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ORIGINAL ARTICLE

Production of biomass, carotenoid and other lipid metabolites by several red yeast strains cultivated on waste glycerol from biofuel production – a comparative screening study

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Abstract This study was focused on a comparison of growth and production properties of seven red yeast strains of the genus *Rhodotorula, Sporobolomyces and Cystofilobasidium* cultivated on glycerol substrate. Production of enriched yeast biomas and specific yeast metabolites (carotenoids, ergosterol, lipids) was evaluated on medium with glucose, pure technical glycerol and/or waste glycerol from biofuel production (40 g/L) and mixture of glycerol and glucose (1:3, 1:1, 3:1; C/N ratio 57 in all cultivations). All tested strains were able to utilize glycerol as the only carbon source. Production of biomass on waste glycerol was in most strains higher than in control as well as in medium with pure technical glycerol and reached 15.97–21.76 g/L. Production of carotenoids and ergosterol was better in glucose medium than in medium with glycerol only. Nevertheless, using glycerol medium with addition of glucose, higher yields of total carotenoids, beta-carotene and ergosterol were obtained than in control. The highest yields of total pigments were reached by *Sporobolomyces roseus* (3.60 mg/g cell dry weight (CDW); glycerol:glucose 1:3), *Sporobolomyces salmonicolor* (2.85 mg/g CDW; glycerol:glucose 1:3) and *Rhodotorula glutinis* (2.80 mg/g CDW; glycerol:glucose 3:1) In glucose medium, most tested strains except *Cystofilobasidium capitatum* (22.6 %) produced neutral lipids in the range of 11–15 %. Production of triacylglycerols in all strains was in 10–30 % better in glycerol medium, in which *Rhodotorula aurantiaca* and *Sporobolomyces shibatanus* also reached intracellular triacylglycerol concentrations up to 20 % of biomass. This study has shown that oleaginous red yeasts could have great potential for converting crude glycerol to valuable lipids and carotenoids in respect of efficient bioresources utilization.

Keywords *Rhodotorula* sp. · *Sporobolomyces* sp. · *Cystofilobasidium* sp. · Carotenoids · Ergosterol · Lipids · Waste glycerol

Introduction

The application of bio-diesel on a large commercial scale is strongly recommended, which could very likely result in the generation of tremendous quantities of glycerol deposit in the market in the near future. Concentrated glycerol containing wastewaters (crude glycerol) are also produced in significant quantities (Jitrwung and Yargeau 2011). Therefore, conversion of crude glycerol to various value-added products by the means of chemical and/or microbial technology attracts enormous interest (Chi et al. 2007; André et al. 2009).

In recent years, some studies on the cultivation of some microbial strains on crude glycerol to obtain products with higher additional value have been published. Many of them

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were focused on production of lipids, fatty acids and lipidic substances (Papanikolaou et al. 2002; Razavi et al. 2007; Rymowicz et al. 2008; Papanikolaou et al. 2008; Mantzouridou et al. 2008; André et al. 2009; Bellou et al. 2012). The important technique of biotechnological valorization of glycerol is related to its biotransformation into 1,3-propanediol (Papanikolaou et al. 2008). Organic acids have also been produced by strains using glycerol (Rymowicz et al. 2008). Nevertheless, citric acid, in spite of being one of the most important metabolites produced via biotechnological methods on an industrial scale, has only rarely been produced using glycerol as starting material (Papanikolaou et al. 2002, 2008).

In spite of the significant number of studies dealing with the production of microbial lipids (also called single-cell oils, SCO) by various microorganisms grown on a plethora of carbon sources and culture configurations, data dealing with growth of oleaginous microorganisms on raw glycerol are quite limited (Saenge et al. 2011; Papanikolaou et al. 2008). SCO present a potential industrial interest mainly due to the capacity of various microorganisms to store lipids with unusual compositions or structures (André et al. 2009; Matsui et al. 2012). For example, the oleaginous yeast *Rhodotorula graminis* has been studied as a bioproducer of lipidic compounds using some wastes including crude glycerol (Galafassi et al. 2012).

Use of raw glycerol led to satisfactory growth of *Yarrowia lipolytica* (André et al. 2009). In nitrogen-limited medium with initial glycerol concentration of 30 g/L, accumulation of lipids at 2 g/L (0.31 g/g d.w.) was observed. Similar results have been described by other authors, who also showed that crude glycerol is suitable as a carbon source for citric acid production by mutants of *Y. lipolytica* (Rymowicz et al. 2008; Papanikolaou et al. 2008). In general, through utilization of raw glycerol discharged from bio-diesel units for the production of lipid, the by-product could be recycled, significantly decreasing the production cost of the whole process.

Glycerol has also been used in some studies focused on carotenoid production (Martelli et al. 1992; Razavi et al. 2007; Mantzouridou et al. 2008). Carotenoids are naturally occurring, lipid-soluble pigments, the majority being C_{40} terpenoids. The most important biotechnologically significant yeast carotenoid pigments include astaxanthin and ß-carotene, lycopen, torulen and torularhodin. They act as membrane-protective antioxidants that efficiently scavenge $^1O_2$ and peroxyl radicals; their antioxidative efficiency is apparently related to their structure (Sandmann 2001). Several types of carotenogenic yeast (mainly of the genus *Rhodotorula*) can be used for converting glycerol into many different types of valuable metabolites (Aksu and Tugba Eren 2005; Saenge et al. 2011; Galafassi et al. 2012; Kitcha and Cheirsilp 2011). Carotenogenic yeasts can assimilate various carbon sources, such as glucose, xylose, cellobiose, sucrose, glycerol, sorbitol, etc., and thus various waste materials can be used as cheap substrates for their cultivation. The red yeasts are able to grow under a wide range of initial pH conditions from 2.5 to 9.5, and over a wide range of temperatures from 5 to 26 °C (Latha et al. 2005; Libkind et al. 2008; Frengova and Beshkova 2009).

Glycerol was applied as the sole carbon and energy source for growing *Rhodotorula lactosa* (Martelli et al. 1992). In this study, the maximum biomass yield (0.53 g/g substrate) was obtained after 20 h with 21.5 g glycerol/L; growth was inhibited with 28.0 g glycerol/L and cell morphology was changed. When cells were grown for 20 h, high yields of biomass and beta-carotene (2.66 mg/g dry cells or 28.0 mg/L) were obtained. Saenge et al. (2011) studied possible use of crude glycerol as the sole carbon source for concomitant production of lipids and carotenoids by oleaginous red yeast *Rhodotorula glutinis* TISTR 5159. Among the factors investigated using response surface methodology, the C/N ratio contributed a significant effect on biomass, lipid content and production of carotenoids. Carotenoid production was also followed in studies dealing with yeast strain *Sporobolomyces ruberrinus* H110 (Razavi and Marc 2006; Razavi et al. 2007). The maximum concentration of total carotenoid was 3.84 mg/g including torularhodin (3.70 mg/g) and beta-carotene (0.14 mg/g) using 19 °C at pH 6, but the highest amount of the maximum specific growth rate was obtained ($p = 0.094\ h^{-1}$) at 27 °C (Razavi et al. 2007).

In the southern region of Thailand, 889 yeast strains were isolated from soils and wastes of palm oil mill and biodiesel plant using glucose or glycerol as carbon source. Among them, 23 strains were identified as potential lipid producers or oleaginous yeasts (Kitcha and Cheirsilp 2011). Production of lipidic compounds from waste substrates was also studied using mixed culture of yeast and algae (Cheirsilp et al. 2011, 2012). Some authors have proposed using yeast lipids as the second generation of diesel (Cheirsilp et al. 2011; Galafassi et al. 2012). The increasing cost of vegetable oils is turning the use of microbial lipids into a competitive alternative for the production of biodiesel fuel.

At this time, natural carotenoids are industrially produced using mainly plant material. Production of these pigments by fermentation can become economically feasible if the cost of production can be minimized by use of cheap industrial by-products such as nutrient sources (Tinoi et al. 2005). A number of researchers have investigated carotenoid production from various grains, lipids and related substances, including cellobiose, sugar cane molasses, grape must, and cheese whey by different strains in shake flask fermentation (Aksu and Tugba Eren 2005; Marova et al. 2011, 2012). The work presented here is focused on screening of growth and metabolic activity of several red yeast strains of the genus *Rhodotorula*, *Sporobolomyces* and *Cystofilobasidium* cultivated on pure and waste glycerol in comparison with a

control glucose medium. The main aim of the current investigation was to assess the potentialities of valorization of bio-diesel-derived waste glycerol. Biotechnological production of carotenoids and ergosterol as well as enriched red yeast biomass on waste glycerol in individual analyzed strains was compared.

