MINI-REVIEW

Rhodotorula sp.–based biorefinery: a source of valuable biomolecules

Cassamo U. Mussagy[1] · Helena F. Ribeiro[2] · Valeria C. Santos-Ebinuma[3] · Boelo Schuur[4] · Jorge F. B. Pereira[2]

Abstract

The development of an effective, realistic, and sustainable microbial biorefinery depends on several factors, including as one of the key aspects an adequate selection of microbial strain. The oleaginous red yeast *Rhodotorula* sp. has been studied as one powerful source for a plethora of high added-value biomolecules, such as carotenoids, lipids, and enzymes. Although known for over a century, the use of *Rhodotorula* sp. as resource for valuable products has not yet commercialized. Current interests for *Rhodotorula* sp. yeast have sparked from its high nutritional versatility and ability to convert agro-food residues into added-value biomolecules, two attractive characteristics for designing new biorefineries. In addition, as for other yeast-based bioprocesses, the overall process sustainability can be maximized by a proper integration with subsequent downstream processing stages, for example, by using eco-friendly solvents for the recovery of intracellular products from yeast biomass. This review intends to reflect on the current state of the art of microbial bioprocesses using *Rhodotorula* species. Therefore, we will provide an analysis of bioproduction performance with some insights regarding downstream separation steps for the extraction of high added-value biomolecules (specifically using efficient and sustainable platforms), providing information regarding the potential applications of biomolecules produced by *Rhodotorula* sp, as well as detailing the strengths and limitations of yeast-based biorefinery approaches. Novel genetic engineering technologies are further discussed, indicating some directions on their possible use for maximizing the potential of *Rhodotorula* sp. as cell factories.

Key points

● *Rhodotorula sp. are valuable source of high value-added compounds.*
● *Potential of employing Rhodotorula sp. in a multiple product biorefinery.*
● *Future perspectives in the biorefining of Rhodotorula sp. were discussed.*

Keywords *Rhodotorula* · Yeast · Biorefinery · Carotenoids · Lipids · Enzymes, Circular bioeconomy

Introduction

The term biorefinery is a broad concept that several authors also describe as a sustainable bio-based circular economy directly linked with the principles of bioeconomy (Leong et al. 2021). The biorefinery is focused on the 3Rs principle

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"Reduce, Reuse and Recycle" of the bioproducts, using bioprocesses with reduced carbon inputs to develop more biocompatible and sustainable technologies (Talan et al. 2022). Furthermore, a biorefinery integrates treatment and valorization of biomass for the efficient production of high added-value commodities, such as carotenoids, biofuels, bioplastics, chemicals, and additives (Leonov et al. 2021). Numerous biorefineries have been commercialized long ago, such as sugar, starch, and pulp and paper industries (Kiss et al. 2016), while in search for sustainable alternatives also new directions are under development, including productions of bio-based plastics (Voet et al. 2021) and bio-based solvents (Brouwer and Schuur 2020). In this sense, several microorganisms have been explored within a biorefinery and/or circular economy precepts (Karamerou et al. 2016).

The microbial technology appears as a helpful tool for the valorization of different wastes, namely, using of a wide range of microorganisms able to convert waste into high added-value compounds and the use of integrative platforms following the biorefinery fundamentals (Shahbazali 2013; Vanthoor-Koopmans et al. 2013; De Souza Mesquita et al. 2020). Microorganisms such as yeast, microalgae, and bacteria produce/accumulate significant amounts of metabolites, such as pigments, lipids, proteins, and polysaccharides, which can be exploited as "natural compounds" for commercial purposes (Wijffels and Barbosa 2010). However, it is clear that the microbial production, i.e., "microbial cell factory," of a single biomolecule is not as sustainable as desirable. First, there are high energy requirements and investment costs for implementing the whole industrial bioprocess and, secondly, significant operational costs related with the large volumes of water, nutritional specificity, and the impact of other processual conditions for microbial growth (Vanthoor-Koopmans et al. 2013). Together, these aspects make a single biomolecule-based biorefinery expensive and energy-intensive and, as a consequence, uncompetitive with traditional industries based on chemical and "extractive" processes (Mussagy et al. 2021c). Fortunately, the biorefinery concept emerged, in which, the use of microbial-based reaction is explored not only to obtain a single product, but a plethora of added-value molecules by recovering, separating, and fractioning all the compounds resulting from the microbial cultivation. A good example was reported by Adarme et al. (2022), who used a biorefinery to produce biogas using sugarcane by-products. In this particular work, anaerobic reactors fed with sugarcane by-products (hemicelluloses hydrolysate, sugarcane bagasse, among others) were used in the production of methane as the main product, reaching a yield of 243 NmL CH_4 g $CODr^{-1}$. The microbial community in the system was also evaluated, revealing structural changes and relationship between the main species. Another remarkable example is referred to the application of a novel bio-based route employing microalgae *Chlamydomonas* sp. using urban wastewater as a low-cost growth media to enhance the biomass production (Malik et al. 2022). In this work, 1.83 mg/g of carotenoids and 480 mg/g of lipids were obtained from the initial biomass and after the extraction procedure, the residual biomass of microalgae (75–100 g/L) was used as a sole feedstock to *Aspergillus* sp. (*niger* and *oryzae*) that biosynthesized α-amylase (131.6 U/mL) and mycoproteins (375–384 mg/g). The solvents used in all stages were recycled to the primary wastewater, leaving no waste at the end of the integrative process (Malik et al. 2022).

A microbial biorefinery is more than the cell growth and the biosynthesis of target-metabolites (i.e., upstream processing — USP), it also includes a series of subsequent downstream processing (DSP) units for separation, fractionation, purification, and formulation of the bio-based products (Elhami et al. 2022). In fact, DSP is responsible for the significant part of manufacturing costs for producing bioproducts (Malik et al. 2022). This is even more critical in bioprocesses where the added-value biomolecules are accumulated intracellularly, requiring adequate methods/technologies for cell disruption and their subsequent recovery, thus, increasing the energy and consumable requirements (Mussagy et al. 2019b; Schuur et al. 2019). Fortunately, bioprocessing platforms are being designed according to more sustainable and circular precepts, in which UPS and DSP units are now become properly integrated to reduce consumable and energy losses during the processing, to maximize profits by valorizing all by-products of the microbial processes (i.e., simultaneous production and fractioning of all metabolites) and, probably the most important, to enhance the "greenness" of the process and reduce the environmental impacts by using alternative eco-friendly and circular methodologies/technologies (Leonov et al. 2021; Talan et al. 2022). As a result, the use of one "microbial bioreaction" to obtain more than one valuable and marketable product will maximize the market value and reduce the costs of production of bioproducts, which is the case of yeast-based biorefineries (Norsker et al. 2011).

