroxytoluene butylate, 0.25 %) and 2 ml NaCl 20 % were added, the mixture was vigorously vortexed for 5 min and centrifuged at 3,500*g* for 5 min. The organic phase containing carotenoids was transferred to a fresh tube. 2 ml DMSO were added to the cell pellet after elimination of the salts and all the steps above described were repeated to obtain a second aliquot of petroleum ether containing carotenoids that was added to the first one. All the stages following the addition of DMSO were carried out in the dark under a red light (Taungbodhitham et al. 1998) to minimize cis–trans isomerization (van den Berg et al. 2000). Total carotenoids concentration was evaluated spectrophotometrically according Frengova et al. (1994) and expressed as β-carotene-equivalents in respect to a calibration curve obtained by utilizing pure β-carotene (Sigma-Aldrich, Italy). UV/Visible scanning spectra of petroleum ether extracts of carotenoids were recorded at λ ranging from 400 and 600 nm using a SmartSpec™ plus spectrophotometer (BioRad, Hercules, CA).

Qualitative analysis of the carotenoids in the extracts was carried out by Thin Layer Chromatography (TLC) on

Table 1 Yeast strains utilized

Yeast strain	Species	Yeast strain	Species
DBVPG 6091	*Rhodotorula mucilaginosa*	CBS 6016	*Rhodosporidium toruloides*
DBVPG 6093	*Rhodotorula mucilaginosa*	CBS 315	*Rhodosporidium toruloides*
DBVPG 6094	*Rhodotorula mucilaginosa*	CBS 6566	*Rhodosporidium paludigenum*
DBVPG 7019	*Rhodotorula mucilaginosa*	CBS 483	*Sporidiobolus salmonicolor*
C71t0	*Rhodotorula mucilaginosa*	CBS 7899	*Sporobolomyces coprosmae*
DBVPG 6081	*Rhodotorula glutinis*	CBS 7501	*Sporobolomyces ruberrimus*
CBS 2366	*Rhodotorula glutinis*	CBS 8619	*Sporobolomyces ruberrimus*
CBS 2367	*Rhodotorula glutinis*	CBS 7228	*Sporobolomyces oryzicola*
C2.5t1	*Rhodotorula glutinis*	CBS 7998	*Rhodotorula cresolica*

DBVPG Industrial Yeast Collection DBVPG, Università di Perugia, Italy, *CBS* Centraalbureau voor Schimmelcultures Utrecht, The Netherlands, *C* Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche, Ancona, Italy

silica gel plates eluted with acetone and hexane (3:7, vol vol^{-1}) according to Kim et al. (2004). Pure β-carotene was used as a standard to identify the corresponding band in the sample, while torulene and torularhodin were identified by Retention factor (Rf) (Kim et al. 2004). Each band was scraped in petroleum ether and analysed by HPLC to identify the retention time of the corresponding peaks.

Reverse Phase HPLC analysis was carried out by using a HPLC Jasco LC 2000 Plus equipped with a UV/Vis UV-2075 Plus detector, on a Supelcosil C18 (150 mm × 4.6 mm) column according to Davey et al. (2006) modified as follows: gradient analysis was carried out by mixing eluents A (methanol: ethylacetate 1:1 vol vol^{-1}, 0,05 % triethylamine and 0.1 % BHT) and B (acetonitrile 0.05 % triethylamine and 0,1 % hydroxytoluene butylate) with an initial linear gradient from 0 to 6 min of 0–20 % solvent B in A, followed by a linear gradient from 6 to 24 min of 20–80 % solvent B in A, and a final gradient, from 24 to 28 min, of 80–0 % solvent B in A; detection was carried out at λ 450 nm. β-carotene (Sigma-Aldrich, Italy) was used as external standard.

Data were obtained in triplicate from at least three biological replicates and subject to ANOVA and Multiple Range test LSD ($P < 0.05$).

Experimental design and process optimization

Response surface methodology (RSM), using a central composite factorial design (CCD) with three factors and two replicates of central point, was performed in duplicate. Factors and their respective levels are summarized in Table 2. The statistical software package JMP 8.0 was used for regression and statistical analyses. The mathematical relationship of the independent variables was determined by fitting the data obtained from 32 runs to the following quadratic polynomial equation:

$$Y = a + bx_1 + cx_2 + dx_3 + ex_1^2 + fx_2^2 + gx_3^2 + hx_1x_2 + ix_1x_3 + jx_2x_3$$

where Y is the predicted response, x_1–x_3 represent the independent variables, a and b–j are the intercept term and the coefficients obtained by the regression analyses of the data. The reliability of the model was estimated by R^2 evaluation and analysis of variance (ANOVA). The optimal crude glycerol, yeast extract and peptone concentrations were obtained by solving the quadratic polynomial equation and analyzing the response surface contour plots.