Materials and methods

Microorganisms and media composition

Following carotenogenic yeasts were used in this study: *Rhodotorula glutinis* – CCY 20-2-26 (abbr. RG), *Rhodotorula rubra* – CCY 20-7-28 (RR), *Rhodotorula aurantiaca* – CCY 20-9-7 (RA), *Cystofilobasidium capitatum* – CCY 10-1-1 (CC), *Sporobolomyces roseus* – CCY 19-6-4 (SR), *Sporobolomyces shibatanus* – CCY 19-20-3 (SSh) and *Sporidiobolus salmonicolor* – CCY 19-4-6 (SSa). All of them were obtained from the Culture Collection of Yeasts (CCY; Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic) and preserved for further use on slant agar, stored at 4 °C. Every 2 weeks, the cultures were re-inoculated and transferred on the new series of 2 % malt extract agar slants. Red yeasts were cultivated on basic glucose medium (as control) and in medium with added technical (pure) or waste glycerol in different ratio in comparison with glucose. Basic control medium was consisted of: glucose (40 g/L), $(NH_4)_2SO_4$ (5 g/L), KH_2PO_4 (5 g/L), $MgSO_4$ (0.34 g/L), yeast autolysate (7 g/L) and tap water. Two-step inoculation was used for red yeast cultivation. In the first step, basic glucose medium was used in all experiments with glucose and glycerol media. The second inoculum contained as a carbon substrate either glucose (control cultivations) or glucose/glycerol (technical or waste according to series of cultivations) mixture (3:1) for cell adaptation to glycerol.

Technical glycerol that was used in the experiment was obtained from Lachema, Neratovice, Czech Republic. Waste glycerol was obtained from the oil mill company VictoriaOil (a member of Victoria group), Šid, Serbia. The sample, defined as a side product of biodiesel production, was previously analyzed on GC/FID as a part of regular control of biodiesel quality. It was found that the composition of waste glycerol mainly consisted of glycerol (83.72 %), water (6.77 %), ash/NaCl (6.5 %) and MONG (1.58 %). MONG symbolize an abbreviation of a variety of other different organic matter which are defined as non-glycerol and found in a crude glycerol. The pH value of waste glycerol was 6.77.

Five sets of experiments were carried out, each for a different yeast strain, in order to gain representative results. In the cultivation medium, as a carbon source, there were used pure technical/waste glycerol (40 g/L), technical/waste glycerol: glucose in ratio 1:3 (10 g/L glycerol and 30 g/L glucose, respectively), 1:1 (20 g/L and 20 g/L of glycerol and glucose, respectively), 3:1 (30 g/L glycerol and 10 g/L glucose, respectively) and a pure glucose as a control medium (40 g/L). The control was prepared individually for both sets of experiments, using technical and waste glycerol. Average values of the parameters of 4 parallel control cultivations are presented in Tables 2, 3, 4 and 5 (see "Results and discussion").

Cultivation of yeasts

Each red yeast strain was cultivated at optimal growth conditions: aerobically at 28 °C, with permanent lighting and shaking (n = 100 rpm). Yeasts were grown in two-step inoculation: all strains were firstly inoculated into INO I, cultivated for 24 h and transferred into INO II. Cultivation in INO II was carried out for 24 h and cells were then poured into minimum production media which is composed as inoculum medium only without yeast autolysate. Both waste and technical glycerol were used in inoculum and production medium in predefined ratio in comparison with common carbon source, glucose (see above). Cultivation in production media was done for 96 h (Marova et al. 2010). The volumetric ratio between INO I and INO II, as well as ratio INO II and production medium, were 1:5 and it was adjusted in all experiments.

During the cultivation, the same volume of available oxygen in the flasks was adjusted in comparison with the present culture broth and was 3.33 (calculated as a quotient between the volume of the Erlenmeyer flasks and the overall volume of culture broth). The growth of yeast culture was determined turbidimetrically using spectrophotometric detection at 630 nm. The amount of the produced biomass was determined using the experimentally obtained relationship between turbidity (A_{630}) and the dry mass of yeast cells in grams per litre (Marova et al. 2010). For measuring turbidity, as a blank, production medium was used.

Cultivation of red yeasts in laboratory fermentor

Selected carotenogenic yeasts (*Rhodotorula glutinis*, *Cystofilobasidium capitatum* and *Sporobolomyces roseus*) were grown in a laboratory fermentor with maximally working volume of 2 L (BioFlo/CelliGen 115 fermentor 2l; New Brunswick, Canada). The yeasts were cultivated in media with glucose as the only carbon source (control media), and with 100 % technical glycerol instead of glucose (glycerol media). The cultivations of inoculum media were made in Erlenmeyer flasks under the same cultivation conditions as described before, except for the addition of glycerol to inoculation media INO II. The whole volume of INO I media (50 mL) was poured into 240 ml of INO II media. After 24 h, the whole INO II was sterile transferred into

1,200 mL of production media. Before starting the new batch, the Biocommand software conditions of cultivation were adjusted. The same conditions were maintained for all batches. The only change was the number of agitations during the time, in order to get the appropriate concentration of dissolved oxygen which is important for yeast cells during their growth. Each batch was monitored by Biocommand Software observing several parameters, such as agitation, temperature, pH value of culture broth and dissolved oxygen. One batch was performed for 75 h, at a temperature of 28 °C and air flow of 5 L min^{-1} with stirring from 150 to 450 rpm.

Several times during the cultivation, samples were taken (about 1–2 mL) for measuring biomass and constructing a growth curve. After approximately 30 h, 50 hand 75 h from the start of the batch, 50 mL of culture was taken for measuring biomass, the metabolites produced and the rest of the carbon source, either glucose or glycerol.

Extraction and analysis of microbial metabolites

After cultivation, culture broth (150 mL) was twice centrifuged (5,000 rpm; 10 min) and biomass samples were collected. Disintegration of yeast cells was carried out by using a mechanical–chemical disruption method with pestle and mortar and simultaneous addition of 50 mL acetone and sea sand. After saponification in the temperature controling water bath (90 °C) by ethanolic solution of potassium hydroxide, carotenoids and other metabolites present were extracted with diethyl ether. The diethyl ether extracts were then collected, pre-concentrated, and dried under vacuum using a rotary evaporator. Later on, the residue was cautiously dissolved in 1 mL of absolute ethanol (UV/VIS grade) and further used for HPLC chromatographic analysis.

Carotenoid pigments, as well as free ergosterol, extracted from yeast cells were individually identified and quantified by RP-HPLC/PDA. Samples (10 μL) were first filtered through PTFE filters (0.45 μm), then centrifuged and injected directly onto a Kinetex C18 column, 2.6 μm, 150 × 4.6 mm with guard column 30 × 4.6 mm, equilibrated with methanol as a mobile phase. Isocratic elution was carried out at 45 °C at a flow rate of 1.0 mL/min. Individual carotenoids as well as ergosterol were quantified using external standards of lycopene (Ψ,Ψ-carotene, concentration range 10–100 μg/mL), β-carotene (β,β-carotene, concentration range 10–100 μg/mL), ergosterol (concentration range 0.1–1 mg/mL), all from Sigma-Aldrich (St.Louis, MO, USA). Carotenes were detected at 450 nm and ergosterol at 285 nm. Individual carotenoids were verified by on-line RP-HPLC/PDA/ESI-MS (detector Finnigan Surveyor PDA, MS detector Finnigan LCQ Advantage Max; Thermo Finnigan, USA) using column type, mobile phase and chromatography conditions as described above. Analysis was performed at the flow rate of 0.5 mL/min and mass spectra were analyzed by ESI ionization in negative mode. Individual and total carotenoids as well as ergosterol were quantified using external standards, constructing appropriate calibration curves.

Glycerol and triacylglycerols were analyzed by a commercial enzyme determination kit (Sigma-Aldrich). Triacylglycerols were first hydrolyzed by lipase to glycerol. Free glycerol was measured using coupled enzyme reactions catalyzed by glycerol kinase, glycerol phosphate oxidase and peroxidase. Lastly, the enzyme catalyzes the coupling of formed H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) *m*-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to the free glycerol concentration of the sample.

Statistical analysis

The results of experiments carried out in the Erlenmeyer flasks on glucose medium (control) were expressed as means ± SD of four measurements. Other results were expressed as an average value of two parallel cultivations. Results were analyzed by Student's *t* test using Statistica for Windows v.5.0 (Statsoft, Tulsa, OH, USA).

