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INGEGNERIA METABOLICA DEL LIEVITO ROSSO OLEAGINOSO *RHODOTORULA TORULOIDES* PER LA SOVRAESPRESSIONE DI GENI CAROTENOGENICI

METABOLIC ENGINEERING OF THE OLEAGINOUS RED YEAST *RHODOTORULA TORULOIDES* FOR CAROTENOGENIC GENES OVEREXPRESSION

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Abstract

La presente Tesi di Laurea Magistrale aveva l'obiettivo di incrementare la produzione di carotenoidi da parte di *Rhodotorula toruloides*. Definito "rosso" ed "oleaginoso", *R. toruloides* è un lievito appartenente al phylum dei Basidiomiceti che è in grado di accumulare al suo interno grandi quantità di lipidi e carotenoidi. Per le sue naturali doti di sintesi in relativa abbondanza di questi o altri composti con importante ruolo nutritivo e funzione benefica sulla salute umana., *R. toruloides* viene ritenuto una potenziale "fabbrica microbica" per la produzione industriale di proteine o biomassa microbica (SCP), olio (SCO), carotenoidi, polioli, o anche enzimi e alcoli grassi. Per una reale applicazione è pero necessario migliorare le sue prestazioni produttive attraverso l'utilizzo di strumenti di ingegneria metabolica e la contemporanea ottimizzazione dei suoi processi di coltivazione. Per tale motivo, in questo lavoro di ricerca sono stati preparati dei costrutti genetici che sovraesprimessero i geni eterologhi t-ylHMG1, mcCrtI e mcCrtYB, famosi per conferire un notevole aumento della sintesi di carotenoidi in specie microbiche simili. Solamente il ceppo SBY161, ottenuto dalla trasformazione di *R. toruloides* CCT 7815, qui SBY29, con il costrutto contenente t-ylHMG1, è stato caratterizzato per la sua migliorata fisiologia.

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Chapter first

INTRODUCTION

1.1 Why we need to change

Climate change is undeniable, and human influence on the climate system is clear: the greenhouse gases (GHG) due to anthropogenic emissions are the highest in history and its warming effect has linked to unprecedent, alarming changes, in particular the increase of average temperature, extreme heat, droughts, sea levels, heavy precipitation events and floods (IPCC Fifth Assessment Report, 2014).

Human-induced warming reached approximately 1°C above pre-industrial levels, increasing at 0.2°C per decade. The target established by the Paris Agreement is keeping the global warming to well below 2°C above pre-industrial levels while pursuing efforts to limit the temperature increase even further to 1.5°C (United Nations, 2015). If all anthropogenic emissions were reduced to zero immediately, the global-mean temperature will still raise in the future, but it would likely be less than 1.5°C. Without additional mitigation efforts, warming by the end of the century will lead to a very high risks of severe, widespread and irreversible impacts for people and ecosystems, that are much higher than the socio-economic challenges of mitigation and adaptation policies.

Over the last two centuries the society has been found its economy on the exploitation of fossil resources: initially, energy and chemical products were coming from coal; later, the discovery of petroleum and natural gas as a cheap and abundant feedstock for the fuel and petrochemical industry has driven the evolution of an indefinite range of commodities, products, and services that we enjoy today (Stevens & Verhe, 2004). The impulsive consumerism that characterizes the modern style of living was embraced as normal way of life, leading to the current situation of land resources depletion, ecosystems destruction, ocean plastic waste pollution and extreme GHG emissions.

Today, the energy and industry sectors accounts for almost 80% of global GHG emissions: transportation (especially road transport), manufactory (from chemical to several other industries) and energy used in buildings provide the largest portion (Ritchie et al., 2020). Agriculture, forestry, land use and waste together represent the remaining 20% or so: breeding, soil fertilization and burning crops are considered the most detrimental practices. Thus, withdrawing from fossil fuels should be our top-priority. Within the energy sector, even if electricity production was 100% renewable (today less than 37%), decarbonization of heating and transport would be much harder because of their heavily reliance on fossil fuel. But even if we could overcome these issues and stop emissions from non-food

sectors right now, the food production system alone would bring us beyond 1.5°C by 2050 to almost the limit of 2°C by 2100, leaving out the CO_2-warming equivalent of just over one year of current fossil fuels emissions for all non-food sectors (Clark et al., 2020). Ignoring alternatives to our diet and neglecting modification to the present food production chain are also not options.