Mutagenesis

Yeast cells were inoculated in 10 ml YEPD and grown at 25 °C with shaking (150 rpm). After 18 h cells were rinsed twice and re-suspended in sterile distilled water to a final concentration of 1×10^6 cell ml^{-1}. Two ml of this suspension were poured in a sterile petri dish, subject to UV irradiation by using a Stratalinker® UV Crosslinker (model 2400—UV lamp 254 nm). Yeast cells were maintained in the dark for 1 h, plated onto YEPD and incubated for 3 days at 25 °C. Viable plate counts were carried out to determine survival rates and irradiation was calibrated at 50,000 micro joules (μJ) cm^{2-1} to obtain about 10 % survival.

Results

Selection of carotenoids producers on YEPGLY

Eighteen yeasts ascribed to the species *Rhodotorula cresolica, Rhodotorula glutinis, Rhodotorula mucilaginosa, Rhodosporidium paludigenum, Rhodosporidium toruloides, Sporobolomyces coprosmae, Sporobolomyces ruberrimus, Sporidiobolus salmonicolor*, and *Sporobolomyces oryzicola* were collected on the basis of their

Table 2 Experimental design, responses based on experimental runs and predicted responses

Run order	x_1 Gly		x_2 YE		x_3 Pep		Biomass[a] ($g\ l^{-1}$)	Predicted Biomass ($g\ l^{-1}$)	Carotenoids ($mg\ l^{-1}$)[a]	Predicted carotenoids ($mg\ l^{-1}$)
	Level	(%)	Level	(%)	Level	(%)				
1	−1	2	−1	0.1	−1	0.2	4.2	4.24	2.69	3.18
2	−1	2	0	0.55	0	1.1	7.8	8.43	6.4	4.87
3	+1	10	−1	0.1	−1	0.2	6.9	7.83	6.85	4.77
4	0	6	−1	0.1	0	1.1	14.5	14.68	10.77	9.75
5	−1	2	+1	1	−1	0.2	7.9	8.40	4.32	3.78
6	+1	10	0	0.55	0	1.1	21.2	20.09	7.27	8.21
7	−1	2	+1	1	+1	2	9.3	9.97	7.45	8.68
8	+1	10	+1	1	−1	0.2	18	17.26	6.94	7.05
9	−1	2	+1	1	−1	0.2	8.8	8.40	4.71	3.78
10	+1	10	+1	1	+1	2	23.5	29.71	14.92	13.78
11	−1	2	−1	0.1	+1	2	10.4	11.39	6.07	6.71
12	−1	2	+1	1	+1	2	11.3	9.97	8.1	8.68
13	+1	10	−1	0.1	−1	0.2	8	7.82	4.9	4.77
14	0	6	+1	1	0	1.1	18.5	18.69	10.81	11.88
15	0	6	0	0.55	−1	0.2	11.2	12.61	5.22	6.53
16	+1	10	−1	0.1	+1	2	23.9	25.85	10.61	10.14
17	0	6	0	0.55	0	1.1	15.6	17.07	8.36	9.60
18	−1	2	−1	0.1	+1	2	11.1	11.39	8.1	6.71
19	+1	10	+1	1	+1	2	35.1	29.71	13.8	13.78
20	−1	2	−1	0.1	−1	0.2	5	4.24	2.9	3.18
21	−1	2	0	0.55	0	1.1	9.1	8.43	3.7	4.87
22	0	6	0	0.55	−1	0.2	13	12.61	5.3	6.53
23	0	6	+1	1	0	1.1	18	18.69	12.5	11.88
24	+1	10	0	0.55	0	1.1	19.4	20.09	7.2	8.21
25	0	6	0	0.55	+1	2	22.6	22.41	13.3	11.67
26	0	6	0	0.55	0	1.1	17.1	17.07	11.1	9.60
27	0	6	0	0.55	+1	2	23.7	22.41	11	11.67
28	+1	10	+1	1	−1	0.2	17.7	17.26	6.8	7.05
29	0	6	0	0.55	0	1.1	17.3	17.07	11	9.60
30	+1	10	−1	0.1	+1	2	27.8	25.86	8.6	10.13
31	0	6	0	0.55	0	1.1	17.4	17.07	11.1	9.60
32	0	6	−1	0.1	0	1.1	16.2	14.68	7.6	9.75