Results and discussion

The study presented here was focused on a comparison of the growth and production properties of selected red yeast strains (including non-traditional ones) when cultivated on glycerol substrate. The aim was to find strains suitable for industrial use of a cheap simple medium with waste glycerol as a carbon source for the production of enriched yeast biomass (e.g., for the feed industry) and specific yeast metabolites—mainly carotenoids.

For screening, the red yeasts were grown in 500-mL Erlenmeyer flasks for 96 h with permanent lighting and shaking. The growth curve of *Rhodotorula glutinis* CCY 20-2-26 as well as the other studied red yeasts exhibited similar typical two-phase characteristics with prolonged stationary phase (Marova et al. 2010), probably due to the ability of the yeast cells to utilize lipid stores formed during growth as an additional energy source, when the limited substrates were depleted or had vanished from the culture broth.

The production of carotenoids during growth fluctuated and some local maxima and minima were observed. The maximum of β-carotene production was obtained in all strains in stationary phase after about 80 h of cultivation (Marova et al. 2011). Cultivation in production media in the presence of some stress factors or using waste substrates is recommended to be carried out for the first production maximum (about 80–90 h) to eliminate potential growth

inhibiton caused by nutrient starvation or the toxic effects of stress (Marova et al. 2012).

In order to compare the production of β-carotene, ergosterol and total carotenoids during 3 and 4 days of cultivation, *Rhodothorula glutinis* was used as an example for preliminary cultivation on technical glycerol, both pure and as a co-substrate with glucose in different mass ratios. Table 1 represents the validation that larger production of total carotenoids, as well as β-carotene, was gained when *Rhodotorula glutinis* was cultivated in flasks for 96 h, instead of 72 h. This produced 4-fold more of total carotenoids and β-carotene when cultivated for 4 days instead of 3 days on pure technical glycerol. The similar increase was also observed in the case of ergosterol.

Based on those results, it was obvious that cultivation of red yeasts was adjusted and held on 4 days with constant lighting and shaking the flasks (see "Materials and methods").

Biomass production

After 96 h of cultivation, the culture was used for determing the biomass, measuring turbidity by spectrophotometry and making the calculation to g/L. Biomass yields on glucose (control) were relatively similar in most of strains (Fig. 1), with the highest production observed in *Cystofilbasidium capitatum* (21.52 g/L). Substantially lower production of biomass (about one-half of the yield of other strains) was repeatedly found in *Sporobolomyces* strains, mainly in *Sporobolomyces roseus* (Table 2).

All tested red yeast strains were able to utilize glycerol as the only carbon source. The biomass production, when cultivated on pure technical glycerol, was less than or equal to control. *Rhodotorula aurantiaca* and *Cystofilobasidium capitatum* gave the biggest biomass amounts in all experiments with pure technical glycerol, more than 20 g/L. The order of the biomass production is the same for all mass ratios when cultivated on technical glycerol. Average yield of biomass cultivated on technical glycerol in all combined ratios did not pass above 20 g/L (Fig. 1). This yield is relatively high and could be considered as evidence that glucose and glycerol are utilized by the tested red yeasts to similar degrees when cultivated for 4 days in shaking flasks.

Biomass obtained during cultivation on waste glycerol was in general greater than on pure technical glycerol at all ratios of glycerol and glucose (Table 2; Fig. 1) and in most strains. Once again, like cultivation on technical glycerol, the *Sporobolomyces* strain, namely *Sporobolomyces roseus*, was a bad producer of biomass when compared to the other carotenogenic strains. In contrast, *Rhodotorula* sp. and *Cystofilobasidium* sp. once again gave the biggest amount of dry biomass. The biggest yield of biomass (21.76 g/L) was obtained in *Cystofilobasidium capitatum* when cultivated on waste glycerol as the only carbon source. This strain generally gave the largest quantity of biomass in total in all ratios of waste glycerol and glucose. In Table 2, data of all obtained biomasses, cultivated on technical and waste glycerol, are shown. From these data, it can be concluded that at most combinations of glucose and waste glycerol in the growth medium, biomass yield is greater in comparison with medium where glucose and technical glycerol were used as carbon source. This effect could be associated either with the additional substance present in waste glycerol or with some stress role of intracellular glycerol in red yeast metabolism (Marova et al. 2004).

In a study by Rywinska et al. (2010) a wild-type strain A-101 of *Y. lipolytica* and its three acetate-negative mutants were compared for the production of citric acid from glucose and glycerol (pure and crude) in batch cultures. The substrates were used either as single carbon sources or as mixtures of glucose and pure or crude glycerol. These mixtures were found to be better carbon sources for the biosynthesis of citric acid than glucose. It is worth pointing out that the yeast first consumed glycerol, andthe after its depletion, glucose was utilized as a carbon source. A similar experiment was conducted by Papanikolaou et al. (2002): in a medium containing 18 g/L of glycerol and 9 g/L of glucose, the strain of *Y. lipolytica* utilized glycerol and glucose simultaneously, but the glucose consumption rate was significantly lower than that of glycerol. In a study by Rywinska et al. (2010), a typical phenomenon of a two-phase growth was observed. After depletion of the glycerol, they noted an increase in biomass concentration when the

Table 1 Production of carotenoids and ergosterol cultivated for 72 and 96 h (*Rhodotorula glutinis*) on pure (technical) glycerol; each cultivation was performed in triplicate

	100 % Glycerol		Glycerol:Glucose 1:1		Glycerol:Glucose 1:3	
	72 h	96 h	72 h	96 h	72 h	96 h
Ergosterol (μg/g biomass)	2,159.7 ± 358.2	3,098.3 ± 485.6	1,681.1 ± 247.5	770.4 ± 147.8	1,264.9 ± 287.8	2,960.7 ± 301.8
B-carotene (μg/g biomass)	105.4 ± 25.6	430.5 ± 82.2	123.9 ± 11.9	783.5 ± 120.2	94.9 ± 10.8	1,197.4 ± 99.2
Total carotenoids (μg/g biomass)	228.1 ± 40.8	868.4 ± 102.4	221.8 ± 30.8	1,103.7 ± 98.7	133.1 ± 12.2	1,944.3 ± 228.2

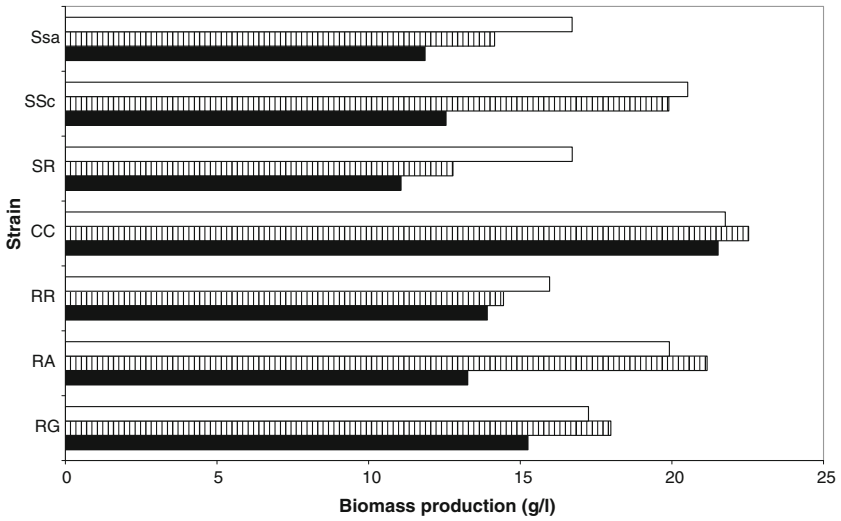

Fig. 1 Production of biomass by red yeasts cultivaded in glucose and glycerol production media for 96 h. Cultivation on waste glycerol □, technical glycerol ▤ and glucose ■. Strains: *RG Rhodotorula glutinis*, *RA Rhodotorula aurantiaca*, *RR* (*RM*) *Rhodotorula rubra* (*Rhodotorula mucilaginosa*), *CC Cystofilobasidium capitatum*, *SR Sporobolomyces roseus*, *SSh Sporobolomyces shibatanus*, *SSa Sporobolomyces salmonicolor*

cells began to consume glucose. A constant concentration of biomass was reached after 50 or 70 h of cultivation, respectively.

In this study, 4 % glucose and glycerol medium, respectively, was used for cultivation in flasks as well as in a laboratory fermentor. According to our results, glycerol was the preferred substrate for *Rhodotorula glutinis* and *Cystofilobasidium capitatum*, while *Sporobolomyces roseus* utilized glucose better than glycerol (see below). Thus, utilization of glycerol by red yeasts is similar to other studies in being strain-specific (Mantzouridou et al. 2008). In some studies, higher crude glycerol concentration was used, mainly when cultivations were performed in a fermentor, e.g., 10 % (Kitcha and Cheirsilp 2011) and 8.5 % (Saenge et al. 2011). In other studies, lower glycerol concetrations (3–5 %) were used (Chatzifragkou et al. 2011; Galafassi et al. 2012) and biomass production was similar or lower than in this work.