Oleaginous red yeasts, in which are included *Rhodotorula* sp., have been regarded as promising sources of natural colorants (i.e., carotenoids) (Kot et al. 2019a; Mussagy et al. 2019b), proteins (especially enzymes), and microbial oils (i.e., fatty acids, mono-, di-, and triglycerides) (Tkáčová et al. 2017; Kot et al. 2019a). These biomolecules have been pointed as valuable raw materials for the production of colored oils, biofuels, and biocatalysts, that are used to obtain valuable chemicals and food/cosmetic additives (Saenge et al. 2011a; Cheng and Yang 2016). Among the oleaginous red yeasts, *Rhodotorula* sp. appear as one of the most studied genus, since these strains are promising sources of: (i) pigments like carotenoids, for instance, β-carotene, torulene, and lycopene (carotenes)

and torularhodin or astaxanthin (xanthophylls), which are applied not only as colorants but also as powerful additives with provitamin A (β-carotene) and antioxidant activities (Mussagy et al. 2019a, 2021b, 2021a, 2022a); (ii) enzymes, such as invertase, epoxide hydrolase, and fructosyltransferase, which can be used as anti-microbial agents and antioxidant aids in the prevention of bacterial infestations, biocatalysts for organic synthesis, and in other food applications (Kronenburg et al. 1999; Rubio et al. 2002; Hernalsteens and Maugeri 2008); and (iii) lipids, such as fatty acids (C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), and C18:2 (linoleic acid)) (Ji and Huang 2019; Vasconcelos et al. 2019), which can be used as feedstock in the cosmeceutical (essential oils) and oleochemical industries (to produce biodiesel, glycerol, and biodegradable lubricating oils) (Saxena et al. 1998). These are some of the most representative examples of biomolecules produced by *Rhodotorula* sp., but it should be noted that even the whole yeast cells can be used as (bio)nutrient or (bio)pharming (e.g., for animal feed), (bio)pharmaceutical (e.g., nutritional supplements), (bio)fertilizer (e.g., plant growth), and (bio) chemical (e.g., alcohols or acids) sources, among others

(Hernández-Almanza et al. 2016; Kot et al. 2016; Mussagy et al. 2019b). A schematic representation of possible products that can be obtained in an integrated *Rhodotorula* sp. yeast-based biorefinery is depicted in Fig. 1.

As shown in Fig. 1, there are a multitude of bioproducts that can be obtained (directly or derived) from *Rhodotorula* sp., which makes this genus of yeast very promising to be used as a microbial producer in an industrial biorefinery. Despite several works published using *Rhodotorula* sp. (Cai et al. 2016; Tkáčová et al. 2017; Santos Ribeiro et al. 2019), as far as we know, there is still no industrial process using these microbial strains. The lack of commercial examples has been associated with the high processing costs that make difficult to commercialize *Rhodotorula* sp. bioproducts (Karamerou et al. 2016). In our opinion, the lack of large-scale knowledge, as well as limited studies of technical-economic and environmental assessment of yeast-based technologies, not well-established processes, and insufficient demand of yeast-based products, contributes negatively to the application of *Rhodotorula* sp. in biorefineries (Mussagy et al. 2020). We foresee a window of opportunity for the future *Rhodotorula* sp. biorefineries, but in order to become an industrial practice and reality, the following primordial
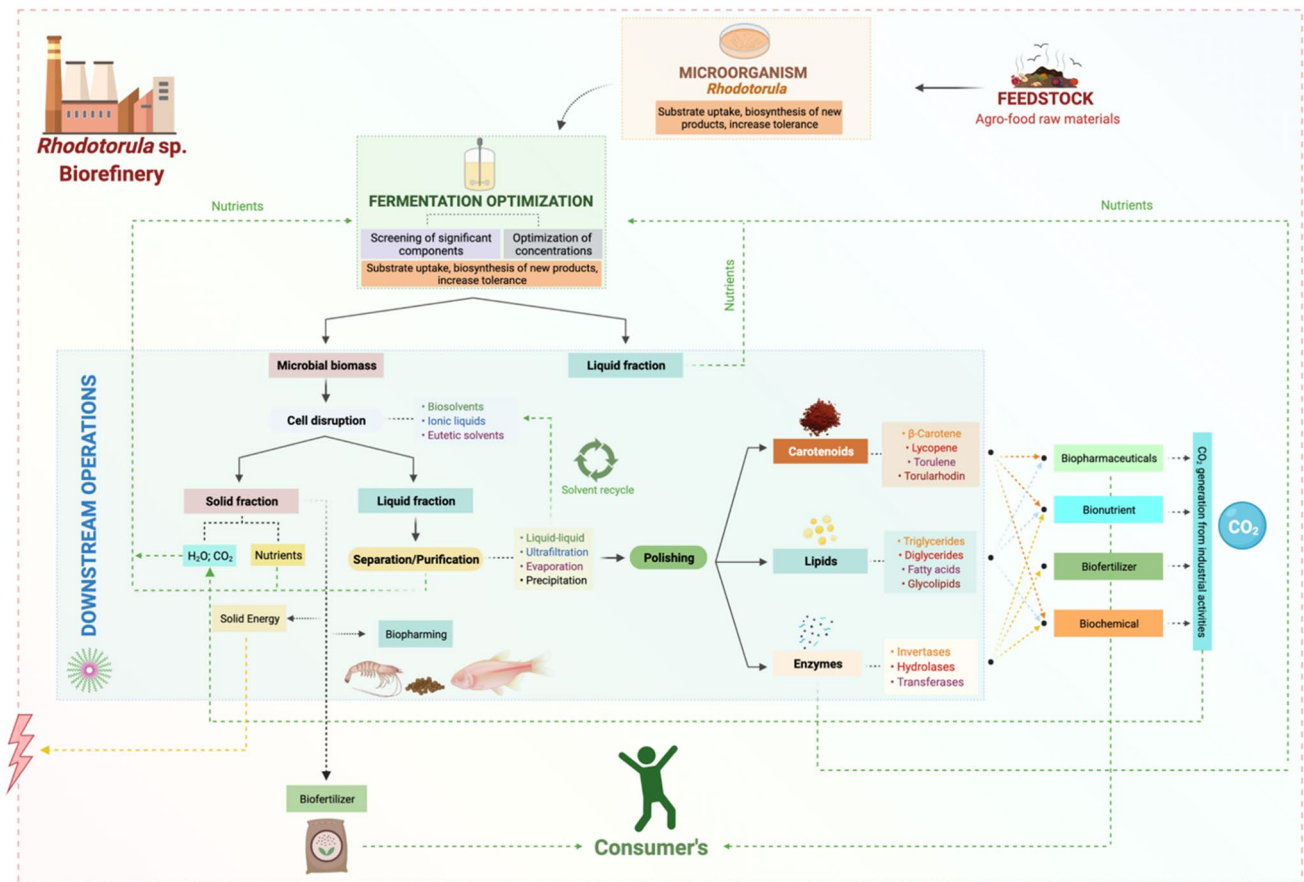

Fig. 1 Schematic representation of an integrated *Rhodotorula* sp.–based biorefinery plant process

factors must be evaluated and properly addressed in complete studies on a laboratory and pilot scale, namely, biorefinery cost-effectiveness; process and product environmental safety; process efficiency and productivity; and process recycling and circularity. These factors are obviously transversal and directly correlated, since it will only be possible to implement an economically and environmentally sustainable biorefinery if recycling, circular, and efficient technologies are considered in the design of the integrated biorefinery platforms. We believe that it is possible to design sustainable biorefinery, namely, using agro-food residues (or other wastes) as nutritional sources for cultivation of *Rhodotorula* sp. and production of added-value metabolites, which can reduce not only the raw materials costs but also contributing for waste valorization; designing integrated and circular DSP platforms using eco-friendlier (e.g., that using biocompatible solvents for extraction, isolation, and purification) and milder (e.g., with low energetic requirements and biocompatible with target molecules) technologies, which will decrease both the costs and solvents requirements (by proper reuse or recycling procedures). These aspects will overcome some of the current environmental and process drawbacks, providing more effective and economic solutions for industrial yeast-based biorefineries.

