

Red yeasts and carotenoid production: outlining a future for non-conventional yeasts of biotechnological interest

Ilaria Mannazzu¹ · Sara Landolfo¹ · Teresa Lopes da Silva² · Pietro Buzzini³

Received: 15 June 2015 / Accepted: 14 August 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Carotenoids are one of the most common classes of pigments that occur in nature. Due to their biological properties, they are widely used in phytomedicine and in the chemical, pharmaceutical, cosmetic, food and feed industries. Accordingly, their global market is continuously growing, and it is expected to reach about US\$1.4 billion in 2018. Carotenoids can be easily produced by chemical synthesis, although their biotechnological production is rapidly becoming an appealing alternative to the chemical route, partly due to consumer concerns against synthetic pigments. Among the yeasts, and apart from the pigmented species *Phaffia rhodozyma* (and its teleomorph *Xanthophyllomyces dendrorhous*), a handful of species of the genera *Rhodospiridium*, *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus* are well known carotenoid producers. These are known as ‘red yeasts’, and their ability to synthesize mixtures of carotenoids from low-cost carbon sources has been broadly studied recently. Here, in agreement with the renewed interest in microbial carotenoids, the recent literature is reviewed regarding the taxonomy of the genera *Rhodospiridium*, *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus*, the stress factors that influence their carotenogenesis, and the most advanced analytical tools for evaluation of carotenoid production.

Moreover, a synopsis of the molecular and “-omic” tools available for elucidation of the metabolic pathways of the microbial carotenoids is reported.

Keywords Basidiomycetous yeast · Carotenoid analysis · Flow cytometry · -omic tools · Transformation

Introduction

Carotenoids are 40-carbon isoprenoids that are generally separated into carotenes (e.g., β -carotene) and xanthophylls (oxygenated carotenoids, such as astaxanthin), and are usually localized within intracellular lipid droplets (Buzzini et al. 2010). Owing to their bright colour, and because of a variety of their properties, carotenoids represent a valuable class of molecules for industrial applications (Johnson and Echavarri-Erasun 2011). In particular, they are widely exploited on an industrial scale as additives in pharmaceutical, chemical, food and feed products, mostly as vitamin A precursors (Johnson and Schroeder 1995; Vachali et al. 2012). Consequently, their industrial market is expected to reach about US\$1.4 billion in 2018 (BCC Research 2011).

To satisfy the increasing demand for carotenoids, they can be produced easily by chemical synthesis. However, due to general consumer concern against synthetic pigments, the biotechnological production of carotenoids (via de-novo microbial synthesis) is becoming an appealing alternative to the chemical route.

Among the microorganisms that can produce carotenoids, there are a number of basidiomycetes yeast species that grow as pigmented colonies, and are for this reason known as ‘red yeasts’. *Phaffia rhodozyma* and its teleomorph *Xanthophyllomyces dendrorhous* are by far the

✉ Ilaria Mannazzu
imannazzu@uniss.it

¹ Dipartimento di Agraria, Università degli Studi di Sassari, Viale Italia 39, Sassari, Italy

² Unidade de Bioenergia, Laboratório Nacional de Energia e Geologia, Estrada do Paço do Lumiar 22, Lisbon, Portugal

³ Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Collezione dei Lieviti Industriali (DBVPG), Università di Perugia, Borgo XX Giugno 74, Perugia, Italy

most well-known representatives of the red yeasts. They have significant biotechnological potential because of their particular carotenoid profiles, which include astaxanthin as the main pigment (Buzzini et al. 2010; Fell and Johnson 2011; Fell et al. 2011). However, other basidiomycetes can also produce different carotenoids. Apart from those ascribed to the genera *Bulleromyces*, *Cystobasidium*, *Cystofilobasidium*, *Mixia* and *Occultifur* (Boekhout 2011; Nishida et al. 2011; Sampaio 2011a; Sampaio and Oberwinkler 2011a, b), which are apparently characterised by traces of intracellular carotenoids, some other species that belong to the genera *Rhodotorula* and *Sporobolomyces*, along with their teleomorphs *Rhodospiridium* and *Sporidiobolus*, are known to produce higher amounts of a mixture of carotenoids (Buzzini et al. 2007, 2010; Hamamoto et al. 2011; Johnson and Echavarri-Erasun 2011; Sampaio 2011b, c, d).

Indeed, the biosynthetic pathway that leads to carotenoid production has been defined for the red yeasts. Moreover, great progress has been achieved in the study of the regulation of the carotenogenic pathway in *X. dendrorhous* (Marcoleta et al. 2011; Martinez-Moya et al. 2015). Therefore, the production of astaxanthin by *X. dendrorhous* has been scaled-up to the industrial level, and for this reason, this yeast will not be considered in this review.

On the other hand, the less characterised red yeast species ascribed to the genera *Rhodotorula*, *Sporobolomyces*, *Rhodospiridium* and *Sporidiobolus* still deserve attention (Mata-Gomez et al. 2014). These yeast have good potential as biocatalysts, due to their bio-transformation of a plethora of carbon sources into a variety of primary and secondary metabolites (Johnson and Echavarri-Erasun 2011; Johnson 2013; Mata-Gomez et al. 2014), and to their marked tolerance to inhibitory compounds that can occur as by-products of the agro-food industry used for their growth (Hu et al. 2009).

Here, in agreement with the renewed interest towards the red yeasts ascribed to the genera *Rhodotorula*, *Sporobolomyces*, *Rhodospiridium* and *Sporidiobolus*, this review provides an update of the most recent literature regarding their taxonomy, the stress factors that affect their carotenogenesis, the most advanced analytical tools for evaluation of their pigment production, and the molecular and ‘-omic’ tools that have been recently proposed for the elucidation of their metabolic pathways.