As a nitrogen source, 0.5 % ammonium sulfate was used in the presented work. Composition of simple cheap mineral medium was optimized in a previous study (Marova et al. 2004), in which amonium sulfate in the range of 0.2–0.8 % was used for *Rhodotorula glutinis* and *Sporobolomyces roseus* cultivation. We observed that production of biomass and pigments increased with increasing amonium sulfate content up to 0.5 %; higher concentrations in the medium exhibited no further influence on production properties of red yeasts. We also used the same simple medium for cultivation of five additional red yeast strains. Other authors have used 20 g/L of ammonium sulfate for production of carotenoids and lipids on glycerol by *Sporobolomyces ruberrinus* H110 (Razavi and Marc 2006; Razavi et al. 2007). They obtained similar yields of lipids (about 13 % d.w.) and about 2–3× higher carotenoid production as in the *Sporobolomyces roseus* strain used in this study (Table 2; Fig. 4).

Table 2 Production of biomass using pure technical glycerol (*A*), waste glycerol (*B*) and mixture of glycerol and glucose

Dry biomass (g/g)	100 % Glycerol		Glycerol:Glucose 3:1		Glycerol:Glucose 1:1		Glycerol:Glucose 1:3		Glucose (Control)
	A	B	A	B	A	B	A	B	
RG	17.99	17.25	16.52	16.40	13.43	15.33	9.72	12.38	15.25 ± 1.98
RA	21.16	19.92	17.70	16.88	12.15	13.08	8.49	11.30	13.27 ± 1.77
RR (RM)	14.45	15.97	13.52	15.28	12.98	13.87	7.80	10.37	13.91 ± 2.34
CC	22.53	21.76	19.39	20.77	19.90	18.94	7.49	10.81	21.52 ± 3.04
SR	12.78	16.71	10.74	9.98	8.76	8.90	9.51	10.71	11.07 ± 1.88
SSh	19.90	20.52	17.14	17.50	14.15	15.16	12.11	12.92	12.55 ± 1.90
SSa	14.15	16.71	12.21	13.82	10.67	11.80	12.87	11.72	11.86 ± 1.89

Results of biomass yield obtained during cultivation on glucose were expressed as average values from four parallel cultivations and statistically evaluated. Other data are expressed as average values of two parallel cultivations

RG Rhodotorula glutinis, *RA Rhodotorula aurantiaca*, *RR* (*RM*) *Rhodotorula rubra* (*Rhodotorula mucilaginosa*), *CC Cystofilobasidium capitatum*, *SR Sporobolomyces roseus*, *SSh Sporobolomyces shibatanus*, *SSa Sporobolomyces salmonicolor*

The molar ratio of C/N in mineral glucose medium was 57, in technical and waste glycerol about 56. Lower concentration of glycerol in the crude preparation used (83.2 %) did not influence final C/N molar ratio of waste glycerol because of the presence of additional nitrogen (0.04 g/kg; analyzed by the Kjeldahl method). In fact, all cultivations (on glucose and both glycerol types) were performed in media with very similar C/N ratios. The reason why production of biomass was the highest in waste glycerol medium (Table 2) could be associated with the presence of some additional organic activators in the waste glycerol, or, more probably, with the content of NaCl in the ash portion of the crude glycerol (about 6.5 %; see "Materials and methods"). Apositively influence of mild salt stress on biomass and metabolite production in red yeasts has been previously described (Marova et al. 2004, 2010). In contrast, in *B.trispora*, the negative effect of salt present in the waste glycerol preparation has been described (Mantzouridou et al. 2008).

In general, production and accumulation of lipids and lipid-soluble metabolites (carotenoids, sterols) as secondary metabolites is considered as better in nitrogen-limited conditions. Saenge et al. (2011) used a similar type of crude glycerol (a by-product of biodiesel plants) as the sole carbon source for concomitant production of lipids and carotenoids by oleaginous red yeast *Rhodotorula glutinis* TISTR 5159. Here, the addition of ammonium sulfate as a nitrogen source and Tween 20 as a surfactant increased the accumulation of lipids and carotenoids. Among the factors investigated using response surface methodology, the C/N ratio had a significant effect on biomass, lipid content and production of carotenoids. The optimum condition for biomass was a glycerol concentration of 8.5 % and C/N ratio of 60, which is quite similar to our study. Nevertheless, studies have been performed in which similar effects of nitrogen depletion on lipid/secondary metabolite production were not confirmed, e.g., in *Cunninghamella bainieri* (Taha et al. 2010).

Carotenoids production

A number of red yeast species, *Rhodotorula, Rhodosporidium, Sporidiobolus, Cystofilobasidium* and *Phaffia*, are known as producers of carotene pigments. Among yeasts, *Rhodotorula* species belong to the main carotenoid-forming microorganisms with predominant synthesis of β-carotene, torulene and torularhodin (Sandmann 2001). Factors such as the nature and concentration of carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light, and stress have a major influence on cell growth and yield of carotenoids (Marova et al. 2011).

Overall yield of carotenoids is directly related to the total biomass yield (Marova et al. 2011). In the presented study, pigment production was accompanied by biomass production in all strains except in *Sporobolomyces roseus*, where an inverse relationship between production of biomass and carotenoids was found. Previous adaptation of yeast cells to glycerol content in medium was necessary (INO II; see "Materials and methods"). Main pigments that were found and identified were torularhodin, torulene, lycopene and beta-carotene and were defined and evaluated as total carotenoids (see "Materials and methods"; Marova et al. 2010). Beta-carotene was evaluated individually and compared with total carotenoid production (Fig. 2a–c)

Production of total carotenoids on glucose was in most tested strains higher than on both technical and waste glycerol (Table 3). Waste glycerol was probably a better carbon source for pigment production than technical glycerol. Addition of glucose to glycerol medium led in some strains and ratios to increased production of pigments than in control: in medium with glucose:waste glycerol ratio of 1:1, about 2× higher total pigment production was observed in *Rhodotorula aurantiaca*, *Cystofilobasidium capitatum* and *Sporobolomyces shibatanus* (Table 3). In medium with glucose:waste glycerol ratio of 3:1, about 3× higher pigment production was observed in *R. aurantiaca* and *S. shibatanus*. Substantially lower effects were exhibited by the addition of technical glycerol to glucose: in medium with glucose:pure glycerol ratio of 3:1, increased pigment production was observed only in *Sporobolomyces* strains. Addition of glucose to technical glycerol in the ratio of 1:3 led to slightly increased production of total carotenoid in *R. glutinis*. The best producers of total carotenoids in glucose medium were *Rhodotorula glutinis* (2.22 mg/g cell dry weight (CDW)) and *Sporobolomyces roseus* (2.01 mg/g CDW). In pure glycerol medium, the highest yields were reached in waste glycerol medium (0.65 mg/g CDW). The best yields of total pigments in glycerol media with the addition of glucose were reached by *Sporobolomyces roseus* (3.60 mg/g CDW; 1:3), *Sporidiobolus salmonicolor* (2.85 mg/g CDW; 1:3), and *Rhodotorula glutinis* (2.80 mg/g CDW; 3:1 – Table 3). Unfortunately, *Sporobolomyces roseus* is typified by extremely low biomass production, so this strain cannot be recommended for industrial applications.

Production of carotenoids in media with glycerol:glucose mixture were studied in the fungus *Blakeslea trispora.* Beta-carotene was accumulated in mould cells in higher amounts as compared to when glucose was used as the sole carbon source, similarly to the present study. Over the range of glycerol levels tested, the highest beta-carotene production (15.0 mg/g of dry biomass) was achieved in cells grown on an initial glycerol concentration of 60.0 g/L.