Taxonomy and morphology of genus *Rhodotorula*

The name *Rhodotorula* derives from the Greek *rhodos* (red) and Latin *torula* (feminine form of *torus-* protuberance) (Kot et al. 2016). The unicellular pigmented *Rhodotorula* genus is part of Fungi kingdom, division *Basidiomycota*, and family of *Sporidiobolaceae* (Kot et al. 2016) that can be naturally isolated from air, soil, human skin, stool, and food (Wirth and Goldani 2012). The genus *Rhodotorula* is considered a phylogenetically mix group with the other red yeasts from the genera *Sporobolomyces*, *Rhodosporidium*, and *Sporidiobolus* (Fell et al. 1984, 1995, 2000). The common species of the genus are characteristic shape of yeast, cf., spheroidal, ovoidal, or elongate, and the reproduction is asexually by multilateral or polar budding (Kot et al. 2016) (Fig. 2b–c). The opposite mating types in the species and some dikaryotic mycelium and chlamydospores have also been described (Harrison 1928).

The identification of *Rhodotorula* species based on phenotype has been described as problematic due to the irregular or unclear results of these procedures. In addition, there are also reports that some of their individual characteristics are highly influenced by cultivation conditions (culture media, pH, temperature, time, among others) (Fell et al. 2000). Molecular identification procedures such as ribosomal RNA gene, DNA-PCR, have been used (Pryce et al. 2003; Tiwari et al. 2021) for a precise identification of *Rhodotorula* species. As previously described by
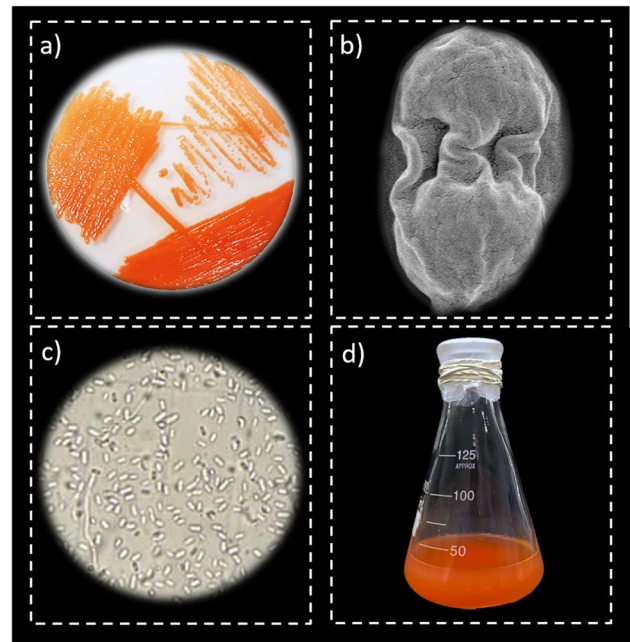

Fig. 2 Representative morphological characterization of *Rhodotorula glutinis* CCT 2186 strain. **a** Red–orange–colored colonies on YPD-agar medium after incubation for 48 h at 30 °C; **b** scanning electron microscopy (SEM) observed at 12,000×magnification; **c** micro-morphology image with rounded blastoconidias observed under 40×magnification; **d** biomass color at the end of the fermentation

Harrinson in 1928 (Harrison 1928), the member of *Rhodotorula* genus revealed the lack of fermentative ability and do not form starch-like compounds. Many strains are aerobic and mesophilic and have a mucous appearance due to the capsule formation, while others are pasty or wrinkled (Fell et al. 1984). The temperature for growing *Rhodotorula* species ranges from 18 to 30 °C (Kot et al. 2019a; Mussagy et al. 2021c). Typically, *Rhodotorula* sp. exhibit characteristic pigmentation that varies according to the species and growing conditions (Mussagy et al. 2019b, 2021c) (Fig. 2a, d). The red–orange pigmentation is attributed to the biosynthesis of large amounts of microbial carotenoids, responsible for the cell protection against the effect of singlet oxygen and radiation of UV–vis light spectrum (Hernández-Almanza et al. 2014).

Most species of the genus *Rhodotorula* are non-pathogenic, while certain species have been considered as emerging yeast pathogens, viz., *Rhodotorula mucilaginosa*, *Rhodotorula glutinis*, and *Rhodotorula minuta*, species that are frequently isolated from human infections. Due to their affinity for plastic materials, they can propagate biofilms in medical devices, cf., plastic catheters and/or dental materials (Lopes Damasceno et al. 2017; Kitazawa et al. 2018; Wang et al. 2019).

Biomolecules production using *Rhodotorula* sp.

Rhodotorula genus is capable to produce distinct added-value biological molecules, some of them with promising properties and commercial potential. *Rhodotorula* sp. are capable to use several compounds as sources of carbon, including glucose, molasses sucrose, whey lactose, galactose, maltose, ethanol, and glycerol (Kot et al. 2019a; Mussagy et al. 2021c). In addition, these microorganisms have the ability to grow using low-cost substrates, presenting tolerance to inhibitory compounds that may be present in these substrates (Hu et al. 2009). Besides that, the main representative characteristic of this genus is the lack of capability to carry out fermentation of sugars (Kot et al. 2016) and the ability to synthesize almost 50% (w/w) of their dry weight in lipids (Mussagy et al. 2021c). Due to its ability to metabolize, in reduced cultivation time, different carbon and nitrogen sources (including low-cost substrates) into added-value metabolites (carotenoids, lipids and enzymes), the *Rhodotorula* genus appears to be an interesting microbial strain to be exploited in the development of sustainable biorefineries. Below, the different strategies and factors that affect the biosynthesis of carotenoids, lipids, and enzymes, i.e., the most valuable compounds, produced by *Rhodotorula* sp. are briefly reviewed.