To hope for a real change, we need to reconsider completely our relationship with the planet, but while we are witnessing growing awareness in community and media, this will not occur in a short time. We need to evolve plenty of solutions to decarbonize the economy and stop climate change, because GHG emissions involve many sectors, and focusing on one alone is not enough. The strategies for a sustainable development aim for the reduction to "net zero emissions" of GHG by 2050 and must integrate effective governments, innovative infrastructures, renewable technologies and more environmental-friendly lifestyle and behaviour (Fankhauser et al., 2022). In that regard, implementing the concepts of biorefinery and circularity into our current production system is essential to fulfil United Nation's sustainability goals (Odei Erdiaw-Kwasie & Monirul Alam, 2023).

1.2 Circular bioeconomy and biorefinery

To achieve sustainable while profitable businesses, the energy and industry sectors must re-evaluate their production processes to adopt the perspective of "circular bioeconomy" (CBE), where the ideas of waste recycling and reuse of by-products are applied together with the employment of biomass as renewable carbon and/or energy source (Ubando et al., 2020). An organizational synergy within the industrial network proposed by CBE aims to tackle social, economic, and environmental issues brought by the current model by reducing waste and raw material generation and minimizing resource consumption, while containing environmental footprint and realizing the conversion of biomass to various high-value bio-based products. In this context, the "biorefinery" concept is an essential mechanism for the expression of a CBE (Cherubini, 2010).

Biorefinery was early defined by the International Energy Agency (IEA) as « the sustainable processing of biomass into a spectrum of marketable products and energy ». A more specific and restricted formulation was later given by the EU Biorefinery Outlook to 2030 (2021), a project study designed to support and facilitate the achievement of a more sustainable development using CBE strategies, where biorefining is termed as a « processing of biomass into a portfolio

of marketable bio-based products, which could include co-production of food and feed, chemicals and materials, and bioenergy ».

Biorefinery system relies on four category groups that describe its core mechanism: feedstocks, platforms, conversion processes and products (Annevelink et al., 2022). In a biorefinery facility, initially a biomass “feedstock” undergoes a specific pre-treatment (primary biorefining) to produce a biocommodity “platforms”, which are later transformed with integrated “conversion processes” (secondary biorefining), including mechanical, thermochemical, chemical, biological and biochemical operations, to generate various bio-based “products”, such as biofuels and bioenergy, bioplastics and biomaterials (composites, fibres, polymers, resins, paints and coatings), biofertilizers (agrochemicals and organic fertilizers), building blocks and other biochemicals (lubricants, solvents, surfactants), food and feed (bionutrients and nutraceuticals, additives, flavours), biocosmeceuticals, biopharmaceuticals (phytochemicals, polyketides and other bioactive compounds, vitamins and antioxidants, vaccines and antibiotics) and other high-value bio-products (enzymes and catalysts).

The classification of biorefineries depends on which of the four categories is considered. According to the types of feedstocks, we can distinguish three generations of biorefineries:

- 1st-generation: it comprises food crops biomass composed of primarily starch/sugars and edible oils; for examples, corn and sugarcane biorefineries for bioethanol production by the model yeast *Saccharomyces cerevisiae*, and rapeseed, soybean, and palm oil for biodiesel by chemical processes, are long-standing biofuel and biochemical industries in some countries (Wagemann & Tippkötter, 2019).
- 2nd-generation: it consists of lignocellulosic biomass of wood, wood residues and grass from forestry, industry and perennial energy crops, and of straws/stovers as non-edible residues from food crops, but it also includes non-homogenous low-valued municipal and industrial solid wastes; biogas, syngas and bioethanol productions from wastes and crop residues are diffuse and settled practises, but biomanufacturing of high value-added products using lignocellulosic substrates is still largely in pilot-scale and pre-commercialization stages (Ho et al., 2014; Yang et al., 2022).
- 3rd-generation: it involves the direct utilization of C1 gases (mainly CO_2 and methane) as feedstock exclusively by microorganisms; photoautotrophic

microalgae- and acetogenic bacteria-based biorefineries have recently entered the commercialization stage to produce biofuels, dietary supplements, food ingredients and other high-value products (Z. Liu et al., 2020).

The original objective(s) of a biorefinery could be manufacturing of valuable bioproducts, functional utilization of primary feedstocks or some by-products, or profit from long-established research on microbial producer strains. A prior technical and socio-economic analysis of project design is essential to establish a successful and sustainable biorefinery industry (S. Y. Lee & Kim, 2015).