Similarly to biomass production, in this case further detailed study is needed for understanding the effects of the simultaneous presence of glucose and glycerol in medium on induction of pigment production in some strains. It can be argued that glycerol in the presence of glucose critically influences cellular metabolism in *Yarrowia lipolytica* (Papanikolaou et al. 2008;

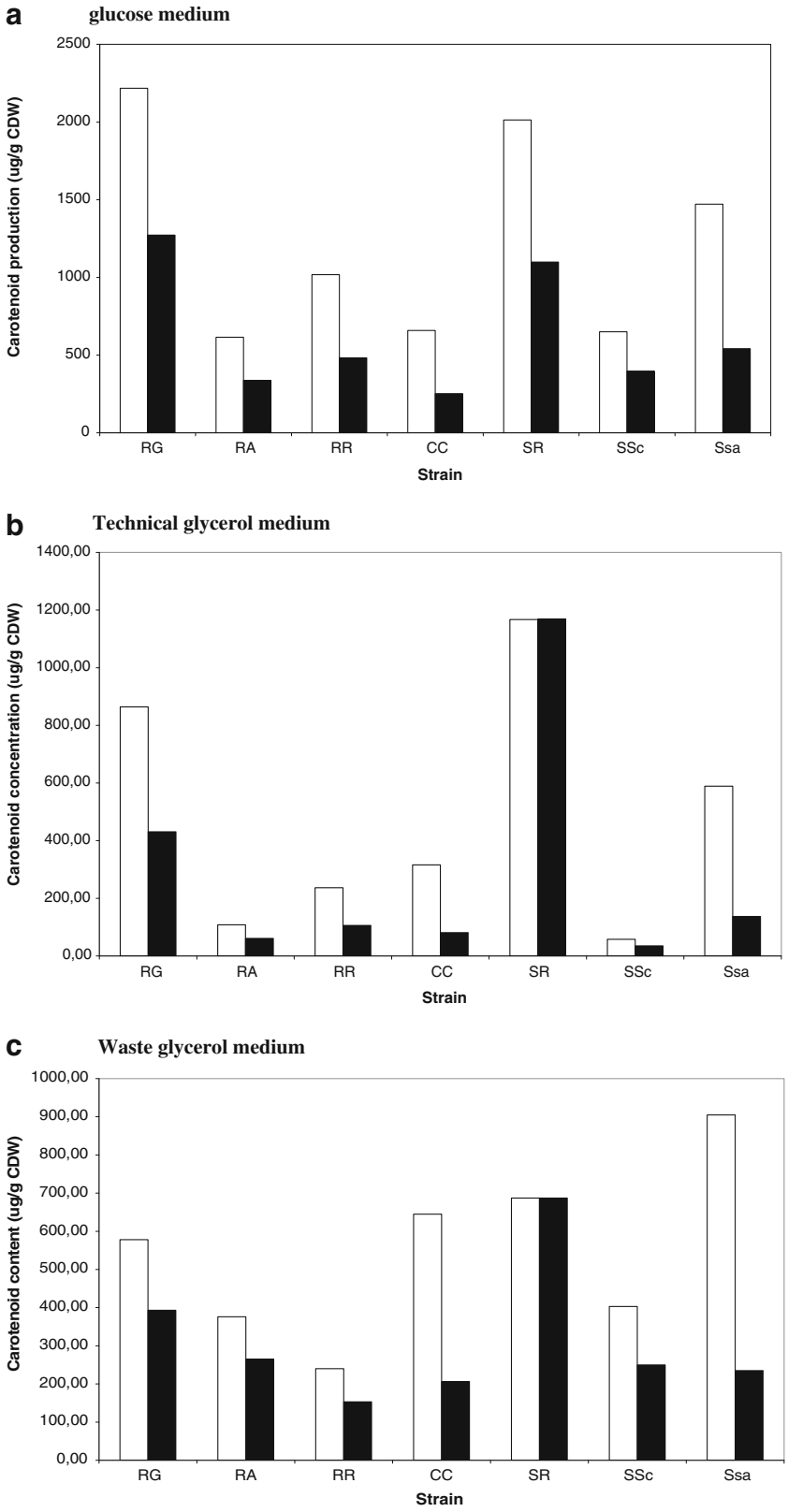

Fig. 2 Production of total carotenoids and beta-carotene by red yeasts grown for 96 h on: **a** glucose medium, **b** technical glycerol medium, **c** waste glycerol medium. Total carotenoids (μg/g of CDW) □, part of beta-carotene in total carotenoids amount (μg/g CDW) ■. Strain abbreviations: see Fig. 1

André et al. 2009). It seems that the key to efficient acetyl-CoA and NADPH production required for increased beta-carotene content may be the energy excess by the catabolism of glycerol via glycolysis that directed glucose toward the pentose phosphate pathway, as also suggested in the case of *Y. lypolytica* growing on glycerol/glucose-based media for citric acid

Table 3 Production of total carotenoids on pure technical glycerol (*A*), waste glycerol (*B*) and mixture of glycerol and glucose

Total carotenoids (μg/g d.w.)	100 % Glycerol		Glycerol:Glucose 3:1		Glycerol:Glucose 1:1		Glycerol:Glucose 1:3		Glucose (Control)
	A	B	A	B	A	B	A	B	
RG	868.4	578.3	2,802.1	731.9	1,103.7	557.5	1,944.3	1,084.2	2,217.1 ± 125.5
RA	107.5	375.6	388.0	618.5	271.6	1,204.6	247.2	1,804.1	614.7 ± 245.0
RR (RM)	237.3	239.8	352.2	1,469.1	1,287.4	1,021.1	942.9	935.6	1,017.5 ± 297.4
CC	315.6	645.3	596.6	1,070.7	477.9	1,368.4	778.9	246.5	658.6 ± 113.1
SR	1,168.6	687.0	421.4	1,658.8	2,037.8	2,051.0	3,599.6	2,561.8	2,012.8 ± 311.1
SSh	58.1	402.8	270.5	616.6	543.6	1,107.2	777.8	1,678.9	650.4 ± 207.0
SSa	589.1	905.2	454.6	591.3	491.1	1,707.6	2,854.4	1,152.8	1,471.2 ± 250.8

Results of total carotenoids yield were approximately quantified as (μg/g d.w.) using total area under chromatogram and calibration curve for beta-carotene. Data obtained during cultivation on glucose were expressed as average values from four parallel cultivations and statistically evaluated. Other data are expressed as average values of two parallel cultivations. Strain abbreviations: see Table 2

production (Makri et al. 2010). Although the lowest concentration of biomass and the lowest maximal specific growth rate with all the strains under investigation were achieved in the culture with pure glycerol, this substrate was found to be a better carbon source for the biosynthesis of citric acid than glucose, both in terms of the quantity of the produced citric acid and product selectivity (Rywinska et al. 2010; Rymowicz et al. 2008).

Because of a potential cross-linked function of glycerol and carotenoids in the stress response of red yeasts, more stress mechanisms are probably involved in this process (Bhosale 2004; Marova et al. 2004). The major fermentation outcome of glucose as substrate is efficient cell growth in parallel with lipid production, wherein almost total glucose consumption occurs within the early growth phase period. Nonetheless, beta-carotene production shows an increasing trend associated with the degradation of the cellular lipids within the late growth phase (Mantzouridou et al. 2008). Considering that carotenoid production is the outcome of the increase of carbon flux through N-lacking secondary routes, selection of an appropriate supplementary carbon source may have a dramatic effect on its yield. Thus, further studies on individual strains are needed to find metabolite regulations by using glycerol and glucose as cosubstrates.

The main pigment produced by red yeasts is beta-carotene. Some strains (mainly *Rhodotorula* sp. and *Cystofilobasidium capitatum*) can further metabolize about 50 % of beta-carotene to more oxidized derivatives such as torulene and torularhodin. Production of beta-carotene exhibited similar trends as the production of total pigments. Using pure technical glycerol substrate, some changes in pigment distribution were observed (Fig. 2a–c). In *Rhodotorula* sp., these changes were only marginal, while a substantial increase of beta-carotene ratio in total pigments was observed in *Sporobolomyces roseus*—practically all total pigments are represented by beta-carotene (Fig. 2b). In *Cystofilobasdidium capitatum* and *Sporobolomyces shibatanus*, some decrease in beta-carotene content in total pigments was observed (Fig. 2b). Using waste glycerol, more intensive changes in individual pigment distribution were observed mainly in *Rhodotorula* strains as well as in *Cystofilobasidium capitatum* (Fig. 2c). In these strains, increased content of beta-carotene (to 60–70 % of total pigments) was found, while the ratio of beta-carotene in *Sporobolomyces* strains was similar to technical glycerol (Fig. 2b, c). The highest yield of beta-carotene in waste glycerol medium enriched by glucose was obtained in *Sporobolomyces roseus* (2.63 mg/g CDW 1:3), while relatively high production was observed in *Rhodotorula glutinis* (1.35 mg/g CDW 3:1) and *Sporobolomyces shibatanus* (1.16 mg/g CDW 1:3).