Taxonomy of the red yeasts

Red yeasts of the four genera *Rhodotorula*, *Sporobolomyces*, *Rhodospiridium* and *Sporidiobolus* constitute a heterogeneous group of basidiomycetous yeast that are

typically isolated from air, the phylloplane, and decaying plant debris, and have a worldwide distribution (Fell and Johnson 2011; Fell et al. 2011; Hamamoto et al. 2011; Johnson and Echavarri-Erasun 2011; Sampaio 2011b, c, d). Although the genera *Rhodotorula*, *Rhodospiridium* and *Sporidiobolus* include a large number of recognized species (Sampaio 2011b, c, d), only a few of these can accumulate significant amounts of carotenoids; their phylogenetic placement is reported in Fig. 1. *Rhodospiridium diobovatum*, *Rhodospiridium toruloides*, *Rhodotorula glutinis*, *Rhodotorula graminis*, *Rhodotorula mucilaginosa*, *Sporidiobolus johnsonii*, *Sporidiobolus metaroseus*, *Sporidiobolus pararoseus* and *Sporidiobolus salmonicolor* are included in Class Microbotryomycetes, while *Rhodotorula minuta* and *Rhodotorula aurantiaca* are included in Class Cystobasidiomycetes (subphylum Pucciniomycotina) (Fig. 1). Finally, *Rhodotorula bacarum* is included in Class Exobasidiomycetes, Order Microstromatales (subphylum Ustilaginomycotina) (Sampaio 2011b, c, d). In contrast, in its current concept, *Sporobolomyces* is a polyphyletic genus. The species known for their carotenogenesis (i.e., *Sporobolomyces patagonicus*, *Sporobolomyces roseus*) belong to the subphylum Pucciniomycotina (Hamamoto et al. 2011) (Fig. 1). These species produce a mixture of carotenoids, namely, β -carotene, γ -carotene, torulene and torularhodin (Buzzini et al. 2007, 2010; Hamamoto et al. 2011; Johnson and Echavarri-Erasun 2011; Sampaio 2011b, c, d), the main properties of which are reported in Table 1. γ -Carotene represents a key precursor and an essential branch point in the synthesis of either β -carotene or torulene (after desaturation at the 3',4' position), and subsequently of torularhodin (Johnson and Schroeder 1995).

Stress factors and carotenogenesis in red yeasts

Within the species indicated above, the yield and composition of their carotenoids vary significantly according to strain, medium components, and environmental conditions (Marova et al. 2011). Therefore, optimisation of culture conditions has been extensively studied, with the aim of improving the yeast performance and reducing the cost of carotenoid production (Bhosale and Gadre 2001a, b; Cutzu et al. 2013a, b; Davoli et al. 2004; Hernandez-Almanza et al. 2014; Maldonado et al. 2008; Marova et al. 2004, 2012; Wang et al. 2008; Yen and Zhang 2011). As carotenoids are apparently involved in stress responses of microorganisms, their production is influenced by several exogenous stress factors. Carotenoids are membrane-protective antioxidant pigments that can efficiently scavenge $^1\text{O}_2$ and peroxy radicals. Their protective effect depends on their chemical structures, and in particular on the length

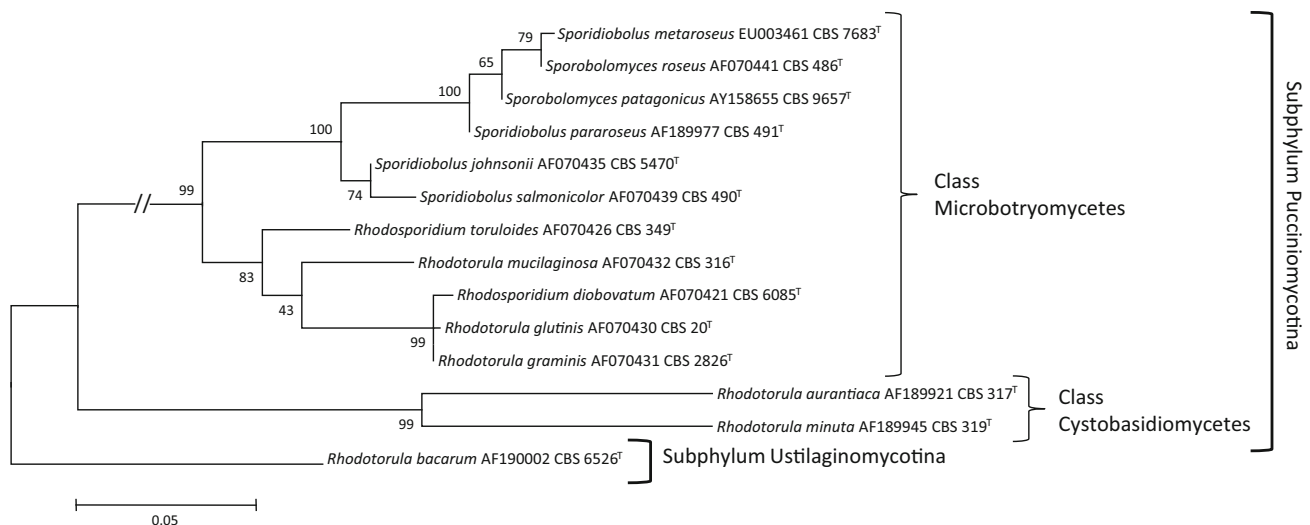


Fig. 1 LSU phylogenetic Maximum Likelihood tree of the type strains of the species recognized for their ability to accumulate significant amounts of carotenoids. The tree was rooted with *Rhodotorula bacarum* type strain. Bootstrap percentages from 1000 replications are shown on the branches (values below 50 % are not shown). GenBank accession numbers of the sequences and strain numbers are indicated. D1/D2 domains of the large sub-unit 26S of the rDNA (Large SubUnit LSU) sequences were downloaded from NCBI website (<http://www.ncbi.nlm.nih.gov>). Phylogenetic analysis

of type strains was performed considering the final Muscle alignment (www.ebi.ac.uk/tools/msa/muscle) based on 623 positions including gaps, implemented in Mega5 software. The final alignment was exported and the best-fit substitution model was determined using Model selection of Mega5. 24 models were evaluated and the phylogenetic tree were reconstructed by maximum likelihood (ML). The robustness of the phylogenetic inference was estimated with the bootstrap method with 1000 replicates generated

Table 1 Characteristics, properties and biotechnological applications of carotenoids produced by red yeasts