Biofuels as well as bio-based chemicals and products such as bioplastics offer real advantages in terms of environmental, economic, and social sustainability, mainly in the form of reduction in GHG emissions, rural development, and energy security (Jeswani et al., 2020). But, on the other hand, there are drawbacks that are not always acknowledged and come from potential competition for land and water use with food crops, and consequent increase of food price, but especially from risks of unaccounted emissions from land-use change (LUC) and its degradation. Considering these concerns, biorefineries employing the 1st-generation feedstocks as renewable biomass are not considered sustainable alternatives in the longer term to fossil-based industry (Sheldon, 2014). On the other hand, the 2nd- and 3rd-

generation feedstocks drew attention of scientific community because they could avoid some of the above issues.

Besides analysis of ecosystem value and natural resources to optimally exploit land, biorefinery has also to carry out an evaluation of sustainable applications. Biomass is made of unique, complex chemical structure and composition which is extremely complicated to obtain synthetically, and its transformation to biofuels as biorefinery product might not be an optimal utilization (Budzianowski, 2017). Additionally, biofuels will have to compete with renewable energies of solar photovoltaic and wind, which are generally more efficient in terms of energy conversion, land use and GHG emissions, and their costs have fallen even below fossil-based energies (Williams et al., 2015). However, neglecting biofuels as renewable energy option is not always more ecological (Sajid et al., 2022). In fact, generation of bioenergy such as biofuel, biopower and bioheat combined with production of higher-value molecules from same feedstock for a complete valorisation of biomass could be essential to achieve feasible biorefinery business model in a more sustainable and integrated socio-economic system (Pickering et al., 2022).

Biorefinery industry could be the key solution to shift the paradigm of economic growth toward a green growth and meet the requirements of CBE, particularly

through the biological transformation carried out by microbes that recover their feed and energy from urban solid waste, wastewater, wood and agro-waste, or industrial and atmospheric C1 gases to recycle them into nutrient-rich food sources or other sustainable biochemicals. Nonetheless, microorganisms are not naturally adapted to quickly integrate nutrients from renewable substrates and efficiently generate desired molecules, and modification of their metabolism is required to turn them into robust biocatalysts (Cho et al., 2022).

1.3 Yeast as microbial cell factory

The promising prospects of biorefinery rely greatly on the optimization of microbial "fermentation", in its broader term of application of microbes for industrial production of target molecules, as main character of conversion process, and particularly systems metabolic engineering for strain development of microbes assumes a central position (J. A. Lee et al., 2023). This emerging discipline, which combines the scientific knowledge of traditional metabolic engineering with systems biology, synthetic biology, evolutionary engineering fermentation and downstream processes, aims to design microbial "cell factories", that are microorganisms modified to acquire new desirable features, for instance optimal production performances (yield, titre and productivity) for a new,

characteristic, or multiple products, inhibition of by-products and/or toxic products, increasing tolerance to environmental stresses and medium inhibitors, secretion of target compounds, utilization of a wider range of substrates, genetical stability over many divisions or resistance to infections (Teng et al., 2021; Gustavsson & Lee, 2016). Efforts on these biotechnologies have allowed to produce on industrial scale several hundreds of microbial-based chemicals and biochemicals commercially available today, opening up the perspective of so-called "precision fermentation", in which microorganisms are developed by metabolic engineering into microbial cell factory to yield large quantity of pure high-value products in a more sustainable process (Chai et al., 2022).

The selection of a candidate microbial host should be based on factors such as utilization of desired carbon substrates and their transformation performances, short generation time, low nutritional requirements, rapid fermentation to avoid contamination risks, ease of metabolic engineering and scaling-up operations, good tolerance to several stresses, and efficient downstream process. Although model organisms like *Escherichia coli* and *S. cerevisiae* have a wider range of available tools for genetic manipulation, sometimes it is more reasonable to employ other species because of their natural ability to consume certain feedstocks and beneficially synthesize valuable biomolecules. Ultimately, latest advances in