Gly crude glycerol, *YE* yeast extract, *Pep* peptone

[a] Responses of experimental runs carried out in duplicate

propensity to assimilate glycerol and produce pigments as reported at http://www.cbs.knaw.nl/ and by Kurtzman and Fell (1998). These strains were grown on YEPGLY, a culture medium containing pure glycerol, and their kinetics of growth were monitored for up to 120 h by evaluating OD600 nm and dry weight at different sampling times. The 18 strains differed markedly in their growth kinetics and specific growth rates (Fig. 1; Table 3). Dramatic differences were observed also for glycerol consumption (Fig. 1). In particular, *R. glutinis* C2.5t1, *R. mucilaginosa* DBVPG 6094, *R. mucilaginosa* C71t0, *S. oryzicola* CBS 7228 and *R. paludigenum* CBS 6566 fully utilized glycerol within the first 48 h of fermentation. *R. toruloides* CBS 315 and CBS 6016 and *R. glutinis* DBVPG 6081, CBS 2366, DBVPG 6081 and CBS 2367 took up to 72 h to consume glycerol while the remaining strains left up to 80 % residual glycerol in the growth medium.

The differences observed in the kinetics of growth and glycerol consumption resulted in significant differences in biomass and carotenoids production (Table 3). The UV/Visible scanning spectra of petroleum ether extracts of carotenoids, carried out as described by Davoli and Weber (2002), indicated a general homogeneity among strains for what concerns the composition of carotenoids and