Pigment	Absorption maxima (nm) in different solvents	Properties	Biotechnological application	References
γ -Carotene	439 461 491 Acetone 446 475 509 Chloroform 440 460 489 Ethanol 437 462 494 Hexane, petroleum ether	Vitamin A precursor		Rodríguez-Amaya (2001)
β -Carotene	429 452 478 Acetone 435 461 485 Chloroform 425 450 478 Ethanol 425 450 477 Hexane petroleum ether	Radical scavenger Vitamin A precursor	Colorant and additive for food, beverages and animal feed Colorant for cosmetic and body care products	Rodríguez-Amaya (2001) Dufossé (2006) Kirti et al. (2014)
Torulene	460 484 518 Petroleum ether	Vitamin A precursor	Proposed as food colorant	Maldonado et al. (2008) Zoz et al. (2015)
Torularhodin	465 492 523 Petroleum ether	Vitamin A precursor Singlet oxygen quencher Antioxidant activity	Proposed as colorant for meat products Colorant for poultry and fish	Maldonado et al. (2008) Sakaki et al. (2001) Zoz et al. (2015)

of their polyene chromophore, the nature of their end groups, and the various substituents that they contain (Marova et al. 2010). Some studies have reported that *R. glutinis*, *R. mucilaginosa* and *S. salmonicolor* can produce increased amounts of carotenoids when they are grown under unfavourable conditions (Marova et al. 2004); in particular, under oxidative, osmotic and salt stress (Marova et al. 2004, 2010), although an excess of NaCl and UV

exposure results in significant decrease in carotenoids biosynthesis in *R. glutinis* (Mahmoud et al. 2014).

Some solvents can act as stress factors and stimulate carotenogenesis in red yeasts, such as ethanol, methanol, isopropanol and ethylene glycol (Bhosale 2004). Similarly, some metal ions can influence the red yeast carotenoid profile, as a probable result of metal-mediated activation (or inhibition) of specific key enzymes involved in

carotenogenesis, or due to activation of a cellular defence mechanism against active oxygen radicals that are generated by some metals (Bhosale 2004; Buzzini et al. 2005; Irazusta et al. 2013; Rapta et al. 2005).

Nicotine and diphenylamine can be used to modify the carotenoid profile. Nicotine is known to inhibit the cyclising steps in β -carotene synthesis, while diphenylamine blocks the desaturation of phytoene, which is a precursor of γ -carotene (Squina and Mercadante 2005). Thus, the addition of 5 μ mol diphenylamine increases the total amount of carotenoids in *R. mucilaginosa* and significantly reduces the torularhodin/torulene ratio in both *R. mucilaginosa* and *R. glutinis*. On the other hand, higher amounts of diphenylamine result in higher β -carotene accumulation in both *R. mucilaginosa* and *R. glutinis*, while nicotine promotes selective biosynthesis of lycopene (Squina and Mercadante 2005).

Advanced analytical tools for evaluation of carotenoid production

Evaluation and quantification of carotenoids produced by microorganisms (including yeast) have gained increasing importance in recent years. The microbial carotenoids are difficult to extract and analyse due to their intracellular compartmentalisation, the variability of their chemical structures, and their poor stability. Indeed, carotenoids can be found both in their free form and in a more stable, esterified form with fatty acids (Herrero et al. 2008). They are subjected to some alternative reactions, such as oxidation and isomerisation (*cis-trans*), especially due to light, heat, acid and oxygen (Provesi et al. 2011). They can also undergo cyclisation, hydrogenation/dehydrogenation reactions, or additions of lateral groups, which results in the formation of an extremely complex variety of compounds that are characterised by the same basic scaffold. In addition, the lack of commercially available standards, the low carotenoid concentrations found in some microorganisms, and the presence of potentially interfering intracellular compounds (e.g., lipids) gives added difficulty to the development of methods for carotenoid analysis in microbial samples (Amorim-Carrilho et al. 2014).

The traditional carotenoid detection methods involve a number of steps, with the need for cell disruption, and carotenoid extraction, separation and quantification (Kaiser et al. 2007). Considering that carotenoids are often tightly bound to cellular membranes, efficient disruption of the whole cell is necessary. Red yeast cells have been disrupted using a mortar and pestle (Maldonado et al. 2008), and sometimes by addition of sand and acetone (Petrik et al. 2013; Somashekar and Joseph 2000). Glass beads have also been used to disrupt *Sporobolomyces ruberrimus*

(Razavi 2006). Chemical disruption using dimethylsulphoxide has also been used to break *R. glutinis* cells (Tinoi et al. 2005). The extraction step should be performed as rapidly as possible, to avoid oxidation or enzymatic degradation. Sometimes cell disruption and carotenoid extraction are carried out simultaneously (Malisorn and Suntornsuk 2008; Ferrao and Garg 2012). Dimethylsulphoxide, acetone and petroleum ether are the most popular solvents used in these steps, and according to Park et al. (2007), the use of multiple solvents increases carotenoid recovery, due to synergistic effects.

The classical method for carotenoid quantification in yeast is spectrophotometry (Aksu and Eren 2005; Park et al. 2007; Tinoi et al. 2005). This method is simple and accurate when there is only one carotenoid in the sample, but when a carotenoid mix is analysed, the identification of the pigment can be hampered by partial overlap of the carotenoid absorption peaks (Soroka et al. 2012).

A more accurate method for carotenoid quantification in yeast uses reverse-phase high-performance liquid chromatography (RP-HPLC). This allows detection and quantification of the individual carotenoids (Malisorn and Suntornsuk 2008), although it has the disadvantage of being more time and cost intensive than spectrophotometry. By coupling HPLC to mass spectrometry (LC-MS), carotenoid identification can be easily confirmed by comparing the mass spectra with published fragment-ion abundances, or mass spectra libraries. In comparison to UV-Vis spectrophotometry, LC-MS allows unambiguous identification of even unknown carotenoids and isoprenoid quinones (Nishijima et al. 1997). In the case of unknown peaks, the coupling of on-line photodiode-array or UV-Vis detectors and LC-MS/MS can provide valuable data for their identification. However, chromatograms are usually very complex, due to the great variety of isomers and structurally related compounds and metabolites. In addition, there are no standards available for all of the carotenoids, and only one isomeric form is commercially available (Amorim-Carrilho et al. 2014).