the field of systems and synthetic biology have finally shortened time to develop valid molecular techniques and allowed therefore to efficiently use more and more "non-conventional" microorganisms as microbial cell factory (Branduardi, 2021). Among microbial cell factories, yeasts have the longest biotechnological tradition. These microorganisms are considered the first ones to be used by humans, with fermentation process of foods and alcoholic beverages dating back to 10.000 BC (Taveira et al., 2021). Although overtaken by bacteria and filamentous fungi in leading advancement of bioprocess technologies in the 20th century while yeast metabolism started to be elucidated about hundred years before, nowadays yeasts are considered more attractive host cells for modern industrial biotechnology than bacteria because of their stability in different environmental conditions, ease of purification processes, absence of phage contamination, and eukaryotic post-translational modification (Mattanovich et al., 2014; Kim et al., 2020). Thus, selected yeast strains are now being designed to produce at large scale not only ethanol from fermentation of simple sugars as the traditional model yeast *S. cerevisiae*, but numerous native and non-native chemicals as platforms for a wide range of bio-products by consumption of alternative substrates (Wu et al., 2017). Yeast fermentation is certainly the main metabolic pathway for fundamental applications like food manufacturing (wine, beer, liquors, and baked goods) and

bioethanol production, and cultivation in anaerobic conditions is known to induce an increase in the synthesis of organic acids and recombinant proteins (enzymes, hormones, antibodies, vaccines, or antibiotics) in Crabtree-negative yeasts like methylotrophic *Komagataella phaffii* (formerly *Pichia pastoris*) and *Ogataea polymorpha* (Baumann et al., 2011). However, many yeasts are capable of functioning in aerobic conditions as well. Normal respiratory metabolism favours yeast cell biomass production in the application of single-cell proteins (SCP), but it also allows formation of metabolites like cytosolic acetyl-CoA or tricarboxylic acid (TCA) cycle intermediates served as precursors for production of lipids in the application of single-cell oils (SCO), terpenoids (e.g., carotenoids), polyketides, fatty acids-derived chemicals (used for examples as emulsifiers, plasticizers, surfactants, and lubricants), and other high-value chemicals (Żymańczyk-Duda et al., 2017).

By 2050, world's population is projected to reach 9.7 billion people from the current 7.9 (United Nations, 2022). Most of the people will inhabit urban areas and great challenges will arouse from feeding them and disposing their wastes (FAO, 2017). Expanding the present intensive agriculture and animal farming to compensate for growing population is not environmentally sustainable for our planet. Half of the world's habitable land is intended to agriculture, and three-

quarters of this is used for livestock (pasture and fodder crops), which accounts for just 37% of global protein and contributes to the GHG emissions (CO_2eq/100 g of proteins) and land use (m^2/100 g of proteins) far more than plant-based food when same amount of these proteins are compared (Poore & Nemecek, 2018). Furthermore, despite meat has high nutritional value, it has been recently classified as carcinogenic to humans, and its excessive consumption is linked to cardiovascular diseases and several common conditions (Bouvard et al., 2015; Papier et al., 2021). In the remaining land left for food crops, 30% is used to grow vegetable oil crops, mainly palm oil, soybean, and rapeseed, whose increase in size tripled over the last half-century is linked to deforestation and biodiversity loss, and the simultaneous rise in consumption of their oils is correlated to several adverse health effects such as cardiovascular disorders and male infertility (Meijaard et al., 2020; Dinicolantonio & O'Keefe, 2018; Reza Safarinejad & Safarinejad, 2012).

As a result, the production of microbial biomass as alternative source of food is probably receiving the greatest attention from research and industry (Ciani et al., 2021). Over the years, microbes have been selected from their immeasurable biodiversity for the natural properties to accumulate proteins or oils in high content along with collection of important nutraceuticals such as vitamins, minerals, and

antioxidant pigments. Since microbial average protein/lipids content is higher than traditional food, especially compared to land use, and their overall controlled cultivation requires less energy and water consumption, fewer GHG emissions, no pesticides or antibiotics, and has also more efficient nutrient utilization and no sensitivity to climate change, these SCP and SCO strains are now being advanced as the new superfood capable to extend the food market and eventually to replace completely the present unsustainable food production, especially of meat and dairy products.