Glucose is described as the best carbon source for red yeasts (Bhosale 2004; Marova et al. 2011). Relatively good production of carotenoids was described in *Rhodotorula mucilaginosa* grown on waste sugar molasses substrate activated by cotton seed oil in a batch system. In general, the increase in sugar concentration increased the growth of yeast and total carotenoid production. The highest carotenoid concentration (89.0 mg/L of total carotenoids) was obtained when 20 g/L molasses sucrose was used as the carbon source (Aksu and Tugba Eren 2005). In another study (Saenge et al. 2011), the addition of ammonium sulfate as a nitrogen source and Tween 20 as a surfactant increased the accumulation of carotenoids (135.25 mg/L in batch culture).

Ergosterol production

Ergosterol is one of the most important components in fungal membranes and can also be used as provitamin D. In this work, it was followed as the additional parameter of biomass quality and also to monitor the competition of two specialized branches of the isoprenoid pathway, which is used for the biosynthesis of both carotenoids and sterols (Marova et al. 2011).

Production of ergosterol was in some strains better on glucose when compared with both technical and waste glycerol medium, while the other strains (*Sporobolomyces roseus*, *Rhodotorula aurantiaca* and *Rhodotorula mucilaginosa*) produced increased amounts of ergosterol (Tables 4, 5). Ergosterol production was increased mainly when cultivated on technical glycerol, especially in *Sporobolomyces roseus*. The biggest amount of provitamin D was found during cultivation on pure technical glycerol (10.93 mg/g CDW) (Table 5). In other combinations of technical glycerol and glucose, the strain *Sporobolomyces roseus* also gave big yield, but it was not dominant. In the ratio 3:1 in advance of glucose, the largest ergosterol producer was *Rhodotorula aurantiaca* (9.86 mg/g CDW), followed by the other two, *Sporobolomyces roseus* and *Cystofilobasidium capitatum* (3.78 and 3.90 mg/g CDW, respectively). Interestingly, in the medium with 75 % of technical glycerol as a carbon source, the amount of obtained ergosterol was bigger than in control, and the best producer were yeasts of *Rhodotorula* strains which were, in the other combination of glucose and technical glycerol, neglectably low (Figs. 3, 4).

Technical glycerol was in most strains a better carbon source for ergosterol production than waste glycerol. It seems that in ergosterol production it plays an important role in its involvement in stress response more than in other metabolites. In our previous studies, we have found that, depending on strain and stress type, some stress factors led to changes of carotenoid and ergosterol production (Marova et al. 2004, 2010, 2012). While carotenoids are produced mainly as antioxidants, ergosterol could participate in stress-exposed membrane remodeling. Intracellular glycerol is also considered as a yeast stress metabolite. Because of potential cross-protection mechanisms, some competition between individual stress metabolites production and/or their presence in cultivation medium (including activation of membrane transport mechanisms) could occur. This is probably the reason why tested strains utilize glycerol in different ways and why pure glycerol led to a different response than crude glycerol containing additional substances.

Production of sterols on glycerol medium in batch cultures under different aeration conditions has been studied in *Saccharomyces cerevisiae* (Mantzouridou et al. 2009). Depending on strain, ergosterol production of 3–5 mg/g d.w. was obtained with biomass production of about 5 g/L. These yields are similar or lower than ergosterol production by particular red yeasts documented in this study (see above). According to the authors of the above-mentioned study, poor supply of oxygen enhanced the selectivity of the bioprocess in favor of ergosterol precursors (mainly squalene). Oxygen is probably the limiting factor for ergosterol as well as carotenoid production in red yeasts (Marova et al. 2011).

Triacylglycerols production

All tested red yeast strains belong to oleaginous yeasts. Biosynthesis of lipids is not related to cell growth, so it is considered as a procedure of secondary metabolism. It was observed that, after exhaustion of nitrogen in the culture, decreased activity of key enzymes coincided with the cessation of lipid accumulation, although glucose was still present (Abdul Hamid et al. 2011). Lipid accumulation in oleaginous yeasts is triggered by cells exhausting the nitrogen, but glucose continues to be assimilated as well as inhibition of ICDH activity within the mitochondrion. This leads to the accumulation of citrate, which is transported into the cytosol and cleaved to acetyl-CoA by ATP:citrate lyase (Papanikolaou et al. 2002; Taha et al. 2010). Generally, NAD+-ICDH was inhibited by two key metabolites, citrate and the ratio of NAD+/NADH, which again illustrates the strategic position of this enzyme in cellular metabolism.

Table 4 Production of beta-carotene pure technical glycerol (*A*), waste glycerol (*B*) and mixture of glycerol and glucose

Beta-carotene (μg/g d.w.)	100 % Glycerol		Glycerol:Glucose 3:1		Glycerol:Glucose 1:1		Glycerol:Glucose 1:3		Glucose (Control)
	A	B	A	B	A	B	A	B	
RG	430.5	393.2	1345.4	340.1	783.5	307.1	1,197.4	516.9	1,272.1 ± 266.4
RA	60.6	265.7	270.7	455.2	232.6	835.8	195.8	1,078.9	338.2 ± 166.7
RR (RM)	106.2	153.4	148.9	564.8	547.9	514.8	444.9	383.2	482.7 ± 100.3
CC	81.4	205.9	123.9	400.6	155.9	516.1	307.2	97.0	252.3 ± 124.3
SR	1,030.6	711.0	357.0	1,659.0	1,458.1	2,051.0	2,631.5	2,066.4	1,098.8 ± 411.1
SSh	35.3	250.0	175.9	440.8	407.1	782.4	531.9	1,161.8	396.7 ± 125.6
SSa	136.7	235.5	164.2	208.5	171.4	552.1	1,093.3	347.1	540.6 ± 200.8

Results of beta-carotene yield were quantified from peak area in HPLC chromatogram and calibration curve for beta-carotene. Data obtained during cultivation on glucose were expressed as average values from four parallel cultivations and statistically evaluated. Other data are expressed as average values of two parallel cultivations. Strain abbreviations: see Table 2

Table 5 Production of ergosterol using pure technical glycerol (*A*), waste glycerol (*B*) and mixture of glycerol and glucose

Ergosterol (μg/g d.w.)	100 % Glycerol		Glycerol:Glucose 3:1		Glycerol:Glucose 1:1		Glycerol:Glucose 1:3		Glucose (Control)
	A	B	A	B	A	B	A	B	
RG	435.85	1,281.5	3,386.9	1,377.6	671.36	1,829.0	2,690.5	2,759.6	1,710.8 ± 178.5
RA	2,853.9	3,719.7	3,891.9	4,466.9	1,110.4	2,017.5	2,723.9	1,875.9	2,240.1 ± 435.9
RR (RM)	2,783.9	762.48	1,889.9	3,161.6	16,754.0	3,591.1	1,882.4	1,858.3	2,256.2 ± 500.4
CC	953.30	1,430.7	2,704.6	2,306.4	1,684.2	2,705.6	3,889.6	4,629.1	2,665.0 ± 257.0
SR	10,926.0	6,080.3	2,766.0	14,998.0	2,564.4	12,012.0	3,778.8	7,766.2	1,553.4 ± 501.0
SSh	1,315.1	1,447.0	2,370.0	2,774.2	2,239.3	1,694.5	2,907.0	3,727.6	2,563.3 ± 264.2
SSa	1,248.6	1,632.6	2,591.8	4,381.9	3,061.3	6,148.9	9,864.8	4,739.4	2,914.6 ± 698.8

Results of ergosterol were quantified using peak area in HPLC chromatogram (285 nm) and calibration curve for ergosterol. Data obtained during cultivation on glucose were expressed as average values from four parallel cultivations and statistically evaluated. Other data are expressed as average values of two parallel cultivations. Strain abbreviations: see Table 2

In red yeast strains in particular conditions, intensive accumulation of lipids up to 60 % of biomass has been described (Taha et al. 2010). In the presented study, cheap mineral medium optimized mainly for biomass and carotenoid production was used. As mentioned above, the C/N ratio used was about 57. All studied strains were able to accumulate triacylglycerols (TAG). Under control conditions, most of the tested strains produced TAG in the range of 11–15 % except *Cystofilobasidium capitatum*, which produced more than 22 % of triacylglycerols. Production of TAG in all strains was about 10–30 % better in glycerol medium. In glycerol medium, not only *C. capitatum* (the best producer of TAG among studied strains) but *Rhodotorula aurantiaca* and *Sporobolomyces shibatanus* also reached TAG production up to 20 % of biomass when compared with glucose medium (Fig. 4). It is necessary to note that in the cultivation conditions used probably a part of the accumulated lipids was used for secondary metabolism according to the above-mentioned facts (Abdul Hamid et al. 2011). As described previously, in red yeasts unsaturated fatty acids formed about one-half of total cell lipids (Marova et al. 2011).