Kaiser et al. (2007) proposed a novel small-scale method for determination of a wide range of carotenoids in yeast and bacteria, with some variations in the above-mentioned steps. For cell disruption, they combined primarily mild techniques (i.e., lyticase, lipase), and for carotenoid extraction, they applied chemical treatment to yeast cells, using dimethylsulphoxide. Separation of the compounds obtained was achieved with HPLC, and the total carotenoids were quantified as *trans*- β -carotene equivalents. However, the procedures used by Kaiser et al. (2007) and most of the other studies that have quantified carotenoids in yeast using these more traditional steps are time consuming, can promote carotenoid degradation, use large amounts of toxic organic solvents, and require

significant amounts of biomass for carotenoid extraction and quantification (Freitas et al. 2014). Therefore, it is crucial to develop rapid carotenoid detection methods that can give accurate results in the shortest time possible, so that the operational growth conditions can be adjusted, to achieve higher carotenoid productivity.

Multi-parameter flow cytometry is a powerful advanced technique that allows evaluation of the intracellular carotenoids content in near real time (at-line), and with a high degree of accuracy (Cutzu et al. 2013a; Freitas et al. 2014; Ukibe et al. 2008). As flow cytometry analyses the cells in vivo immediately after sampling, carotenoid degradation is reduced. In addition, flow cytometry allows evaluation of other cellular parameters during the yeast cultivation. Cutzu et al. (2013a) used multi-parameter flow cytometry for β -carotene quantification in *R. glutinis*. In their study, the *R. glutinis* autofluorescence intensity showed good correlation with its β -carotene content detected by traditional analytical methods. Furthermore, there was also the possibility to determine cell-membrane permeability, and cell size and granularity, which provided a better understanding of the yeast physiology during β -carotene accumulation. More recently, Freitas et al. (2014) used flow cytometry to quantify for the first time the total carotenoid content in a *R. toruloides* strain that produces β -carotene, torulene and torularhodin, and γ -carotene at low concentrations.

Despite the benefits of using flow cytometry for microbial carotenoid at-line detection, this technique only gives information on the total carotenoid content, although this information is crucial when optimising the total carotenoid production bioprocess.

Molecular and ‘-omic’ tools for definition of metabolic pathways and management of carbon fluxes within red yeast cells

A survey of the most recent literature indicates that there is growing interest in the development of effective molecular and -omic tools that are aimed at the definition of red yeast metabolic pathways, and also for management of carbon fluxes within the cells. The first attempt at DNA delivery into red yeasts dates back to the middle 1980s, when a *R. toruloides* polyethylene glycol/protoplast transformation method was described (Tully and Gilbert 1985). A relatively long time later, other studies developed more effective transformation methods, with the perspective of studying gene function in red yeasts. Great impulse was given by the study of Ianiri et al. (2011), who described the construction of plasmids containing the *URA3* and *URA5* auxotrophic markers (Table 2). These were delivered into *Sporobolomyces* spp. through biolistic or *Agrobacterium-*

tumefaciens-mediated transformation (ATMT) methods and targeted disruption of the *LEU1* gene by homologous recombination, thus paving the way for development of functional genetics in *Sporobolomyces* spp. Two years later, Abbott et al. (2013) evaluated the possibility of using common plasmids and transformation methods for *R. kratochvilovae*, *R. sloffiae*, *R. graminis* and *Sporobolomyces* spp., with their aim being to increase the range of red yeast species that are amenable to genetic engineering. Based on these results, they concluded that the efficiency of transformation of these species is largely affected by the origin of the DNA and the recipient species. In particular, they observed that genes from *Sporobolomyces* cannot be transformed in *R. kratochvilovae* and *R. graminis*, but serve as selection markers in *R. sloffiae*. Conversely, genes from *R. graminis* can be transformed in all of these species, with the exception of *R. sloffiae*. According to this study, the possibility to use common selection markers in different species might be precluded by the marked differences in G+C content and in codon use preference. In agreement with this hypothesis, Liu et al. (2013) reported that ATMT of *R. toruloides* only succeeds when a codon-optimised version of the hygromycin phosphotransferase (*HYG*) gene is used, while no transformants were obtained when the original *HYG* gene was used. ATMT was subsequently used by Lin et al. (2014) for the integration of multiple genes into *R. toruloides* haploid and diploid strains, while at the same time, Takahashi et al. (2014) described electroporation of a cassette that integrated randomly into the *R. gracilis* genome. Finally, based on the assumption that targeted gene deletion is crucial to determine gene function, Koh et al. (2014) reported on the successful generation of *R. toruloides* *KU70*-deficient strains. *KU70* codes for a subunit of the KU heterodimer, a DNA-binding protein that is involved in non-homologous end joining. *KU70* deletion results in impairment of non-homologous end joining, with a consequent increase in the frequency of homologous recombination. Targeted gene deletion was obtained by transforming a *KU70*-deficient mutant with the set of gene deletion plasmid vectors reported in Table 2. ATMT was also used in this case, thus indicating that it is the favoured transformation method, at least for *R. toruloides*, although alternative methods are still under study.

The development of plasmid vectors for red yeast transformation (Table 2) and of DNA transformation methods is a prerequisite for the construction of genetically modified strains for the production of metabolites of interest. This has also been accompanied by the release of an increasing number of red yeast genome sequences. At present the whole genome sequences of *R. graminis*, *R. minuta*, and a few strains of *Rhodotorula* sp. and *Sporobolomyces* sp. are available at <http://genome.jgi-psf.org/>. The genome sequences of some strains of *R. toruloides*