Carotenoids

The most common carotenoids naturally synthesized by *Rhodotorula* sp. are β-carotene, γ-carotene and torulene (corresponding to the class of carotenes), and torularhodin, which is a xanthophyll (Mata-Gómez et al. 2014; Mussagy et al. 2019b). The quantities and yields of carotenoids are not constant, mainly because the production efficiency is influenced by several factors, including the yeast strain, nutritional composition of the medium, pH, temperature, light irradiation, agitation, and aeration rate. Table S1 from Supplementary Material compiles several examples of studies that used *Rhodotorula* sp. to produce carotenoids, including the species strain, main cultivation conditions, and carotenoids' production yields.

From Table S1 from Supplementary Material, it is evident that the most important factors that affect the carotenoid production are carbon and nitrogen sources. Buzzini and Martini (Buzzini and Martini 1999) investigated the production of carotenoids using *R. glutinis* strains in media containing different carbon sources, achieving a maximum yield (5.95 mg/L of total carotenoids) when concentrated grape must was used as the sole carbon source. Under optimized conditions, the ratio of β-carotene, torulene, and torularhodin reached 9.3:9.4:78.9. Interestingly, when a glucose syrup medium was used, the *R. glutinis* yeast was unable to biosynthesize β-carotene. Under these conditions, the yield

of carotenoids was the lowest (1.80 mg/L), and the ratio of torulene and torularhodin was 10.8:85.0. Recently, Elfeky and co-workers (Elfeky et al. 2019) studied the effect of different nitrogen sources on carotenoid production, obtaining the highest value of total pigments with ammonium sulfate, followed by yeast extract and peptone. These authors also investigated the production of carotenoids at different C/N ratios, observing an increase of total pigments concentration with the decrease of C/N ratio.

There are studies indicating that the supplementation with metal ions is a good strategy to favor the carotenoids biosynthesis (Bhosale and Gadre 2001b; El-Banna et al. 2012; Elfeky et al. 2020). Elfeky et al. (Elfeky et al. 2019) studied the influence of different metals on carotenoid production using *R. glutinis*. The highest content of carotenoids (102.4 µg/g) was obtained for 1-mM $NiSO_4$ (an increase of 53% in relation to the control). Relatively to the individual carotenoid profile, the control group detected 63% of torulene and 30% of γ-carotene, while the addition of 1-mM $NiSO_4$ increase torulene production to 88.9%. Irazusta et al. (Irazusta et al. 2013) studied the effect of copper (Cu(II)) and hydrogen peroxide (H_2O_2) in the cultivation of *R. mucilaginosa* RCL-11. The copper supplementation increased carotenoid biosynthesis in this yeast (from 3.8 mg/L in control medium to 8.65 mg/L in copper supplemented medium), modifying at the same time the relative proportion of the pigments produced. The exposure to copper caused an increase in the β-carotene, torulene, and torularhodin production as well as the appearance of γ-carotene. The effect of hydrogen peroxide resulted in an increase of total carotenoid production (18.01 mg/L) compared to copper supplemented medium. The relative proportion of the pigments increased for the different compounds compared to copper supplemented medium, except for torularhodin which decreased from 57.37 to 28.89%, although with similar concentration in both culture media.

Temperature is another important parameter for stimulating yeast growth and corresponding production of carotenoids. Temperature affects the activity of the enzymes involved in the carotenoid's synthesis and, consequently, their composition and yields (Korumilli and Mishra 2014; Massoud and Khosravi-Darani 2017). For instance, Kot et al. (Kot et al. 2020) produced carotenoids at 20 °C and 28 °C. At low temperature, the highest carotenoid content (304.2 ± 17.9 µg/$g_{d.w.}$) was obtained after 120 h of culture, resulting in a production of 41.6% of β-carotene and 55.8% of torulene, while torularhodin was synthesized in negligible amounts. With increasing temperature, carotenoid content decreased to 260.3 ± 15.9 µg/$g_{d.w.}$, while torulene increased to 62.8% and β-carotene decreased to 34.5%. The content of torularhodin remained the same. Frengova et al. (Frengova et al. 1995) observed that low temperatures favored the production of carotenes, obtaining at 20 °C, a β-carotene

concentration of 19.0%, which is about 10% higher than that obtained at 35 °C (i.e., 9.6% of β-carotene). On the other hand, higher temperatures favored the biosynthesis of torularhodin (a xanthophyll). In that case, at 35 °C, the concentration of torularhodin was of 78.3%, contrasting with the 56.0% of torularhodin obtained at 20 °C. These results clearly demonstrate that increasing the temperature, the production of torularhodin is favored, while the concentrations of torulene and β-carotene decreased.

Other parameters, such as, pH, light exposure, and aeration, are also critical for maximizing the production of carotenoids. For example, Kot and co-workers (Kot et al. 2017) investigated how the pH of culture media (i.e., initial pH from 3 to 7) affects the concentration and type of carotenoids produced by *R. glutinis*. At pH 4–7, β-carotene (34.7–38.6%) and torulene (43.5–47.7%) were preferentially produced, with minor amount of torularhodin (12.1–16.8%). Under acidic conditions, pH 3, the torulene biosynthesis increased to 56.2%, torularhodin decreased to 4.5%, and β-carotene remained similar (36.8%). Similarly, Cheng and Yang (Cheng and Yang 2016) also observed a decrease in torularhodin biosynthesis by *R. mucilaginosa* at low pH. At pH 4, torularhodin content was 20.1, increasing to 36.0% when cultivated in a medium at pH 7. In addition to pH effect, light exposure has also been pointed out as a critical factor for stimulating carotenoids' biosynthesis. The increase of carotenoids results from the higher expression of genes that codes the enzymes involved in their biosynthetic pathway (Frengova and Beshkova 2009). A good example is the study of Sakaki et al. (Sakaki et al. 2001), in which the production of all carotenoids was intensified by light exposure, mainly, torularhodin that exhibited a twofold increase. Since *Rhodotorula* genus are aerobic, the adequate aeration is also critical to obtain high production yields (Frengova and Beshkova 2009). Tinoi et al. (Tinoi et al. 2005) evaluate the effect of

the shaking rate in the carotenoid production by *R. glutinis*, observing that low agitation rates reduced the cell growth due to the low availability of nutrients on the cells surface. However, these authors stated that an increase of agitation should be carefully performed, since high agitation rates can lead to cell membrane disruption.

There are other strategies for stimulating and tailoring the biosynthesis of carotenoids in *Rhodotorula* genus, such as the use of additives (different chemicals and solvents) as carotenoids' production intensifiers. For instance, the supplementation of culture media with 2% of ethanol results in an increase of β-carotene and torulene, limiting the torularhodin synthesis (Bhosale 2004). Saenge et al. (Saenge et al. 2011a) reported that the presence of Tween 20 increased the production of carotenoids (108.94 mg/L). Alternatively, the adjustment of incubation time can be used to adjust the carotenoids profile. A good example is the work of Mussagy et al. (2021c), in which *R. glutinis* yeasts were cultivated in a bioreactor for 72 h, achieving production yields of β-carotene (297.84 mg/L) and torularhodin (286.06 mg/L), with a lowest concentration of torulene (37.35 mg/L). However, increasing the cultivation time from 72 to 120 h, a full conversion of torulene to torularhodin was observed (Mussagy et al. 2021c). A completely different approach to adjust the production of carotenoids is the use of microbial consortia, instead of only using *R. glutinis* (Frengova et al. 1995, 2004; Buzzini 2001). Buzzini (Buzzini 2001) studied the production of carotenoids using a co-cultivation of *R. glutinis* DBVPG 3853 + *Debaryomyces castellii* DBVPG 3503, obtaining, after 120 h of cultivation, an increase of threefold of total carotenoids produced by consortia, in comparison with the single yeast cultivation (under same cultivation and nutritional conditions).