Previous work with oleaginous microorganisms (Fakas et al. 2007) showed that under carbon-limiting conditions, the previously accumulated lipid was utilized for biosynthetic purposes. This lipid was hypothesized to be TAG, though this was not checked by lipid analysis. Similar or higher lipid production than in the present study (30–58 %) was observed according to the aim of particular studies. Some of them were focused on production of yeast lipids as a biofuel of the second generation. In the Thailand study (Kitcha and Cheirsilp 2011) on 23 strains identified as potential lipid producers or oleaginous yeasts, the lipid contents were compared in crude glycerol-based medium. It was found that strain BY4-523 accumulated highest lipid content (up to 53.28 %), while JU4-57 grew fastest and gave comparable high lipid content (41.50 %). Lipid biosynthesis and fatty acids composition of oleaginous Zygomycetes, namely *Cunninghamella bainieri* 2A1, cultured in media with excess or limited nitrogen were

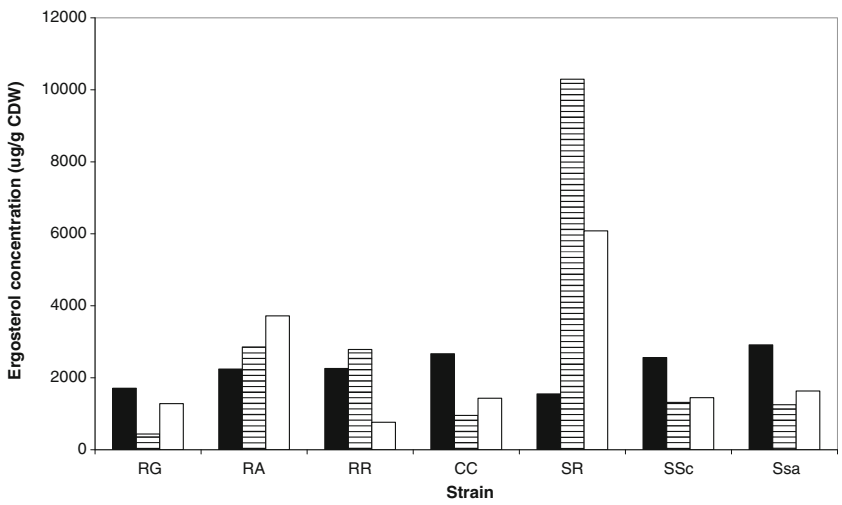

Fig. 3 Production of ergosterol by red yeasts grown on glucose and glycerol for 96 h. Cultivation on waste glycerol □, technical glycerol ▤ and glucose ■. Strain abbreviations: see Fig. 1

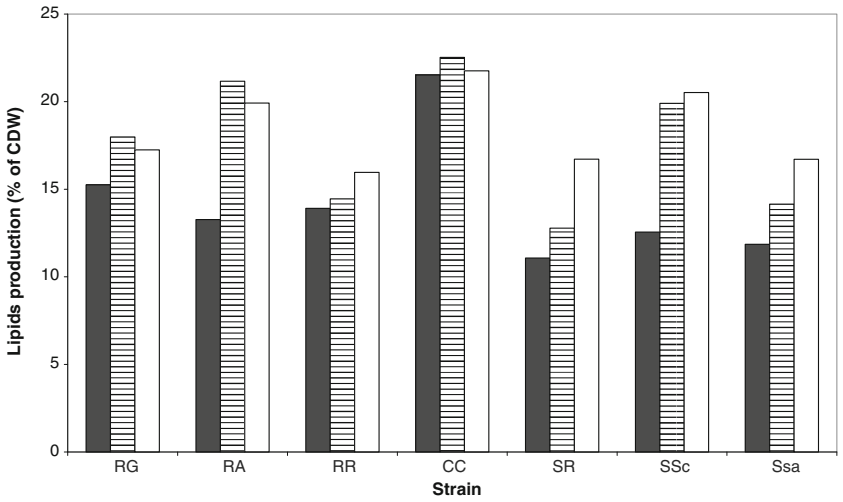

Fig. 4 Production of triacylglycerols by red yeasts grown on glucose and glycerol for 96 h. Triacylglycerols are expressed as % of CDW (cell dry weight). Cultivation on glucose ■, technical glycerol ▤ and waste glycerol □. Strain abbreviations: see Fig. 1

quantitatively determined at different times of culture growth (Taha et al. 2010). Another study in 2002 demonstrated that the initial C:N ratio is important for lipid accumulation (Papanikolaou et al. 2002). The role of the C:N ratio in lipid accumulation has been shown to depend on the microorganism and the fermentation liquid medium.

Raw glycerol, a by-product from the bio-diesel production process, was used as carbon substrate in production of 1,3-propanediol by *Clostridium butyricum* in batch anaerobic cultures, while the substrate uptake rate increased with increase in glycerol concentration in the medium (Papanikolaou et al. 2008). In the same study, *Yarrowia lipolytica* ACA-DC 50109 was grown in nitrogen-limited aerobic cultures on raw glycerol and it exhibited remarkable biomass production even at high glycerol concentration media, while rS decreased with the increase in glycerol concentration. Citric acid was produced after nitrogen depletion in the medium, with the highest quantity of 62.5 g/L, and the yield on the glycerol consumed was 0.56 gg/L. Fatty acid analysis of total cellular lipids showed that a glycerol concentration increase in the growth medium somehow increased the cellular unsaturated fatty acids content of lipids, similar to Taha et al. (2010). Further, crude glycerol was also used for cultivation of *Mortierella isabellina* ATHUM 2935, which exhibited satisfactory growth in nitrogen-limited aerobic cultures with raw glycerol used as sole substrate. When high initial glycerol quantities were employed (100 g/L), 4.4 g/L of lipid were accumulated corresponding to around 51 % (wt/wt) of lipid in dry weight (Papanikolaou et al. 2008).

The oleaginous yeast *Rhodotorula graminis* was also studied as a bioproducer of lipidic compounds using some wastes including crude glycerol as carbon sources. During a preliminary experiment performed in a 1-L fermenter on crude glycerol, a biomass (about 48 g/L) rich in lipids, representing 54 % of its weight, very close to the one obtained with pure glycerol, and almost the same yield (0.26 g/g) and productivity (0.15 g/L/h) were obtained (Galafassi et al. 2012). As mentioned above, Saenge et al. (2011) and Cheirsilp et al. (2012) studied the possible use of crude glycerol as the sole carbon source for production of lipids and carotenoids by the oleaginous yeast *Rhodotorula glutinis* TISTR 5159. The synergic effects of the C/N ratio with glycerol concentration and Tween 20 concentration were observed in the accumulation of lipids. The optimum conditions for lipid content and carotenoids production were glycerol concentration of 9.5 % and C/N ratio of 85. In fed-batch fermentation, the highest lipids production of 6.05 g/L with a cellular lipid content of 60.7 %. The yeast lipids obtained have shown the favorable properties for being used as feedstock in the production of biodiesel.

In an other study (Chatzifragkou et al. 2011), 15 eukaryotic microorganisms were tested for their ability to assimilate biodiesel-derived waste glycerol and convert it into value-added metabolic products. For this purpose, yeast and *Zygomycetes* strains were cultivated in nitrogen-limited raw glycerol-based media (initial glycerol concentration 30 g/L). The yeasts tested accumulated restricted lipid quantities (up to 22 %, wt/wt, in the case of *Rhodotorula* sp.), while differences in their fatty acid composition were recorded in relation to the yeast strains employed and the fermentation time. It should be noted that, in all yeast strains, regardless of the quantity of lipid accumulated into the cells, lipids were mainly composed of C16 and C18 fatty acids (Marova et al. 2011). This fat is not suitable for some food applications, but it can be used as starting material for the synthesis of "2nd generation" biodiesel (Chatzifragkou et al. 2011). Because of increasing demand and utilization of the "1st generation biodiesel" (biodiesel deriving from trans-esterification of plant oils), the cost of various foodstuffs has also been increasing. This situation has led to the need to discover novel sources of oils that ccan be subsequently converted into biodiesel, with the oleaginous yeasts (due to their easy handling and good large-scale cultivation potentialities) being considered as perfect candidates for this purpose.

Cultivation of carotenogenic yeasts in a bioreactor

In a further part of the presented study, a laboratory scale-up process under controlled conditions was tested. Strains *Rhodotorula glutinis*, *Cystofilobasidium capitatum* and *Sporobolomyces roseus* were cultivated in a laboratory fermentor with a maximum capacity of 2 L. Two series of experiments were performed using different carbon sources: cultivation in basic glucose medium and in medium with technical glycerol (see "Materials and methods"). During cultivation, biomass and pigments production, glucose and glycerol changes in medium and intracellular glycerol concentrations were monitored.