Table 2 Vectors designed for genetic engineering of red yeast

Vector	Description		Source
	Backbone	Characteristics	
pAIS1	TOPO pCR2.1	<i>URA3</i> and <i>URA5</i> auxotrophic markers for genetic manipulation and gene replacement in <i>Sporobolomyces</i> sp.	Ianiri et al. (2011)
pAIS2			
pAIS3	pZPK-201BK		
pAIS4			
pEK627 and derivatives	TOPO pCR2.1	<i>car2::URA5</i> cassette for <i>CAR2</i> gene deletion in <i>Sporobolomyces</i> sp.	Abbott et al. (2013)
pPZP627 and derivatives	pZPK-201BK		
pRS2	pRS426	<i>URA5</i> auxotrophic markers of <i>Sporobolomyces</i> sp. for transformation of <i>Sporobolomyces</i> sp. and <i>Saccharomyces cerevisiae</i>	
pEKWU3	TOPO pCR2.1	<i>URA3</i> and <i>URA5</i> auxotrophic markers of <i>R. graminis</i> , for genetic manipulation of different red yeast species	
pEKWU5			
pPZWU3	pZPK-201BK		
pPZWU5			
pGI1	TOPO pCR2.1	<i>NAT</i> resistance marker under the control of β -tubulin promoter and terminator of <i>R. graminis</i> for transformation of different red yeast species	
pGI2	pZPK-201BK		
pRH203Pxxx-RtGFP	pPZP200	<i>GFP</i> under the control of <i>GPDH</i> promoter of <i>R. toruloides</i> for genetic manipulation of <i>R. toruloides</i>	Liu et al. (2013)
pEC3Pxxx-HPT3		<i>HPT-3</i> resistance marker under the control of <i>GPDH</i> promoter of <i>R. toruloides</i> for genetic manipulation of <i>R. toruloides</i>	
pPGK1E/rD/ME	pPICZ-E	<i>ME</i> under the control of <i>PGK</i> promoter of <i>S. cerevisiae</i> , and 5.8S rDNA and 26SrDNA of <i>R. glutinis</i> for homologous integration of the exogenous gene into the <i>R. glutinis</i> genome	Li et al. (2013)
PZPK-pPGK-HYG-Tnos	pZPK	<i>HPT-3</i> , <i>NAT</i> , and <i>BLE</i> resistance markers under the control of <i>GPDH</i> or <i>PGK</i> promoters of <i>R. toruloides</i> , for integration and expression of exogenous genes in <i>R. toruloides</i>	Lin et al. (2014)
PZPK-pGPD-HYG-Tnos			
PZPK-pGPD-NAT-Tnos			
PZPK-pPGK-BLE-Tnos			
pKOKU70	pEX2	Homologous sequences of target gene at each side of hygromycin selection cassette (<i>HPT-3</i> under the control of <i>GPDH</i> promoter of <i>R. toruloides</i>) for targeted gene deletion in <i>R. toruloides</i>	Koh et al. (2014)
pKOCAR2			
pKOSTE20			
pKOURA3			
pBKSTZP	pBluescript II KS(+)	<i>URA3</i> flanking regions and <i>Sh ble</i> resistance marker for targeted gene deletion in <i>R. gracilis</i>	Takahashi et al. (2014)

BLE bleomycin resistance marker gene, *CAR2* phytoene synthase and carotene cyclase encoding gene, *GFP* green fluorescent protein encoding gene, *GPDH* glyceraldehyde phosphate dehydrogenase promoter, *HPT-3* Hygromycin phosphotransferase encoding gene, *ME* malic enzyme encoding gene, *NAT* nourseothricin resistance marker gene, *PGK* phosphoglycerate kinase promoter, *Sh ble* zeocin resistance marker gene, *STE20* mating type specific gene, *URA3* orotidine 5-phosphate decarboxylase encoding gene, *URA5* orotate phosphoribosyltransferase encoding gene

(Kumar et al. 2012; Zhu et al. 2012), *R. glutinis* (Debarati et al. 2014) and *R. mucilaginosa* (Deligios et al. 2015) have been deposited at NCBI, and the draft genome sequence of another strain of *R. toruloides* has been deposited in the European Nucleotide Archive (Morin et al. 2014).

The availability of whole genome sequences of red yeasts is crucial for the analysis of the -omic datasets. As an example, due to the lack of genomic data for *R.*

toruloides in 2009, Liu et al. proceeded to identify proteins that were differentially expressed by *R. toruloides* during lipid accumulation, in comparison with the database of the yeast *Saccharomyces cerevisiae*. This strategy markedly hampered the impact of the investigation, as only highly homologous proteins were identified. Accordingly, only 184 proteins were identified, 46 of which were differently expressed during lipid production. In 2012, Zhu et al. re-

analysed the proteomic dataset obtained by Liu et al. (2009) in comparison with a protein dataset obtained from the assembly of the available genomic data of *R. toruloides*. They thus identified 3108 proteins, 538 of which were differentially expressed during lipid accumulation. The combination of proteomic and transcriptomic analyses led Zhu et al. (2012) to determine the central and lipid metabolism of *R. toruloides*, while also producing information that was useful for the study of the regulation of carotenoid biosynthesis. For instance, they reported that in *R. toruloides* under nitrogen starvation, ATP citrate lyase is highly expressed, while acetyl-CoA hydrolase and acetyl-CoA C-acetyltransferase expression is decreased. These results indicate that under nitrogen starvation, acetyl-CoA is deviated towards the production of fatty acids instead of isoprenoids, and it highlighted possible molecular targets to increase carotenoid production. Moreover, genome sequencing and annotation of *R. toruloides* led to the identification of two genes that code for carotenogenic enzymes: phytoene synthase and phytoene dehydrogenase (Zhu et al. 2012). These genes were recently isolated also from *R. diobovatum* (Guo et al. 2014, 2015). It was thus shown that a single gene of *R. diobovatum* (*crtYB*) encodes a bifunctional phytoene synthase/lycopene cyclase enzyme that is similar to that observed in *X. dendrorhous* (Verdoes et al. 1999), and in other fungal species. Similarly, the phytoene dehydrogenase of *R. diobovatum* showed significant similarities with these genes of other fungi (Guo et al. 2015).

Indeed, most of the studies carried out so far have mainly focussed on *R. toruloides*. However, other red yeast are also gaining increasing attention, as shown by Irazusta et al. (2012) and Wang et al. (2013). Their studies included proteomic analyses of *R. mucilaginosa* and *Rhodotorula taiwanensis*, respectively, to determine the cell responses to metal stress, and they produced two different protein datasets. At present, the number of proteins identified in these two yeast is relatively low, although the amount of information generated by these analyses is expected to increase upon the release of the genomic datasets for these yeast.

Concluding remarks

Yeast species ascribed to the genera *Rhodospiridium*, *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus* are characterised by marked taxonomic and phenotypic heterogeneity. Thus, although it is generally recognized that different stress factors and culture conditions can influence carotenogenesis, and can result in a diversity of carotenoid structures (Marova et al. 2004), much work is

still needed to understand how to enhance the biosynthesis of carotenoids. Indeed, due to renewed interest in these yeasts in view of their biotechnological potential and to the recent development of molecular and -omics tools, our knowledge of red yeasts is expected to increase. On the one hand, over the past 4 years, transformation, gene targeting, and random insertional mutagenesis have also started to be possible in some red yeast species. Even though these techniques are in their infancy, they can now be used for the production of collections of gene knock-out strains of great importance for our understanding of the function of single genes and gene networks, and for the management of carbon fluxes. On the other hand, the generation of proteomic data is leading to good coverage of the red yeast metabolic pathways.