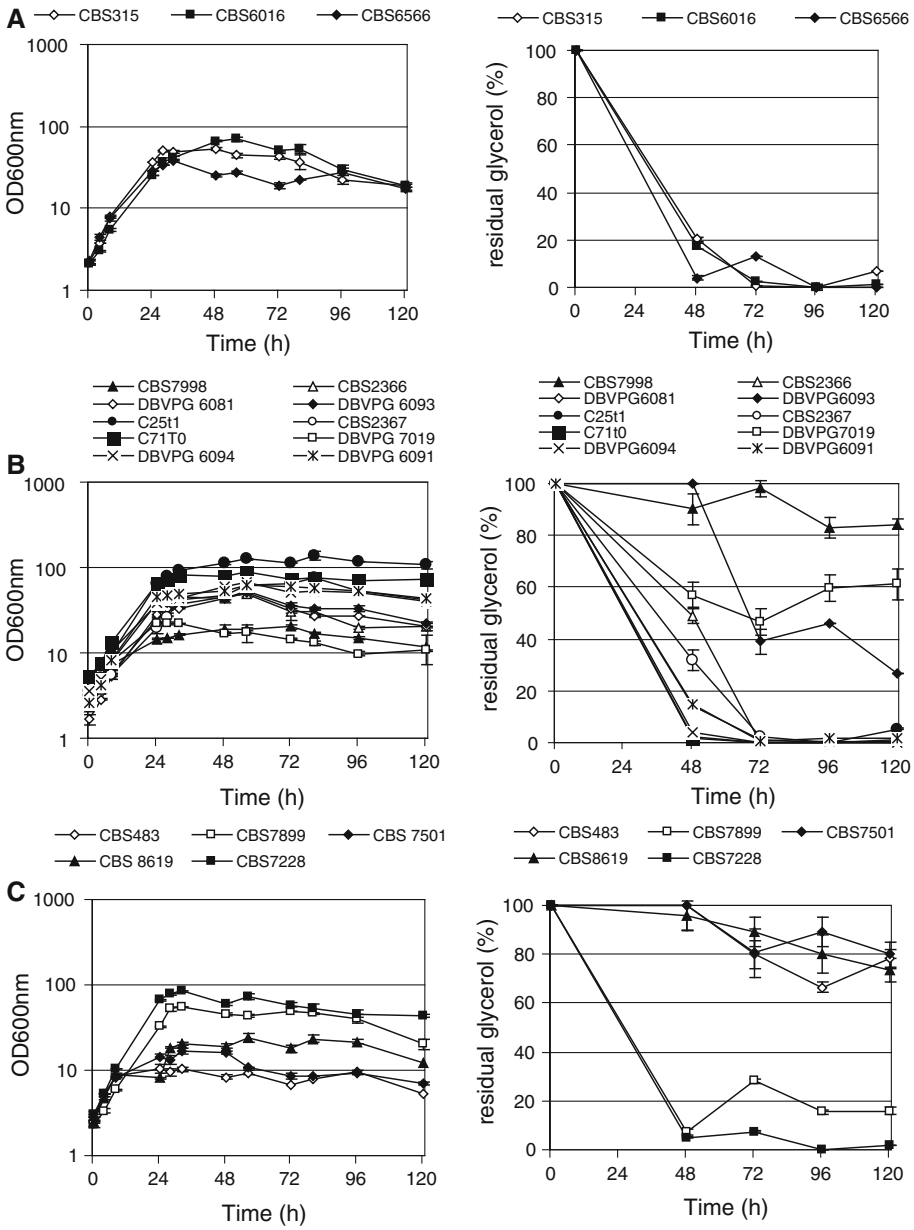

Fig. 1 Growth kinetics and glycerol consumption of (**a**) *Rhodosporidium* spp.; (**b**) *Rhodotorula* spp.; (**c**) *Sporobolomyces* spp. and *Sporidiobolus salmonicolor*. Data are representative of three independent experiments. Where not visible, bars lye within the symbols

highlighted the production of β-carotene, torulene and torularhodin. These results were confirmed by thin layer chromatography. Similar to that found by Kim et al. (2004) β-carotene and torulene migrated at 0.92 and 0.91 Rf, respectively and torularhodin at 0.31 Rf (data not shown).

R. glutinis C2.5t1, *S. oryzicola* CBS 7228 and *R. toruloides* CBS 6016 showed the highest volumetric (mg l^{-1}) and cellular (mg g^{-1}dry weight) carotenoids productivities although with significant differences (Table 3). Carotenoids quantification by means of HPLC confirmed C2.5t1 as the best producer of pure β-carotene (0.45 ± 0.06 mg l^{-1}) while *S. oryzicola* CBS 7228 and *R. toruloides* DBVPG 6016 showed productions of 0.32 ± 0.02 and 0.35 ± 0.07 mg l^{-1}, respectively.

Biomass and carotenoids yields on glycerol and glucose

The three strains showing the best performances on YEPGLY were cultured on YEPD for 120 h. While C2.5t1 showed comparable biomass yields on the two culture media, CBS 7228 and CBS 6016 showed higher biomass yields on YEPGLY. Carotenoids yields were invariably significantly higher in YEPGLY than in YEPD (Table 4). Thus, in accordance with other authors (Chatzifragkou et al. 2011; Taccari et al. 2012) and contrary to that expected considering that glucose is a preferred carbon source for red yeasts (Latha et al. 2005), glycerol proved to be a well-suited substrate for carotenoids production.