In this sustainable view of future, yeasts will consequently play a vital role. If algae and bacteria are being optimized to use municipal wastes and 3rd generation resources as feedstock, yeasts can be domesticated to make the most out of low-value lignocellulosic materials, which could be derived from rational forest management, sawmill residues, agro-industrial waste, maintenance of urban greenery, pulp and paper industry by-products, or woodworking factories scrapes. Terrestrial plants store energy as carbon via photosynthesis from the sun 10 times the world usage, and every year they produce an amount of new biomass equal to about two-thirds of global energy demand, a significant portion of which can potentially be destined for the biorefinery industry (Demain et al., 2005). Lignocellulose is made up of cellulose (35–50%), hemicellulose (23–32%) and

lignin (15–30%), thus comprising a relevant carbohydrate portion (sometimes more than 80% of the dry mass) that can be utilized by microorganisms to yield convenient products (Y. Liu, Tang, et al., 2021). But lignocellulosic biomass is naturally recalcitrant to microbial degradation due to the molecular architecture of its components and their complex interactions, so it requires physical and/or chemical pre-treatment processes (Kim et al., 2021). At this point, only some bacteria and filamentous fungi are capable to efficiently degrade the resulted lignocellulose. Instead, saccharification by enzymatic hydrolysis can be carried out to free simple sugars for yeast applications. Since these sugars consist predominantly of glucose (60-70%) and xylose (30-40%), yeast strains that consume both monosaccharides are preferred in order to maximize the conversion of lignocellulosic biomass (Jin et al., 2015). Metabolism of xylose is not native or highly inefficient to many producer strains, so they need to be engineered to utilize this C5 sugar (Jagtap & Rao, 2018). Among native xylose-utilizing yeasts, *Rhodotorula toruloides* is a well-known oleaginous yeast that has recently emerged as a model organism for lipid production (Wiebe et al., 2012).

1.4 The oleaginous red yeast *Rhodotorula toruloides*

Due to the adaptation to a wide range of temperatures (8-37°C) and pH (2-12), and to high concentrations of toxic metals, the basidiomycetous yeast species *R. toruloides* can be isolated from seawater to soil and plant materials, or even in extreme environments like acidic mine waters and hydrothermal vents (Sampaio, 2011; Z. Liu et al., 2021; Gao et al., 2019). Formerly known as *Rhodosporidium toruloides*, it is now classified into the *Rhodotorula* genus, *Sporidiobolaceae* family, *Sporidiobolales* order, *Microbotryomycetes* class and *Basidiomycota* division (Wang et al., 2015). Most of its strains are heterothallic, dimorphic yeasts: after the conjugation of two opposite mating-type spore cells (e.g., A and a), clamp-connected hyphae develop with terminal and/or lateral teliospores; the germination of basidia from teliospores produces haploid or diploid basidiospores, whose reproduction by budding, namely the yeast phase, represents the biotechnological stage of present microbial cell factories (Banno, 1967; Abe & Sasakuma, 1986).

Oleaginous yeasts, like *R. toruloides*, are microbial species that are capable of synthesis and accumulation of over 20% w/w of lipids on a cell dry weight (Ratledge & Wynn', 2002). Under optimized growth conditions and/or as a consequence of genetic improvement, the concentration of lipids can exceed even

70% (Q. Li et al., 2008). Early comparisons study of different oleaginous microorganisms, including yeasts, fungi, bacteria, and microalgae, have shown faster growth rate and higher lipid content for oleaginous yeasts, and *R. toruloides* can provide larger biomass and higher lipid yield and content than other important oleaginous yeasts such as *Yarrowia lypolytica* and *Lipomyces starkeyi* (Mota et al., 2022).

Oleaginous yeasts generally produce more lipids than other oil-producing microorganisms because their assimilation of carbon is quicker than its conversion in new cells, and resulting carbon excess is stored as lipid molecules into lipid droplets (LDs), intracellular organelles that occupy almost the entire cell in *R. toruloides* under conditions favourable to lipid accumulation (Zhu et al., 2015). LDs are fundamental for biosynthesis, mobilization, and trafficking of intracellular lipids, but they are considered non-essential for yeast growth (Sandager et al., 2002). However, expansion of LDs is an important survival mechanism of yeasts during element deficiency events such as those for carbon, nitrogen, magnesium, zinc, iron, phosphorus, or sulphur, or other unpleasant environmental conditions, like osmotic or oxidative stresses (Beopoulos et al., 2011; Henne & Hariri, 2018; Bonatto, 2022). The dichotomy between surplus of carbon sources and lack of nitrogen ones, known as carbon-to-nitrogen (C/N) ratio

imbalance, triggers the largest lipid build-up in *R. toruloides*: in order to make for nitrogen shortage in the case of high C/N ratio, adenosine monophosphate (AMP) is broken down by AMP deaminase (AMPD) to release ammonia; resulting shrinkage of AMP limits the activity of isocitrate dehydrogenase, a TCA cycle enzyme whose AMP is an allosteric activator; TCA is therefore dysregulated because isocitrate is not converted to α-ketoglutarate, but isocitrate increase in mitochondria is balanced by isomerization to citrate, and liberation of this into cytoplasm via malate/citrate antiport; here, the characteristic enzyme of oleaginous yeasts ATP-citrate lyase (ACL) cleaves citrate to form cytosolic acetyl-CoA, the main precursor of fatty acids (FAs), thus redirecting the carbon excess towards lipid biosynthesis (Lopes et al., 2020; Rodriguez et al., 2016). The Fig. 1 shows the main reactions and key genes connected to the metabolism of lipids in *R. toruloides*.