The identification of genes coding for key enzymes in the carotenogenic pathway has recently been achieved in *R. toruloides* and *R. diobovatum*, although more will be done now with the increase in the number of red yeast genome sequences that are available. The information derived from these studies can then be coupled with further studies of the culture and environment conditions that induce carotenogenesis, the development of analytical tools for at-line quantification of carotenoids, and the development of functional genomics in these yeast. This combination can be expected to provide useful tools for optimisation of carotenoid production at an industrial level, and to convert these non-conventional yeast into convenient 'cell factories'.

Acknowledgments This study was partially supported by Regione Autonoma della Sardegna (LR7/07-2010; grant to I.M. and fellowship to S.L.). The authors are grateful to Benedetta Turchetti for valuable help in drawing the phylogenetic tree of red yeasts.

References

- Abbott EP, Ianiri G, Castoria R, Idnurm A (2013) Overcoming recalcitrant transformation and gene manipulation in Pucciniomycotina yeasts. *Appl Microbiol Biotechnol* 97:283–295
- Aksu Z, Eren AT (2005) Carotenoids production by the yeast *Rhodotorula mucilaginosa*: use of agricultural wastes as carbon source. *Process Biochem* 40:2985–2991. doi:10.1016/j.procbio.2005.01.011
- Amorim-Carrilho KT, Cepeda A, Fente C, Regal P (2014) Review of methods for analysis of carotenoids. *TrAC Trends Anal Chem* 56:49–73
- BCC Research (2011) The global market for carotenoids. <http://www.bccresearch.com/market-research/food-and-beverage/carotenoids-global-market-fod025d.html>. Published Sept 2011
- Bhosale P (2004) Environmental and cultural stimulants in the production of carotenoids from microorganisms. *Appl Microbiol Biotechnol* 63:351–361
- Bhosale PB, Gadre RV (2001a) Production of β -carotene by a mutant of *Rhodotorula glutinis*. *Appl Microbiol Biotechnol* 55(4): 423–427