According to growth data on glucose, *Rhodotorula glutinis* entered the stationary phase after 24 h from the beginning of cultivation and stayed unchanged until 70 h, when further local maximum was observed (data not shown). Similar growing properties of carotenogenic yeasts were found in other studies (Marova et al. 2004, 2010) containing several local maxima with extended stationary phase. Specific growth rates in glucose controls were relatively similar in *Cystofilobasidium capitatum* and *Rhodotorula glutinis,* while in *Sporobolomyces roseus* lower values were found.

When technical glycerol was used as carbon source, some type of intracellular osmotic stress was probably induced. As a consequence, the exponential phase was doubled in comparison to the control series. In control cultivations, the lag phase lasted 20 h, but when glycerol was introduced, the lag phase was from 43 h in *Cystofilobasidium capitatum* and even 47 h in *Rhodotorula glutinis*. Prolonged lag phase resulted in higher biomass production at the end of exponential phase and also in overall biomass production.

During the cultivation on glycerol and glucose, samples were taken regularly from the bioreactor at defined times from the beginning of cultivation: 28, 50, and 75 h. Table 6 illustrates growth characteristics for carotenogenic yeasts cultivated on glucose and glycerol (see "Materials and methods"). Intracellular glycerol contents measured in yeast cells at the end of cultivation (75 h) are also shown in Table 6. As expected, glycerol was intensively utilized mainly by *Cystofilobasidium capitatum*. During the first 28 h from the beginning of cultivation, more than one-half of the overall amount of glycerol was consumed, leaving in the culture broth the unused amount of glycerol (12.05 g/L). Between 28 and 50 h, almost all the remaining glycerol was utilized by *C. capitatum* and it stayed constant until the end of cultivation. At the late stationary phase, the concentration of the remaining glycerol was the same and very low, not exceeding 1.00 g/L. *Rhodotorula glutinis* also utilized glycerol, but not as fast as was found in *Cystofilobasidium capitatum.* In the first phase (lag and beginning of the exponential phase), the remained glycerol was 16.47 g/L. It is interesting that in the next 22 h the concentration of free glycerol decreased only 5 g/L and the biomass was doubled. At the end of cultivation (75 h), residual glycerol was, as in the case of *Cystofilobasidium capitatum*, very low (1.76 g/L). In these two strains, glucose was utilized more slowly, and thus glycerol could be considered as the preferred substrate. In contrast, *Sporobolomyces roseus* utilized glycerol relatively slowly. In the lag and exponential phases, only one-third of available glycerol was used. Observing the produced biomass, which was very small (4.14 g/L at 28 h and 7.31 g/L at 50 h), glycerol is probably not the preferred carbon source for *S. roseus* biomass production when compared with gluocse. At the end of cultivation, 11.50 g/L of glycerol remained in the medium. That large amount of free glycerol represents approximately the same amount as when *Rhodotorula glutinis* or *Cystofilobasidium capitatum* was cultivated for 50 h.

Cultivation of *Blakeslea trispora* on raw glycerol also showed a prolonged lag phase (18 h) accompanied by non-significant substrate uptake. Then, an efficient cell growth with simultaneous cellular lipid accumulation and utilization of glucose and nitrogen substrate took place (Mantzouridou

Table 6 Changes of concentration of carbon source in medium (c/glucose, c/glycerol), production of biomass (*X*), pigments and intracellular glycerol changes during cultivation of yeasts in laboratory fermentor

Supernatant	$c_{glucose}$ (mg/L)	$c_{glycerol}$ (g/L)	x (g/L) glucose	x (g/L) glycerol	Total carotenoids/ glucose, mg/L	Total carotenoids/ glycerol, mg/L	Glycerol, mg/g biomass
RG (27 h)	13.45	16.47	15.11	17.11	–	–	–
RG (50 h)	12.24	11.69	23.21	29.53	–	–	–
RG (72 h)	13.53	1.76	28.98	32.16	64.95	48.24	1.46
CC (28 h)	18.86	12.05	16.54	14.42	–	–	–
CC (50 h)	10.83	0.96	29.41	22.49	–	–	–
CC (75 h)	10.70	0.93	35.46	30.81	53.82	29.65	0.69
SR (28 h)	210.36	27.47	4.14	3.14	–	–	–
SR (50 h)	17.07	20.70	7.31	4.85	–	–	–
SR (75 h)	8.57	11.50	9.48	7.23	26.58	13.01	16.70

Strain abbreviations: see Table 2

et al. 2008). Higher yields of lipids than in the present work are probably caused by different strain and cultivation conditions. In *Yarrowia*, higher assimilation rates of glycerol were also observed compared with those of glucose. Glycerol was exhausted from the culture medium whereas signicant quantities of glucose remained unconsumed (Papanikolaou et al. 2008).

The residual concentration of intracellular glycerol determined at the end of cultivation corresponded to the concentration in appropriate supernatants (Table 6). As could be expected, the highest concentration of free glycerol was found in *Sporobolomyces roseus* (16.70 mg/g). On the other hand, a relatively minor concentration of glycerol was observed in both *Rhodotorula glutinis* and *Cystofilobasidium capitatum* cells. During cultivation in basic glucose medium, all red yeasts strains utilized glucose almost at the beginning of growth. After 24 h, all red yeasts entered the stationary phase, where biomass production was stopped and metabolite production was started.

Total carotenoid production was measured at the end of cultivation and expressed as mg per litre of culture (Table 6). In glucose media, about 1.5× higher yields of total pigments were observed in all tested strains when compared with technical glycerol media. Total production of pigments in the laboratory fermentor was more intensive than in Erlenmeyer flasks (see Table 4). The reason is probably better aeration and controlled fermentation conditions.

Conclusions

The presented study focused on a comparison of growth and production properties of seven red yeast strains of the genus *Rhodotorula*, *Sporobolomyces* and *Cystofilobasidium*, when cultivated on glycerol substrate (technical and waste) as well as in mixed glycerol:glucose media. The aim was to find strains suitable for industrial use of cheap simple medium with waste glycerol as a carbon source for production of enriched yeast biomass and specific yeast metabolites (carotenoids, ergosterol, triacylglycerols).

All tested strains were able to utilize glycerol as the only carbon source. The biomass production, when cultivated on pure technical glycerol, was less or equal to control. Using waste glycerol as the only carbon source, most of strains exhibited higher biomass production than in control as well as in medium with pure technical glycerol. Production of carotenoids and ergosterol was better in glucose medium than in medium with only glycerol. Nevertheless, using glycerol medium with the addition of glucose, higher yields of total carotenoids, beta-carotene and ergosterol were obtained than in control. The highest yields of pigments were reached in the *Sporobolomyes roseus* strain, which is typified by low biomass production.

All tested red yeast strains belong to oleaginous yeasts. In the presented study, poor cheap mineral medium optimized mainly for biomass and carotenoid production was used (C/N ratio about 57). All studied strains were able to accumulate triacylglycerols (TAG). Under control conditions, most of the tested strains produced triacylglycerols in the range of 11–15 % except *Cystofilobasidium capitatum*, which produced more than 22 % of neutral lipids. Production of TAG in all strains was about 10–30 % better in glycerol medium. In glycerol, not only *Cystofilobasidium capitatum* (the best producer of lipids among studied strains) but also *Rhodotorula aurantiaca* and *Sporobolomyces shibatanus* reached TAG production up to 20 % of biomass when compared with glucose medium

With regard to growth and production stability, the suitable candidates for biotechnological applications in the area of carotenoid rich biomass and pigment production on glycerol substrate are mainly *Rhodotorula glutinis*, *Cystofilobasidium capitatum* and *Sporobolomyces shibatanus*. These strains take advantage of the utilization of the whole biomass (complete nutrition source), which is efficiently enriched for carotenoids (provitamin A, antioxidants) and also ergosterol (provitamin D). Such a product could serve as an additional natural source of significant nutrition factors in feed and food industry.

In conclusion, we can say that this study has shown that oleaginous red yeasts could have great potential for converting crude glycerol to valuable lipids and carotenoids in view of efficient bioresource utilization. Because of the potential role of glycerol in red yeast stress response, the use of individual strains should be optimized for potential industrial production of enriched yeast biomass or specific yeast metabolites. The type of crude glycerol and its composition are also important factors substantially influencing the biotechnological process. The potential significant increase of bio-diesel production in the near future is already resulting in the need for the discovery of various integrated bioprocesses for valorization of this residue.

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