There are several factors that influence the ability of *Rhodotorula* sp. to produce carotenoids, particularly, in favoring

Fig. 3 Main (bio)process conditions to produce specific carotenoids by *Rhodotorula* sp
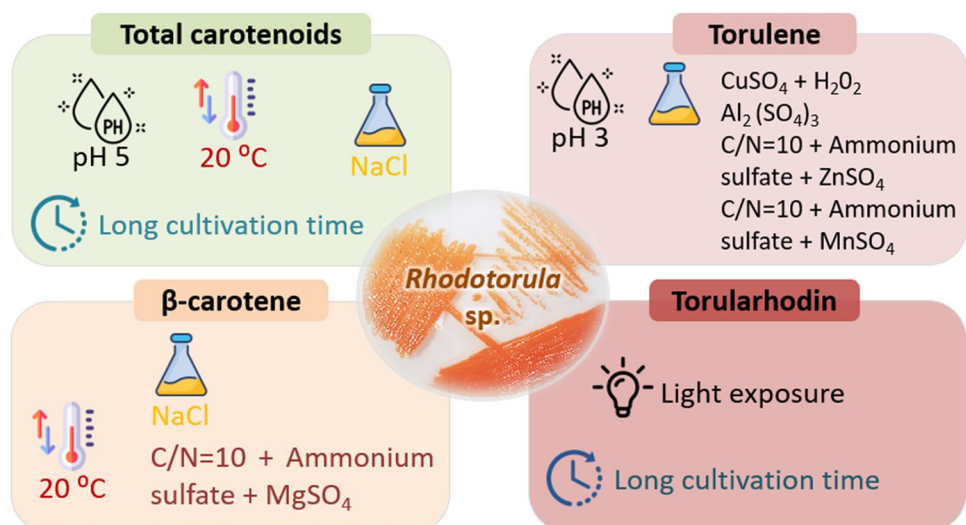
one or other carotenoids' biosynthesis pathway. Figure 3 summarizes the best conditions to maximize the production of total carotenoids. As can be seen, the presence of additives that induce oxidative cell stress, such as H_2O_2, NaCl, and/or metals, or the light exposure are key parameter for adjusting the carotenoid production (i.e., β-carotene, torulene, and torularhodin) and their corresponding composition profile.

The highest costs associated to the raw materials, chemicals, and technology of the bioprocess scale is the main limiting factor in the carotenoid production. The strategies for yeast cultivation should be improved, making the industrial production of carotenoids feasible. These strategies involve the optimization of the different factors influencing carotenogenesis, employing different statistical methods to optimize the use of low-cost substrates and making the production of these compounds more efficient and cost-effective.

Lipids

Rhodotorula sp. are oleaginous microorganisms considered promising candidates for the production of microbial lipids. Oleaginous yeasts can accumulate more than 20% of their biomass as intracellular lipids. The most abundant fatty acids produced by these yeasts are C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), and C18:2 (linoleic acid) (Ji and Huang 2019; Vasconcelos et al. 2019). These yeasts have as main advantages the shorter cultivation time, higher growth rate, substrate diversity, and high lipid productivity (Caporusso et al. 2021). Microbial lipids can be used as a feedstock to produce biodiesel, biolubricant, and jet fuel through different methods including enzymatic and chemical catalysis (Kot et al. 2016; Vasconcelos et al. 2019).

As with carotenoid biosynthesis, the production of lipids is influenced by several factors (cf., Table S2 from Supplementary Material), especially the C/N ratio, which is probably the most important (Mussagy et al. 2019a). At a low C/N ratio, the carbon flux is distributed to allow cellular proliferation, resulting in a large number of cells but a low lipid content. On the other hand, at a high C/N ratio, less growth is observed and, when cells run out of nitrogen, they cannot multiply, leading to the assimilation of excess carbon to produce storage lipids. In any case, it is important to note that despite the high lipid content in each individual cell, the overall lipid productivity is highly dependent on cell density, i.e., low productivity yields with low number of cells. Therefore, it is evident to make a balance between cell growth and lipid accumulation and, therefore, to determine the optimal cultivation condition for maximizing both parameters (Li et al. 2006). Saenge et al. (Saenge et al. 2011a) used glycerol and ammonium sulfate as C/N sources at a ratio of 35/85 for production of lipids by *R. glutinis*. The higher lipid content

was obtained using 9.5% of glycerol and a C/N of 85 (the highest C/N ratio studied). The production of lipids was carried out in a bioreactor using a medium with a C/N of 85 for 72 h of *R. glutinis* cultivation, reaching a lipid content of 60.7%, corresponding to a lipid yield of 6.10 g/L. Oleic acid (45.75%) and linoleic acid (17.92%) were found as the most abundant fatty acids. The type of carbon used as substrate also influences the production of lipids. Easterling et al. (Easterling et al. 2009) evaluated the effect of culture media containing different carbon sources, such as glucose, xylose, glycerol, glucose + xylose, glucose + glycerol, and xylose + glycerol, at an initial C/N ratio of 10, to produce lipids. They observed a variation of the lipid content with the different carbon sources, ranging from 10% using glucose and xylose, as main carbon sources, to 34% when using a mixed culture media containing glucose + glycerol.

As other microbial metabolites, the lipid production is highly affected by the pH of the cultivation media. Kot and co-workers (Kot et al. 2019a) investigated the effect of different initial pH (3–7) of culture media on production of lipids. Although the lipid content was similar in all assays (i.e., 10.2–12.7 g/100 $g_{d.w.}$), there was an evident change in the lipidic profile. In this case, oleic acid had the highest concentration at an initial pH of 3 (60%), whereas in the remaining cases (i.e., pH from 4 to 7), the amount of oleic acid decreased to 48.1–53.4%. Like pH, temperature is a processual parameter that influences not only yeast growth, but also the lipid production. Kot et al. (Kot et al. 2020) showed that by reducing the cultivation temperature from 28 to 20 °C, the lipid biosynthesis is favored. At the lowest temperature, oleic acid was the most abundant fatty acid, while the content of unsaturated fatty acids, mainly linoleic and linolenic acids, increased at the highest temperature.