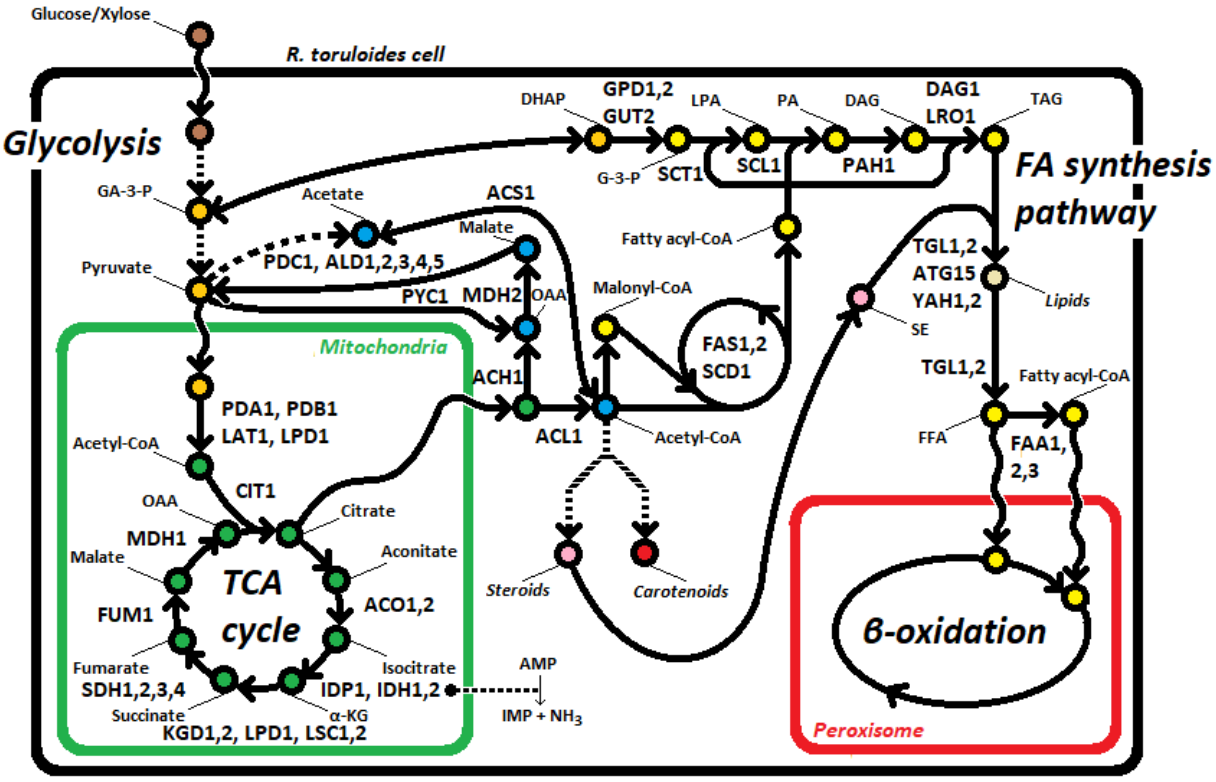

Figure 1. Metabolic pathways in cytoplasm, mitochondria and peroxisome related to the biosynthesis of lipids in R. toruloides. Inspired from Wen et al., 2020. Solid lines: single step; dotted lines: multi-steps. Abbreviations: GA-3-P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; SE, steryl ester; FFA, free fatty acid; OAA, oxaloacetate; α-KG, α-ketoglutarate; AMP, adenosine monophosphate; IMP, inosine monophosphate.