Table 3 Productive parameters on YEPGLY at 25 °C

Strains	Growth rate (h^{-1})	Biomass (mg ml^{-1})	Total carotenoids (mg g^{-1}dw)	(mg l^{-1})
DBVPG 6091	0.11 ± 0.000^{bc}	10.80 ± 0.42^{abcd}	0.11 ± 0.02^{fg}	1.11 ± 0.20^{def}
DBVPG 6093	0.10 ± 0.002^{fg}	7.30 ± 0.14^{ef}	0.31 ± 0.07^{bc}	2.25 ± 0.56^{bc}
DBVPG 6094	0.10 ± 0.003^{ef}	11.37 ± 1.04^{ab}	0.26 ± 0.02^{cd}	3.03 ± 0.42^{b}
DBVPG 7019	0.01 ± 0.001^{i}	4.65 ± 0.78^{gh}	0.03 ± 0.00^{h}	0.09 ± 0.02^{fg}
C71t0	0.11 ± 0.000^{cd}	12.02 ± 1.31^{a}	0.16 ± 0.04^{e}	2.16 ± 0.14^{c}
DBVPG 6081	0.11 ± 0.002^{cd}	8.15 ± 0.21^{ef}	0.15 ± 0.02^{ef}	1.21 ± 0.15^{de}
CBS 2366	0.09 ± 0.003^{fg}	9.30 ± 3.25^{bcde}	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}
CBS 2367	0.08 ± 0.007^{hi}	8.04 ± 0.00^{de}	0.23 ± 0.01^{d}	1.95 ± 0.08^{cd}
C2.5t1	0.13 ± 0.000^{a}	10.52 ± 2.08^{abc}	0.43 ± 0.02^{a}	4.58 ± 1.11^{a}
CBS 7998	0.05 ± 0.000^{l}	4.40 ± 0.14^{gh}	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}
CBS 6016	0.10 ± 0.001^{def}	9.57 ± 0.40^{bcde}	0.33 ± 0.03^{b}	3.11 ± 0.18^{b}
CBS 315	0.11 ± 0.000^{cde}	8.85 ± 0.35^{cde}	0.10 ± 0.04^{fg}	0.93 ± 0.40^{defg}
CBS 6566	0.09 ± 0.001^{gh}	5.70 ± 0.14^{fg}	0.08 ± 0.03^{g}	0.45 ± 0.13^{efg}
CBS 483	0.03 ± 0.007^{m}	3.20 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}
CBS 7899	0.11 ± 0.002^{c}	8.60 ± 0.14^{cde}	0.14 ± 0.01^{efg}	1.16 ± 0.05^{de}
CBS 7501	0.04 ± 0.003^{l}	2.35 ± 0.21^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}
CBS 8619	0.02 ± 0.000^{n}	3.60 ± 0.00^{gh}	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}
CBS 7228	0.12 ± 0.001^{ab}	11.75 ± 0.35^{ab}	0.37 ± 0.05^{b}	4.29 ± 0.42^{a}

Total carotenoids are expressed as β-carotene equivalents. Biomass production is expressed as dry weight after 120 h growth; Data are mean ± standard deviation of 3 independent experiments. Different letters within each column indicate significant different values ($P < 0.05$)

Table 4 Biomass and carotenoids yields on YEPGLY and YEPD at 25 °C

Culture medium	Strain	Biomass yield	Carotenoids yield
YEPGLY	C2.5t1	52.60 ± 10.39^{a}	22.89 ± 5.57^{a}
	CBS7228	58.75 ± 1.77^{a}	21.44 ± 2.10^{ab}
	CBS6016	47.83 ± 2.02^{a}	15.53 ± 0.89^{bc}
YEPD	C2.5t1	57.83 ± 7.22^{a}	$14{,}23 \pm 1.78^{c}$
	CBS7228	48.25 ± 2.36^{b}	7.93 ± 1.77^{d}
	CBS6016	34.75 ± 5.55^{b}	10.34 ± 2.81^{cd}

Biomass yield (%): mg dry weight g^{-1} glycerol or glucose consumed). Carotenoids yield (%): (mg β-carotene equivalents g^{-1} glycerol or glucose consumed) × 100. Data are mean ± SD of at least 3 independent experiments. Different letters in each column indicate significantly different values ($P < 0.05$)

Mutagenesis of *R. glutinis* C2.5t1

R. glutinis C2.5t1 was subject to UV mutagenesis to obtain mutants with increased carotenoids production. Of 18 mutants selected on the basis of the color of the colonies, 10 were light pink, 5 albino, 2 orange-yellow and 1 yellow. The petroleum ether carotenoids extracts of the light pink and albino mutants showed low or null carotenoids production. The two orange-yellow (20A5 and 20A3) and the yellow mutants (400A15) increased carotenoids production in respect to the parental strain *R. glutinis* C2.5t1. In particular, the yellow mutant 400A15 produced 5.8 mg l^{-1} total carotenoids and differed markedly from parental in the composition of carotenoids. The UV/visible scanning of the pigments extracted showed the disappearance of the peak at 515 nm relative to torularhodin, and an increase of the peak at 450 nm relative to β-carotene. TLC analysis of the pigments extracted confirmed these results (data not shown).

Characterization of mutant 400A15

At 25 and 30 °C 400A15 and C2.5t1 showed comparable growth kinetics and biomass production. However, they differed markedly for what concerns carotenoids production, as indicated by HPLC analysis. In particular, at 25 °C total carotenoids consisted of 25.14 % β-carotene, 40.78 % torulene and 34.08 % torularhodin in C2.5t1 and of 73.06 % β-carotene and 26.94 % of torulene in 400A15. At 30 °C 400A15 showed a further increase of total carotenoids production. At this temperature 400A15 produced 7.18 ± 0.16 mg l^{-1} total carotenoids and showed a 280 % increase in β–carotene production in respect to the parental strain, possibly due to a mutation in the biosynthetic pathway that affects torulene and torularhodin productions.