Figure 4 summarizes the main conditions that allowed an increase in the production of lipids using *Rhodotorula*
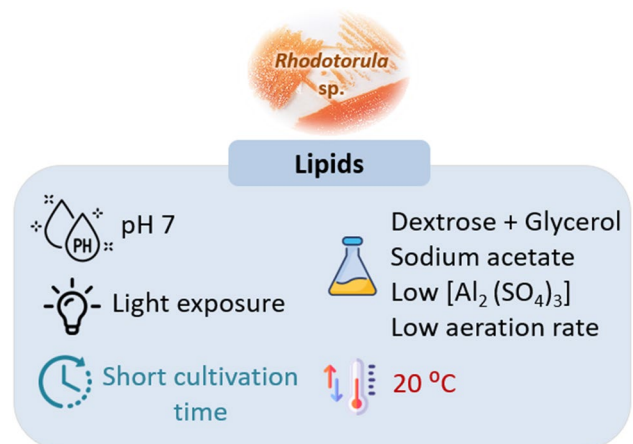

Fig. 4 Main (bio)process conditions to produce lipids using *Rhodotorula* sp

sp. Unlike carotenoid content, the lipid content was higher at neutral pH (7), with low or no metal concentration. Short cultivation times and low aeration rates, as well as the fed-batch cultivation were preferable for lipogenesis. Similar to the production of carotenoids, the lipid production using *Rhodotorula* sp. still needs to be optimized to reduce costs and increase yields and, consequently, turn these bioprocesses industrially competitive.

Other added-value compounds produced by *Rhodotorula* sp.

Carotenoids and lipids are the two main classes of biomolecules produced by *Rhodotorula* sp., but these yeasts can naturally synthesize other compounds with commercial value, namely, enzymes, exopolysaccharides, and sterols. A list of the other compounds that can be produced by *Rhodotorula* sp. is presented in Table S3 from Supplementary Material, detailing not only the main compounds, but also the process conditions corresponding to their production. Like other yeast species, *Rhodotorula* sp. can successfully produce several enzymes such as lipase, thymine hydroxylase, invertase, tanase, polygalacturonase, and phenylalanine ammonia lyase. The production of these enzymes occurs in bioprocesses under acidic conditions (pH of 4.5) or in the presence of different compounds, such as tannic acid, L-phenylalanine, or casein. For instance, Taskin et al. (Taskin 2013) demonstrated the co-production of tanase and polygalacturonase by free and immobilized cells of *R. glutinis* MP-10 isolated from tannin-rich persimmon fruits. Barbosa et al. (2018) reported the production of invertase in submerged cultivation of *R. mucilaginosa*. The highest invertase production rate was of 4.21 U/mL after 168 h in the extracellular environment, while the intracellular production was only of 23 U/mL after 72 h.

Few reports indicated the sterol and exopolysaccharide production using *Rhodotorula* sp. An example is the work of Marova et al., which reported a maximum ergosterol production of 83.46 mg/L using *R. glutinis* strain CCY 20–2-26 [188]. Hamadi and co-workers (Hamidi et al. 2020) reported the production of an exopolysaccharide (around 28.5 g/L) by *R. mucilaginosa* using a culture medium containing sucrose and ammonium sulfate. The exopolysaccharide was characterized as a highly branched beta-D-glucan having glucose and mannose residues (85:15 mol%, respectively) with an average molecular weight of 84 kDa.

Recovery and separation of biomolecules from *Rhodotorula* sp.

The production of most high added-value biomolecules (e.g., carotenoids, lipids, and enzymes) by *Rhodotorula* sp. is intracellular, requiring, after USP (production stage), a sequential series of cell disruption, extraction, and separation/purification units (DSP) for the effective isolation of biomolecules from yeast cells (Mussagy et al. 2019a; Mussagy et al. 2022a). Among the USP improvements needed to make *Rhodotorula*-based biorefinery a reality, the design and integration of the initial cell disruption/extraction operations are crucial for the efficient recovery intracellular biomolecules from complex yeast matrix. A good example of the complexity of these processes is the recovery of intracellular carotenoids. The extraction of carotenoids is strongly associated with the recovery of other intracellular biomolecules, i.e., lipids and enzymes, requiring an adequate process integration for guaranteeing a subsequent selective fractionation. In addition, it is crucial to consider the physical and chemical properties of the target intracellular molecule, since some of these biomolecules are sensitive to light, heat, oxygen, acids, and alkaline bases (Boon et al. 2010). Conventional extraction procedures employed in the recovery nonpolar carotenoids or lipids from *Rhodotorula* yeast biomass frequently uses nonpolar volatile organic compounds (VOCs) such as ethyl acetate, petroleum ether, and hexane (Hernández-Almanza et al. 2017; Mussagy et al. 2021c). For the recovery of enzymes, some polar solvents are usually employed, viz., acetone, dimethyl sulfoxide, and ethanol (Hernández-Almanza et al. 2017; Martínez et al. 2020a). The process intensification using mechanical-/physical-assisted procedures, like maceration or shaking (+ glass beads) with VOC-based extractions, is widely applied at lab scale, but other procedures involving the use of sonication, supercritical fluids, or high-pressure homogenization have been also studied (Saini and Keum 2018). Table S4 from Supplementary Material includes some examples of the most efficient techniques using VOCs applied in the recovery carotenoids from *Rhodotorula* biomass. Tkáčová et al. (2017) evaluated the simultaneous extraction of carotenoids and lipids from *R. glutinis*, and the maximum concentration of carotenoids and lipids recovered was 12.9 mg/L and ~ 6.5 g, respectively, using mixture of chloroform and methanol (2:1 v/v) combined with maceration (with sea sand) for 60 min. Similarly, Da Silva et al. (2020) evaluated the use of VOCs (dimethyl sulfoxide + chloroform + acetone (for carotenoids) and chloroform + methanol (for lipids)) combined with shaking + glass beads procedures (at 60 °C for 15 min) for the recovery of carotenoids and lipids from *R. mucilaginosa*. In this work, concentrations of 92.44 µg/g and 0.54 g/L for total carotenoids and lipids were achieved, respectively. Martínez et al. (2020b) applied an ultrasound-assisted extraction (UAE) (20 kHz, 96 µm amplitude, 200 kPa) for 3 min at 630 °C with water to recover carotenoids (267 µg/g$_{dw}$) from *R. glutinis*. The same authors also proposed the use of a more complex and innovative approach, involving the use of supercritical fluid extraction (SFE) with pure SC-CO$_2$ (50 MPa) (15 min, 80 °C) with ethanol as co-solvent for

the recovery of carotenoids (67.29 μg/g$_{dw}$) from *R. glutinis.* Another class of "green" solvents such as deep eutectic solvents (DES) and protic ionic liquids (PILs) have been fascinating the scientific community due to peculiar properties including the low toxicities and reduced adverse environmental effects. Recently, a pioneer work was performed by Mussagy et al. (2019a), in which the intracellular carotenoids were recovered from *R. glutinis* using a highly concentrated aqueous solution of ammonium-based PILs. PILs were effective for the recovery of 206.65, 112.82, and 17.21 μg/mL of β-carotene, torularhodin, and torulene, respectively. The use of DES for the carotenoid's recovery is quite new, for example, Da Costa et al. (2020) have reported the extraction of carotenoids (0.11 mg/mL) from *R. mucilaginosa* using DES composed of choline chloride and glycerol.