- Bhosale PB, Gadre RV (2001b) β -Carotene production in sugar cane molasses by a *Rhodotorula glutinis* mutant. *J Ind Microbiol Biotechnol* 26(6):327–332
- Boekhout T (2011) *Bulleromyces* Boekhout & Fonseca (1991). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1391–1394
- Buzzini P, Martini A, Gaetani M, Turchetti B, Pagnoni UM, Davoli P (2005) Optimization of carotenoid production by *Rhodotorula graminis* DBVPG 7021 as a function of trace element concentration by means of response surface analysis. *Enzyme Microb Technol* 36(5–6):687–692
- Buzzini P, Innocenti M, Turchetti B, Libkind D, van Broock M, Mulinacci N (2007) Carotenoid profiles of yeasts belonging to the genera *Rhodotorula*, *Rhodospiridium*, *Sporobolomyces*, and *Sporidiobolus*. *Can J Microbiol* 53:1024–1031
- Buzzini P, Goretti M, Branda E, Turchetti B (2010) Basidiomycetous yeasts for production of carotenoids. In: Flickinger MC (ed) *Encyclopedia of industrial biotechnology: bioprocess, bioseparation, and cell technology*, vol 1. Wiley, New York, pp 469–481
- Cutzu R, Clemente A, Nobre B, Mannazzu I, Roseiro J, Lopes da Silva T (2013a) Assessment of β -carotene content, cell physiology and morphology of the yellow yeast *Rhodotorula glutinis* mutant 400A15 using flow cytometry. *J Ind Microbiol Biotechnol* 40:865–875
- Cutzu R, Coi A, Rosso F, Bardi L, Ciani M, Budroni M, Zara G, Zara S, Mannazzu I (2013b) From crude glycerol to carotenoids by using a *Rhodotorula glutinis* mutant. *World J Microbiol Biotechnol* 29(6):1009–1017
- Davoli P, Mierau V, Weber RWS (2004) Carotenoids and fatty acids in red yeast *Sporobolomyces roseus* and *Rhodotorula glutinis*. *Appl Biochem Microbiol* 40(4):392–397
- Debarati P, Magbanua Z, Arick M II, French T, Bridges SM, Burgess SC, Lawrence ML (2014) Genome sequence of the oleaginous yeast *Rhodotorula glutinis* ATCC 204091. *Genome Announc* 2(1):e00046-14. doi:10.1128/genomeA.00046-14
- Deligios M, Fraumene C, Abbondio M, Mannazzu I, Tanca A, Addis MF, Uzzau S (2015) Draft genome sequence of *Rhodotorula mucilaginosa*, an emergent opportunistic pathogen. *Genome Announc* 3(2):e00201–e00215. doi:10.1128/genomeA.00201-15
- Dufossé L (2006) Food grade pigments. *Food Technol Biotech* 44(3):313–321
- Fell JW, Johnson EA (2011) *Phaffia* M.W. Miller, Yoneyama & Soneda (1976). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1853–1856
- Fell JW, Johnson EA, Scorzetti G (2011) *Xanthophyllomyces* Golubev (1995). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1595–1601
- Ferrao M, Garg S (2012) Shake flask optimization of β -carotene production in *Rhodotorula graminis* RC04. *Afr J Biotechnol* 11(52):11431–11437
- Freitas C, Nobre B, Gouveia L, Roseiro J, Reis A, Lopes da Silva T (2014) New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodospiridium toruloides* NCYC 921 batch growth. *Proc Biochem* 49:554–562
- Guo W, Tang H, Zhang L (2014) Lycopene cyclase and phytoene synthase activities in the marine yeast *Rhodospiridium diobovatum* are encoded by a single gene crtYB. *J Basic Microbiol* 54:1053–1061
- Guo W, Liu Y, Yan X, Liu M, Tang H, Liu Z, Zhang L (2015) Cloning and characterization of a phytoene dehydrogenase gene from marine yeast *Rhodospiridium diobovatum*. DOI, A van Leeuw J Microb. doi:10.1007/s10482-015-0394-6
- Hamamoto M, Boekhout T, Nakase T (2011) *Sporobolomyces* Kluyver & van Niel (1924). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1929–1990
- Hernandez-Almanza A, Montanez JC, Aguilar-Gonzalez MA, Martinez-Avila C, Rodriguez-Herrera R, Aguilar CN (2014) *Rhodotorula glutinis* as source of pigments and metabolites for food industries. *Food Biosci* 5:64–72
- Herrero M, Cacciola F, Donato P, Giuffrida D, Dugo G, Dugo P, Mondello L (2008) Serial coupled columns reversed-phase separations in high-performance liquid chromatography: tool for analysis of complex real samples. *J Chromatogr* 1188:208–215
- Hu C, Zhao X, Zhao J, Wu S, Zhao ZK (2009) Effect of biomass hydrolysis by-products on oleaginous yeast *Rhodospiridium toruloides*. *Bioresour Technol* 100:4843–4847
- Ianiri G, Wright SAI, Castoria R, Idnurm A (2011) Development of resources for the analysis of gene function in Pucciniomycotina red yeasts. *Fungal Genet Biol* 48:685–695
- Irazusta V, Estévez C, Amoroso MJ, de Figueroa LJ (2012) Proteomic study of the yeast *Rhodotorula mucilaginosa* RCL-11 under copper stress. *Biometals* 25:517–527
- Irazusta V, Nieto-Penalver CG, Cabral ME, Amoroso MJ, de Figueroa LIC (2013) Relationship among carotenoid production, copper bioremediation and oxidative stress in *Rhodotorula mucilaginosa* RCL-11. *Process Biochem* 48:803–809
- Johnson EA (2013) Biotechnology of non-*Saccharomyces* yeasts—the basidiomycetes. *Appl Microbiol Biotechnol* 97:7563–7577
- Johnson EA, Echavarri-Erasun C (2011) Yeast biotechnology. In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts. A taxonomy study*, vol 1. Elsevier, New York, pp 21–44
- Johnson EA, Schroeder WA (1995) Microbial carotenoids. In: Fiechter A (ed) *Adv Biochem Eng Biotechnol* 53:119–178
- Kaiser P, Surmann P, Vallentin G, Fuhmann H (2007) A small-scale method for quantitation of carotenoids in bacteria and yeasts. *J Microbiol Method* 70:142–149
- Kirti K, Amita S, Priti S, Kumar AM, Jyoti S (2014) Colorful world of microbes: carotenoids and their applications. *Adv Biol* ID837891
- Koh CMJ, Liu Y, Moehninsi MD, Ji L (2014) Molecular characterization of KU70 and KU80 homologues and exploitation of a KU70-deficient mutant for improving gene deletion frequency in *Rhodospiridium toruloides*. *BMC Microbiol* 14:50
- Kumar S, Kushwaha H, Bachhawat AK, Raghava GPS, Ganesan K (2012) Genome sequence of the oleaginous red yeast *Rhodospiridium toruloides* MTCC 457. *Eukaryot Cell* 11(8):1083–1084
- Li Z, Sun H, Mo X, Li X, Xu B, Tian P (2013) Overexpression of malic enzyme (ME) of *Mucor circinelloides* improved lipid accumulation in engineered *Rhodotorula glutinis*. *Appl Microbiol Biotechnol* 97:4927–4936
- Lin X, Wang Y, Zhang S, Zhu Z, Zhou YJ, Yang F, Sun W, Wang X, Zhao ZK (2014) Functional integration of multiple genes into the genome of the oleaginous yeast *Rhodospiridium toruloides*. *Yeast* 14:547–555
- Liu H, Zhao X, Wang F, Li F, Li Y, Jiang X, Ye M, Zhao ZK, Zou H (2009) Comparative proteomic analysis of *Rhodospiridium toruloides* during lipid accumulation. *Yeast* 26:553–566
- Liu Y, Chong Mei JK, Longhua S, Mya Myintzu H, Minge Du, Ni P, Lianghui J (2013) Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RtGPD1 and development of genetic transformation method by dominant selection in oleaginous yeast *Rhodospiridium toruloides*. *Appl Microbiol Biotechnol* 97:719–729
- Mahmoud AGY, Abo-Shady MA, El-Sheekh MM, Hamza TW (2014) The role of some stress factors including hydrogen peroxide, methylen blue, sodium chloride and ultraviolet on *Rhodotorula glutinis* DBVPG# 4400 total carotenoids production. *Int J Biosci (IJB)* 4(9):10–19
- Maldonado IR, Rodriguez-Amaya DB, Scamparini ARP (2008) Carotenoids of yeasts isolated from Brazilian ecosystem. *Food Chem* 107(1):145–150