The most abundant class of lipids that is produced by oleaginous yeasts is triacylglycerols (TAGs), which can even exceed 90% of the total lipids in *R. toruloides*, with at least 36 different kinds (Wei et al., 2017). The other lipids present in *R. toruloides* in a low content are, in descending order, phosphatidylcholine (PC), phosphatidylethanolamine (PE), steryl esters (SE),

ergosterol (ES), phosphatidylinositol (PI), and phosphatidylserine (PS). FAs that characterize these TAGs of *R. toruloides* are slightly more often unsaturated than saturated and consist predominantly of C16-C18 long-chain FAs (Zhang et al., 2022; Krikigianni et al., 2022). The main FAs of *R. toruloides*, with concentrations and unsaturation level that depend on selected strain and environmental conditions, are listed here according to highest content: oleic acid (OA, C18:1), palmitic acid (PA, C16:0), linoleic acid (LA, C18:2), stearic acid (SA, C18:0), palmitoleic acid (POA, C16:1), α-linolenic acid (ALA, C18:3) (Fei et al., 2016).

Besides being classified as oleaginous, *R. toruloides* is also labelled as "red yeast", which identifies a varied group of basidiomycetes that are able to produce carotenoids (Watcharawipas & Runguphan, 2023). Carotenoids are tetraterpene pigments ($C_{40}H_{64}$) that protect red yeasts from free-radical damages originated from UV irradiation and serve as precursors for other secondary metabolites, while giving a yellow, orange, red or even purple colour to the basidiomycetes due to the absorption spectrum ranging from 400 to 500 nm of their long unsaturated isoprenoid chains (Lin & Xu, 2020; Scott, 2001). Like lipids, due to the hydrophobic nature carotenoids biosynthesis takes place in endoplasmic reticulum (ER) and ends up with storage in LDs, from where they are transferred to cell membrane as structural and functional molecules associated with lipids,

preventing their oxidation to preserve membrane stability and fluidity (Eman, 2019).

R. toruloides generates the so-called building blocks of carotenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and later their major precursor, geranylgeranyl diphosphate (GGPP), via the mevalonate (MVA) pathway from cytosolic acetyl-CoA, the same used by lipid biosynthesis pathway, thus competing with each other. At this stage, only three enzymes are known so far catalysing condensation, dehydrogenation and cyclization reactions that make up the following carotenoid pathway: geranylgeranyl diphosphate synthase (CrtE), phytoene dehydrogenase (CrtI), and phytoene synthase/lycopene cyclase (CrtYB) (Zhu et al., 2012). The Fig. 2 displays in detail the reactions and key genes that characterize the MVA pathway and the carotenoid biosynthesis pathway of *R. toruloides*.