Optimization of carotenoids production on crude glycerol

A central CCD with three factors (glycerol, yeast extract and peptone) and two replicates of central points was

carried out at 30 °C. The three factors were utilized at low (−1), middle (0) and high (+1) concentrations, according to the experimental design matrix given in Table 2. The experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of culture media and incubated at 150 rpm for 120 h and the results obtained are reported in Table 2. The quadratic polynomial equations for biomass (Yb) and carotenoids (Yc) production as a function of crude glycerol (x_1), yeast extract (x_2) and peptone (x_3) concentrations were the following:

$$Yb = 17.06 + 5.83x_1 + 2.00x_2 + 4.90x_3 + 1.32x_1x_2 + 2.72x_1x_3 - 1.39x_2x_3 - 2.80x_1^2 - 0.38x_2^2 + 0.45x_3^2;$$

$$Yc = 9.60 + 1.67x_1 + 1.06x_2 + 2.57x_3 + 0.42x_1x_2 + 0.46x_1x_3 + 0.34x_2x_3 - 3.05x_1^2 + 1.22x_2^2 - 0.50x_3^2.$$

Regression analyses ($R^2 = 0.94$ and 0.87, respectively) suggested that the model is reliable and significant ($P < 0.0001$) in the prediction of biomass and carotenoids production on the basis of the concentrations of the three factors. The signs and the values of the coefficients of the factors in the model equations indicate the effects of the factors and their interactions on biomass and carotenoids production. The three linear terms x_1 (glycerol), x_2 (yeast extract) and x_3 (peptone), the interaction terms x_1x_2, x_1x_3 and x_2x_3 and the quadratic term x_1 were significant for biomass production ($P < 0.0001$). The linear terms x_1, x_2 and x_3 and the quadratic terms x_1 were significant for carotenoids production ($P < 0.0001$).

The quadratic polynomial equations were employed to draw the response surface and contour plots reported in Fig. 2. The optimum values for the selected variables were obtained by solving the quadratic polynomial equations reported above. It resulted that the three factors should be maximized to optimize biomass production. To maximize carotenoids biosynthesis crude glycerol should be used at 7.5 % while yeast extract and peptone concentrations should be maximized. Thus, mutant 400A15 was cultured in YEPCGLY made as follows: x_1 (crude glycerol) 7.5 %; x_2 (yeast extract) 1 %; x_3 (peptone) 2 %. Under these conditions the carotenoids production obtained (14.07 ± 1.45 mg l^{-1}) was not statistically different from the maximal predicted (14.64 ± 1.57 mg l^{-1}) ($P < 0.05$).

Discussion

With the aim of selecting a biocatalyst for the valorization of crude glycerol, a number of red yeast strains were screened on the basis of their ability to convert glycerol into added-value carotenoids. With the exception of five strains that did not produce pigments on YEPGLY, the remaining strains showed cellular and volumetric productivities of carotenoids comparable to those reported for other red yeasts, although under different culture conditions (Buzzini 2000; Buzzini et al. 2007; Buzzini et al. 2010; Frengova et al. 2003; Maldonade et al. 2008; Tinoi et al. 2005; Valduga et al. 2009). As already reported by Buzzini et al. (2007) there was a good correlation ($R^2 = 0.90$) between carotenoids cellular (mg g^{-1} dry weight) and volumetric productivities (mg l^{-1}). On the contrary, cell dry weight and cellular carotenoids production seemed not to be related ($R^2 = 0.54$). Considering that acetyl-CoA is a key precursor for the biosynthesis of biomass and carotenoids (Lee and Schmidt-Dannert, 2002), it can be hypothesized that biomass and carotenoids productions compete for the utilization of the acetyl-CoA derived from the aerobic assimilation of glycerol.