- Malisorn C, Suntornsuk W (2008) Optimization of α - and β -carotene production by *Rhodotorula glutinis* DM28 in fermented radish brine. *Bioresour Technol* 99:2281–2287
- Marcoleta A, Niklitschek M, Wozniak A, Lozano C, Alcaíno J, Baeza M, Cifuentes V (2011) Glucose and ethanol-dependent transcriptional regulation of the astaxanthin biosynthesis pathway in *Xanthophyllomyces dendrorhous*. *BMC Microbiol* 11:190
- Marova I, Breierova E, Koci R, Friedl Z, Slovak B, Pokorna J (2004) Influence of exogenous stress factors on production of carotenoids by some strains of carotenogenic yeasts. *Ann Microbiol* 54:73–85
- Marova I, Carnecka M, Halienova A, Koci R, Breierova E (2010) Production of carotenoid/ergosterol supplemented biomass by red yeast *Rhodotorula glutinis* grown under external stress. *Food Technol Biotech* 48:56–61
- Marova I, Certik M, Breierova E (2011) Production of enriched biomass by carotenogenic yeasts—application of whole-cell yeast biomass to production of pigments and other lipid compounds, biomass—detection, production and usage. Darko Matovic (ed), ISBN:978-953-307-492-4
- Marova I, Carnecka M, Halienova A, Certik M, Dvorakova T, Haronikova A (2012) Use of several waste substrates for carotenoid-rich yeast biomass production. *J Environ Manag* 95:S338–S342
- Martinez-Moya P, Niehaus K, Alcaíno J, Baeza M, Cifuentes V (2015) Proteomic and metabolomic analysis of the carotenogenic yeast *Xanthophyllomyces dendrorhous* using different carbon sources. *BMG Genomics* 16:289
- Mata-Gomez LC, Montañez JC, Méndez-Zavala Aguilar CN (2014) Biotechnological production of carotenoids by yeasts: an overview. *Microb Cell Fact* 13:12
- Morin N, Calcas X, Devillers H, Durrens P, Sherman DJ, Nicaud J-M, Neugebuse C (2014) Draft genome of *Rhodospiridium toruloides* CECT1137, an oleaginous yeast of biotechnological interest. *Genome Announc* 2(4):e00641-14
- Nishida H, Robert V, Sugiyama J (2011) *Mixia* C.L. Kramer emend. H. Nishida, K. Ando, Y. Ando, Hirata & Sugiyama (1995). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1499–1502
- Nishijima M, Araki-Sakai M, Sano H (1997) Identification of isoprenoid quinones by frit-FAB liquid chromatography–mass spectrometry for the chemotaxonomy of microorganisms. *J Microbiol Methods* 28:113–122
- Park PK, Kim EY, Chu KH (2007) Chemical disruption of yeast cells for the isolation of carotenoid pigments. *Sep Purif Technol* 53:148–152
- Petrik S, Marova I, Haronikova A, Kostovova I, Breierova E (2013) Production of biomass, carotenoid and other lipid metabolites by several red yeasts strains cultivated on waste glycerol from biofuel production—a comparative screening study. *Ann Microbiol* 63:1537–1551
- Provesi JG, Dias CO, Amante ER (2011) Changes in carotenoids during processing and storage of pumpkin puree. *Food Chem* 128:195–202
- Rapta P, Polovka M, Zalibera M, Breierova E, Zitnanova I, Marova I, Certik M (2005) Scavenging and antioxidant properties of compounds synthesized by carotenogenic yeasts stressed by heavy metals—EPR spin trapping study. *Biophys Chem* 116:1–9
- Razavi SH (2006) UV-HPLC/APCI/MS method for separation and identification of the carotenoids produced by *Sporobolomyces ruberrimus* H110. *Iran J Chem Chem Eng* 25:1–10
- Rodríguez-Amaya D (2001) In: Rodríguez-Amaya D (ed) *A guide to carotenoid analysis in foods*. ILSI Press, Washington DC
- Sakaki H, Nakanishi T, Tada A, Miki W, Komemushi S (2001) Activation of torularhodin production by *Rhodotorula glutinis* using weak white light irradiation. *J Biosci Bioeng* 92:294–297
- Sampaio JP (2011a) *Cystofilobasidium* Oberwinkler & Bandoni (1983). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1423–1432
- Sampaio JP (2011b) *Rhodospiridium* Banno (1967). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1523–1540
- Sampaio JP (2011c) *Rhodotorula* Harrison (1928). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1873–1928
- Sampaio JP (2011d) *Sporidiobolus* Nyland (1949). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1549–1562
- Sampaio JP, Oberwinkler F (2011a) *Cystobasidium* (Lagerheim) Neuhoﬀ (1924). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1419–1422
- Sampaio JP, Oberwinkler F (2011b) *Oocultifur* Oberwinkler (1990). In: Kurtzman CP, Fell JW, Boekhout T (eds) *The yeasts, a taxonomic study*. Elsevier, London, pp 1515–1518
- Somashekar D, Joseph R (2000) Inverse relationship between carotenoid and lipid formation in *Rhodotorula gracilis* according to the C/N ratio of growth medium. *World J Microbiol Biotechnol* 16:491–493
- Soroka IM, Narushin VG, Turiyansky YD, Tyurenkov AA (2012) Spectroscopy analysis for simultaneous determination of lycopene and β -carotene in fungal biomass of *Blakeslea trispora*. *Acta Biochim Pol* 59:65–69
- Squina FM, Mercadante AZ (2005) Influence of nicotine and diphenylamine on the carotenoid composition of *Rhodotorula* strains. *J Food Biochem* 29(6):638–652
- Takahashi S, Okada H, Abe K, Kera Y (2014) Genetic transformation of the yeast *Rhodotorula gracilis* ATCC 26217 by electroporation. *Appl Biochem Microbiol* 50(6):624–628
- Tinoi T, Rakariyatham N, Deming RL (2005) Simplex optimization of carotenoid production by *Rhodotorula glutinis* using hydrolyzed mung bean waste flour as substrate. *Proc Biochem* 40:2551–2557
- Tully M, Gilbert HJ (1985) Transformation of *Rhodospiridium toruloides*. *Gene* 36(3):235–240
- Ukibe K, Katsuragi T, Tani Y, Takagi H (2008) Efficient screening for astaxanthin-overproducing mutants of the yeast *Xanthophyllomyces dendrorhous* by flow cytometry. *FEMS Microbiol Lett* 286:241–248
- Vachali P, Bhosale P, Bernstein PS (2012) Microbial carotenoids. In: Barredo J-L (ed) *Microbial carotenoids from fungi: methods and protocols*. *Methods Mol Biol* 898:41–59
- Verdoes JC, Krubasik KP, Sandman G, van Ooyen AJ (1999) Isolation and functional characterization of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. *Mol Gen Genet* 262:453–461
- Wang SL, Sun JS, Han BZ, Wu XZ (2008) Enhanced β -carotene production by *Rhodotorula glutinis* using high hydrostatic pressure. *Korean J Chem Eng* 25(3):513–516
- Wang C, Wang CY, Zhao XQ, Chen RF, Lan P, Shen RF (2013) Proteomic analysis of a high aluminium tolerant yeast *Rhodotorula taiwanensis* RS1 in response to aluminium stress. *Biochim Biophys Acta* 1834:1969–1975
- Yen HW, Zhang Z (2011) Enhancement of cell wall growth rate by light irradiation in the cultivation of *Rhodotorula glutinis*. *Bioresour Technol* 102:9279–9281
- Zhu Z, Zhang S, Liu H, Shen H, Lin X, Yang F, Zhou YJ, Jin G, Ye M, Zou H, Zhao ZK (2012) A multi-omic map of the lipid-producing yeast *Rhodospiridium toruloides*. *Nat Commun* 3:1112. doi:10.1038/ncomms2112
- Zoz L, Carvalho JC, Soccol VT, Casagrande TC, Cardoso L (2015) Torularhodin and torulene: bioproduction, properties, and prospective applications in food and cosmetics—a review. *Braz Arch Biol Technol* 58(2):278–288