Additionally, the use of biocompatible and sustainable VOCs combined with simple procedures such as centrifugation (4,000 × *g*, 5 °C, 10 min) allowed the effective recovery of enzymes (930 U/mL) from *Rhodotorula* biomass (Hernalsteens and Maugeri 2008). Invertases (0.04 μkat/mg protein) were also recovered from *R. glutinis*, using ballistic disintegrator with acetic acid-sodium acetate buffer + 2-mercaptoethanol as extractant solvents (Rubio et al. 2002).

Since the mixtures obtained after cell disruption/extraction steps are complex, including not only the target biomolecule but also other added-value compounds, a proper fractionation and separation of these compounds are crucial for designing *Rhodotorula*-based biorefineries. The separation/fractionation processes of carotenoids, lipids, and/or proteins from extracts are generally complex and must consider the polarity and functional groups of each molecule, which determine its relative hydrophilic/hydrophobic character (Meléndez-Martínez et al. 2019). The separation methods are chosen according to the physical and chemical properties of the target compound, with chromatographic methods being the most used (Mussagy et al. 2021c). However, there are some examples using alternative approaches for the selective separation of intracellular molecules produced by *Rhodotorula* sp. For instance, after cell disruption, a precipitation (using cold acetone or ethanol) can be applied to separate enzymes from carotenoids and lipids, followed by a saponification unit using alkali treatment, to isolate carotenoids from lipids (Mussagy et al. 2020; Mussagy et al. 2022b). Depending on the final commercial application, other steps may be included using suitable high-resolution procedure for purification of specific carotenoids, lipids, or enzymes, which must be selected according to the nature of the "contaminants" and purity of the target compound. Sharma and Ghoshal (2021) carried out a chromatographic purification of β-carotene from torulene and torularhodin extracted from *R. mucilaginosa* using a nonpolar stationary phase composed of C$_{18}$-coated silica. Ungureanu et al. (Ungureanu et al. 2013) evaluated

the efficiency of centrifugal partition chromatography for the selective recovery of torularhodin from *R. rubra* extracts, achieving an extraction yield of approximately 91%, demonstrating also the potential of this procedure for specific carotenoids' purification. The purification of enzymes (cf., fructosyltransferase) from *Rhodotorula* sp. extracts was achieved using a sequential DSP, namely, cell disruption, centrifugation, followed by precipitation using ethanol precipitation, and purification by anion exchange chromatography (Hernalsteens and Maugeri 2008).

Summing up, the biorefinery or circular bioeconomy concept can be fully accomplished if a proper design and integration of upstream (production) and downstream (extraction and purification) procedures will be performed. Specifically, a good integration of all USP and DSP bio-fabrication steps is essential to minimize energy consumption and reduce the number of operation units and, consequently, the costs of the integrative platforms. Of course, it is important to note that process design must include operations to ensure complete recycling and reuse of solvents used in DSP, recovery of by-products, and polishing of target solutes, thus ensuring the economic viability of the biomanufacturing process. After each specific polishing step (e.g., lyophilization, spray-drying), purified fractions/formulations of carotenoids, lipids, and/or enzymes are obtained, which can be commercialized and applied in the nutraceutical, cosmeceutical, food, feed, and pharmaceutical industries.

Market opportunities and challenges for the implementation of a biorefinery based on *Rhodotorula* sp.

Carotenoids produced by *Rhodotorula* sp. have many applications and their global market is impressive. In 2019, it reached the value of 1.5 billion USD, and based on the expectations of experts, it should reach 1.8 billion USD by 2027, with a compound annual growth rate of 3.4% during the forecast period (Research 2022). Astaxanthin, β-carotene, capsanthin, lutein, lycopene, and canthaxanthin represent about 90% of the total market value of carotenoids. The most prominent sector is animal feed, accounting for about 41% of total revenue, followed by food and dietary supplements (Research 2022; Saini and Keum 2019). The ability of *Rhodotorula* sp. to produce oils with different lipid profiles should be also explored, as the production of oils with different profiles could be directed toward the production of different commercial products, such as biodiesel, vegetable oil substitutes, food additives, biopolymers, and pharmaceutical and cosmetic industries.

The examples above are representative of the expanding market for yeast-produced biomolecules, which can result in a significant improvement in the economy of these compounds and in the fulfillment of the biorefinery's expected

outcomes (Vasconcelos et al. 2019). Of course, there is still a lot of work to be done. As a first and key objective, it is to improve sustainability and the cost of the biorefinery process, that is, increasing the production yields of metabolites, optimizing production with low-cost raw materials (Karamerou et al. 2016), metabolic engineering for *Rhodotorula* strain improvement (Shanmugam et al. 2020), as well as designing more sustainable extraction and purification approaches (Mussagy et al. 2021b). Recently, synthetic biology and omics tools (genomics, proteomics, transcriptomics, and metabolomics) have been applied in the improvement of yeast-based production of different biomolecules (Horgan and Kenny 2011). Advances in genetic engineering technologies, such as the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene (Cas) system, have gained widespread attention for genetic engineering, providing an essential tool to rapidly integrating genetic modifications into non-conventional yeast strains, as opposed to the conventional genetic modifications performed using the cellular DNA repair system (Pi et al. 2018). So far, the only work reported in the literature on the use of CRISPR to *R. glutinis* engineering was developed by Pi et al. (2018) which focused on the transformation of β-carotene biosynthesis genes (*crtI*, *crtE*, *crtYB*, and *tHMG1*) and cellulase genes (*CBHI*, *CBHII*, *EgI*, *EgIII*, *EglA*, and *BGS*), achieving production yields of β-carotene (27.13 ± 0.66 mg/g) higher than the wild type, as well as including further cellulase activity. Since this technology is quite recent and underexplored, there are needed further works to optimize this technology, but it is already clear that significant advances in this fields will be obtained, being expected to save time on cell engineering and to reduce the complexity in the construction of plasmids and the cost (Shanmugam et al. 2020). These strain engineering improvements will be crucial not only to increase the production yields but also to increase the sustainability of industrial processes, especially by reducing the complexity of the DSP and the number of operation units required to obtain commercial-grade products using *Rhodotorula* yeasts as cell factories.