Since the color of pigmented yeasts varies depending on the relative amounts of the different carotenoids produced (An et al. 2000; Molldrem et al. 2004; Moran and Jarvik 2010), the screening of carotenoids over-producing mutants was based on their color on YEPGLY plates. This approach led to the isolation of mutant 400A15 that shows significant increases in carotenoids production ($P < 0.05$) and differs markedly from the parental strain in the composition of carotenoids. In particular this mutant greatly increases the production of β-carotene at the expenses of torularhodin. Moreover, the application of a factorial approach showed that this strain may significantly increase carotenoids production under optimized growth conditions. The factors considered, together with crude glycerol, for the implementation of the central composite design, were yeast extract and peptone that are considered effective enhancers of total carotenoids production in *R. glutinis* (Bhosale and Gadre, 2001). The resulting response surface plots indicated that crude glycerol and peptone affect biomass and carotenoids productions more than yeast extract. Moreover it was shown that crude glycerol concentration plays a key role in driving 400A15 metabolism towards biomass or carotenoids production. In particular, glycerol concentrations above 7.5 % decrease carotenoids production while increasing biomass production. Thus, as shown by Saenge et al. (2011) biomass and carotenoids productions are not related and their optimization occurs under different culture conditions.

In conclusion, mutant 400A15 of *R. glutinis*, that privileges β–carotene production, showed, under optimized culture conditions, about 100 % increase in total carotenoids production. Thus, although further increases in carotenoids production may be expected under controlled growth conditions in bioreactor, *R. glutinis* mutant 400A15 shows a potential in the bioconversion of crude glycerol and may represent a biocatalyst of choice for the valorization of this by-product of the biodiesel industry.

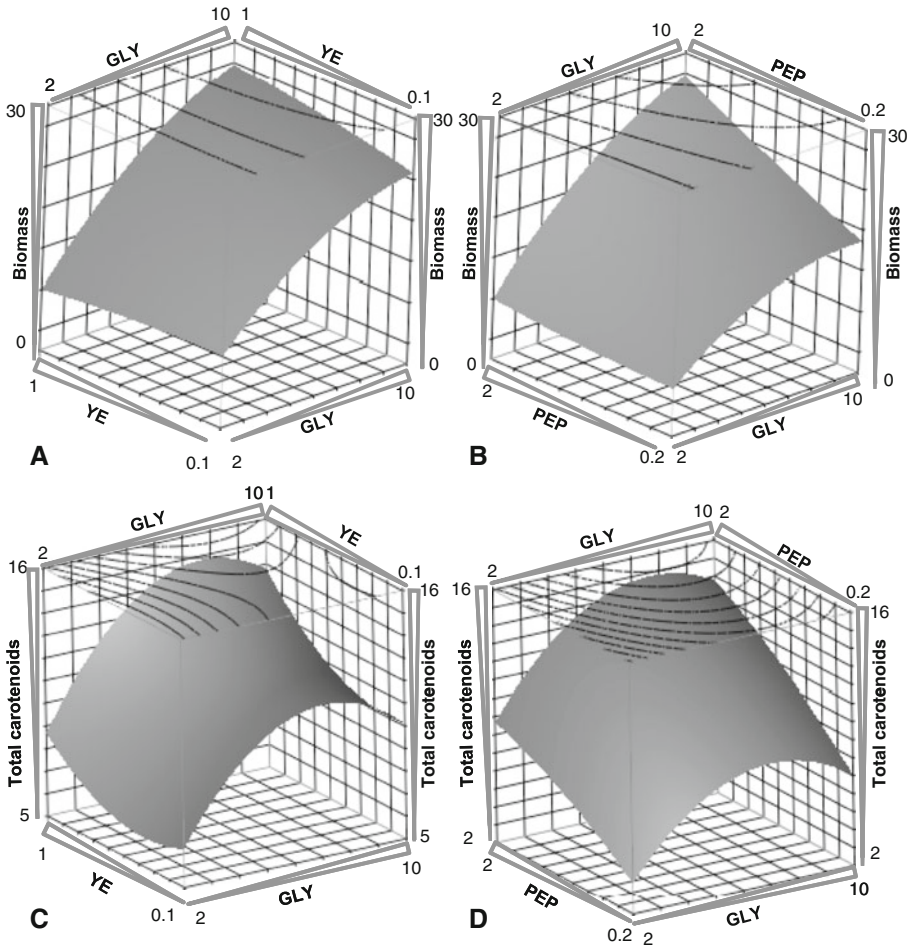

Fig. 2 Response surface and contour plots for the effect of glycerol (GLY, x_1), yeast extract (YE, x_2) and peptone (PEP, x_3) on the production of biomass (**a**, **b**) and total carotenoids (**c**, **d**). The concentration of each factor is expressed as % weight vol^{-1}

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