The development of new biotechnology strategies is very promising (Kot et al. 2016), but the high processing costs of these still limit commercial implementation for obtaining chemicals and other food-grade products on a large scale (Mussagy et al. 2019b). In addition, the genetic improvement of *Rhodotorula* sp. and, like other well-implemented yeast-based biorefineries, the optimization of the industrial fermentation are very important (Aksu and Eren 2007; Hernández-Almanza et al. 2014). The last one has a strong cost impact due to the need for large bioreactors, preservation of strain, aeration, pH control, and energy requirements (Karamerou et al. 2016; Mussagy et al. 2021b). Among the improvements in cultivation processes necessary to make

the industrial production of carotenoids, lipids, and enzymes a reality, the use of low-cost cultivation media appears as a critical factors for designing sustainable *Rhodotorula*-based biorefineries (Santos Ribeiro et al. 2019). As cited before *Rhodotorula* sp. are yeasts with high substrate versatility (Kot et al. 2016; Mussagy et al. 2021c) that can use several raw materials as substrates for the production of carotenoids (Braunwald et al. 2013), lipids (Saenge et al. 2011a), and enzymes (Kot et al. 2016). Lignocellulosic materials, such as sugarcane bagasse, rice straw, and corncob, are abundant and renewable in nature and regarded as a potential feedstock for microbial cultivation (Liu et al. 2015). Alternatively, the use of agri-food residues such as olive mill wastewater (Ghilardi et al. 2020), tomato waste (Chandi et al. 2010), or crude glycerol (Saenge et al. 2011a) have also been proposed as low-cost feedstocks.

Cai et al. (2016) proposed an integrated biorefinery process using *R. glutinis* P 14 for the production of biofuels, in which microbial lipid and bioethanol were co-generated using corn cob bagasse as feedstock. They showed, in the optimized condition, a production of about 131.3 g of bioethanol, while about 11.5 g of microbial lipids were co-generated from 1 kg raw material. Karthikeyan et al. (Karthikeyan et al. 2018) proposed a bio-strategy for food waste-valorization through the integration of *R. glutinis* cultivation and anaerobic digestion for bioenergy and fuel recovery. After several pre-treatments of food waste, the hydrolysates were used as culture medium for cultivation of *R. glutinis*, while the residual solids were subjected to anaerobic digestion, achieving maximal *R. glutinis* dry weight biomass (5.18 g/L) and total fatty acid contents (1.03 g/g $DW_{biomass}$). An interesting demonstration of simultaneous biosynthesis of lipids and carotenoids by *Rhodotorula* yeast strains using raw material was shown by Kot et al. (Kot et al. 2019a). These researchers demonstrated that *R. glutinis* cultivation in media with potato wastewater supplemented with 3% to 5% glycerol allowed the simultaneous production of carotenoids and lipids (i.e., 230 $\mu g/g_{d.w}$ and 15 $g/100 g_{d.w}$, respectively).

Regardless these examples for the simultaneous production of carotenoids, lipids, and enzymes using *Rhodotorula* sp. yeast, from our knowledge, there is no work using integrated platform for the industrial production of these metabolites. In our opinion, this is a result of the complexity and hardness of optimizing the production stage for simultaneous and efficient production of the three main biomolecules. In addition, studies with low-cost substrates are also lacking, requiring additional efforts to demonstrate the feasibility of production and subsequent pilot and large-scale implementation.

The development of efficient, simple, and sustainable cell disruption/extraction operations, which allow not only an effective cell wall rupture, but also the solubilization and maintenance of biological activities of intracellular solutes, is

also one of the main challenges of the process (Mussagy et al. 2021b). Currently, the recovery of carotenoids and lipids from *Rhodotorula* sp. is still not environmentally friendly, as the use of significant amounts of VOCs such as hexane, chloroform, methanol, petroleum ether, dimethyl sulfoxide, acetone, chloroform, and hexane are still the most applied (Park et al. 2007; Hernández-Almanza et al. 2014; Martínez et al. 2020a; Mussagy et al. 2021b). The use of VOCs, in addition to the low extraction efficiency, presents some undesirable economic and environmental impacts for the process; namely, many of these solvents have been shown to be highly toxic to the environment and the European Union start to implement stricter rules for their use, resulting in increased costs for storage and disposal, even downright prohibition (Alfonsi et al. 2008). In view of these environmental regulations and health concerns, the search for more biocompatible extraction methodologies has become imperative to ensure the sustainable development of yeast-based biorefineries (Martínez et al. 2020b). To replace non-ecological and toxic solvents with their benign equivalents, the chemical and biotechnological industries have been searching for "greener alternatives," including conventional bio-based solvents such as ethanol, ethyl acetate, isopropanol, and others (Mussagy et al. 2021b) that can be partially or fully derived from renewable sources, ionic liquids (Mussagy et al. 2019b), and non-conventional assisted methods, such as ultrasound-assisted extraction (UAE) (Martínez et al. 2020b), microwave-assisted extraction (MAE) (Chuck et al. 2014), and solvent-based pulsed electric fields (PEF) (Martínez et al. 2020b).

Finally, regardless of the final application of the biomolecule, all processes capable of generating new compounds need to overcome some of current industrial drawbacks, especially, implementing circular systems (De Souza Mesquita et al. 2020). In our opinion, the industrial implementation of *Rhodotorula*-based biorefinery will be a reality if the following factors will be considered as crucial for increasing the commercial viability of their metabolites: productivity, efficiency, recyclability, cost-effeciency, sustainability. These aspects will be crucial to guarantee both economic and environmental sustainability of the integrative process, maintain the biological properties of the target-compounds, and thus obtain high-quality and pure commercial bioproducts.

Final remarks

Rhodotorula species are increasingly reported in the literature for their biotechnology applications, especially, due to their ability to convert a wide range of carbon sources into valuable compounds including microbial lipids, carotenoids, enzymes, and biomass. Therefore, with these species biorefineries with economic performance and sustainability are considered feasible, bringing new environmental and social opportunities.

The production of microbial high-added value biomolecules has attracted rising interest from several industries as a sustainable alternative to substitute the synthetic counterparts, representing an expansion in the market. Regarding the potential of *Rhodotorula* sp., a few of the most comprehensive studies concerning the carotenoids, enzymes, and lipid production are presented herein. However, the applications of this genus revolve in more fields of the scientific community than those depicted in this paper, which recognize the potential of this yeast for the development of *Rhodotorula*-based bio-refineries. Recently, the genome of *R. mucilaginosa*, *R. glutinis*, and *R. graminis* species have been sequenced, providing for the industries strains an extended nutritional spectrum and well-known tolerance, particularly important for the biorefinery applications. The selection of the appropriate "raw material" for the production is an important aspect to be considered, evaluating the appropriate C:N balance and understanding the metabolic pathways in order to increase the economic turnover of a biorefinery concept. The development of biocompatible and sustainable downstream process platforms is also crucial to efficiently extract and polish the target compounds considering the complex chemical composition of *Rhodotorula* species. In this line, the next level of investigations should focus on the cost-effective continuous processing in laboratories and pilot-plant scale-up for the production of *Rhodotorula* biomolecules, due the great benefits of this technology including process control, enhanced productivity, solvent recycle, and improvement of quality and yields of final product. Finally, it is important to understand that the new biorefinery models using non-conventional yeasts such as *Rhodotorula*-based refineries are intrinsically associated to the sustainable progress goals set by a number of industries and nations, in order to reduce the emissions of CO_2 and also create new business opportunities for the companies and new jobs for the society.

Declarations

Ethics approval and consent to participate This article does not contain any studies involving human or animal participants conducted by any of the authors.

Conflict of interest The authors declare no competing interests.

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