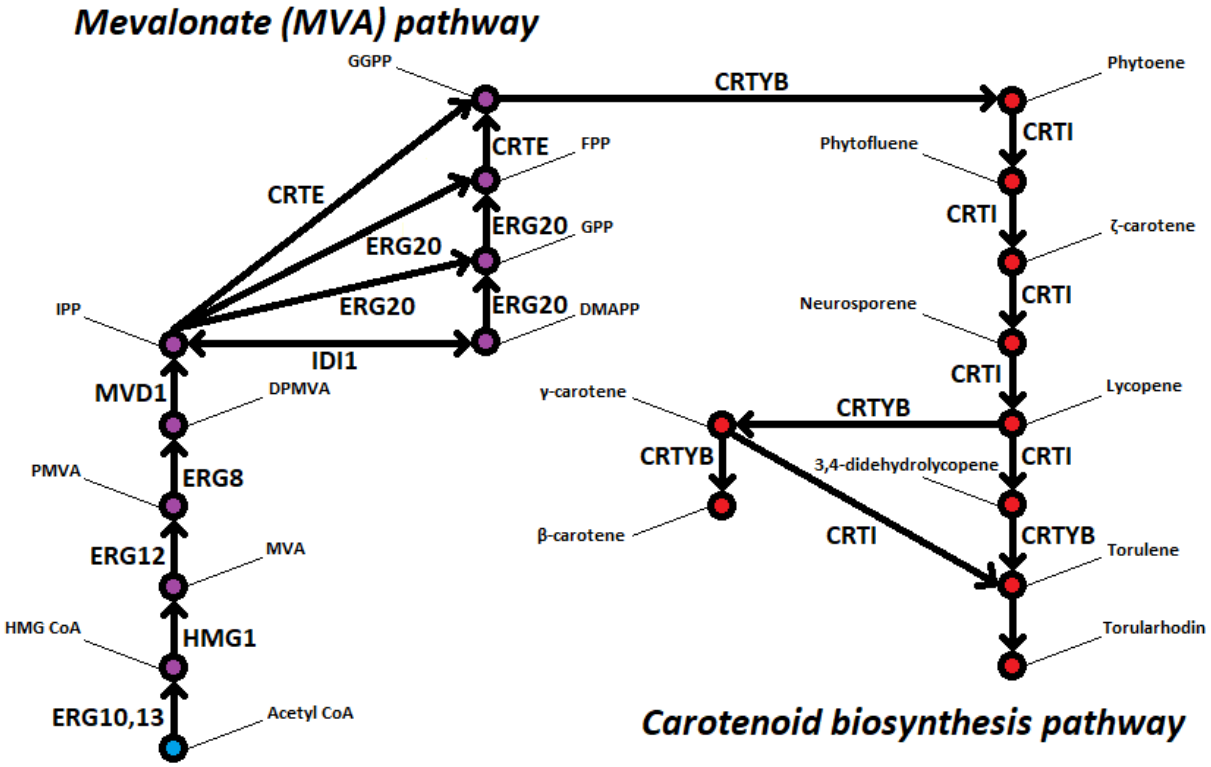

Figure 2. MVA and carotenoid biosynthesis pathways of *R. toruloides* outlined according to the iRhtoC model (Dinh et al., 2019). Abbreviations: HMG CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; PMVA, mevalonate-5-phosphate; DPMVA, mevalonate-5-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

Same as lipids, production of certain carotenoid compounds or their different quantity depends upon distinct yeast species or strains, and environmental factors such as C/N ratio imbalance or elements depletion, osmotic stress, metal ions or certain chemical reagents, oxygen availability, light irradiation, temperature and pH variations, or competition with other microorganisms (Buzzini et al., 2007; C.

Liu et al., 2021). Carotenoids that are naturally produced to a higher degree by *R. toruloides* are:

- torularhodin: cyclic carboxylic acid carotenoid ($C_{40}H_{52}O_2$) with 12 conjugated double bonds in the polyene chain, and a carboxylic acid group in the beta-ionone ring (retinyl group) present at one end.
- torulene: cyclic carotenoid ($C_{40}H_{54}$) with 12 conjugated double bonds in the polyene chain, and the retinyl group at one end.
- γ-carotene: cyclic carotenoid ($C_{40}H_{56}$) with 11 conjugated and 1 non-conjugated double bonds in the polyene chain, and the retinyl group at one end.
- β-carotene: isomer of γ-carotene with 11 conjugated double bonds in the polyene chain, and 2 retinyl groups at both ends.

The enzyme(s) responsible for the conversion of torulene to torularhodin through hydroxylation and oxidation is(/are) still being investigated, and the possible synthesis of astaxanthin by some *R. toruloides* wild-type (WT) strains is unclear (Tran et al., 2019).

1.5 Biotechnological applications of *R. toruloides*

R. toruloides is an oleaginous red yeast that can rapidly grow to high cell density, efficiently utilize multiple carbon sources derived from low-cost feedstocks like lignocellulosic hydrolysate and food waste, and well tolerate their toxic compounds, such as furfural, 5-hydroxymethyl furfural, phenolic compounds, and organic acids, mainly formed during pre-treatment processes (Li et al., 2007; Osorio-González et al., 2019). Moreover, omics data are available for many strains, metabolism is being elucidated, genome-scale metabolic model (GEMs) is reconstructed and being actively updated, bioprocess scale-up is under intensive optimization, conventional engineering techniques are successfully applied, and synthetic biology tools and elements are increasingly accessible (Jagtap et al., 2021; Dinh et al., 2019; Tiukova et al., 2019; Bonturi et al., 2022; Reķēna et al., 2023; Yu & Shi, 2023).

Random mutagenesis, delivered through atmospheric room temperature plasma (ARTP), ultraviolet (UV) light or chemical mutagens, and adaptive laboratory evolution (ALE) are the two classical approaches for creating new strains as they are capable of inducing random mutations that might result in improved phenotypes. They are intended to address problems such as resistance to fermentations conditions, incomplete substrate utilization or susceptivity to

medium inhibitors, and ultimately result in increased production performances of the adapted strain because of the higher activation of genes linked to lipid and/or carotenoid biosynthesis that improves robustness (Qi et al., 2014; Yamada et al., 2017; C. Zhang et al., 2016; Díaz et al., 2018). These techniques are simple, effective, and economical, do not require huge knowledge about genetic background of the selected microorganism and are not subjected to the regulation imposed on GMO (Arora et al., 2020). *R. toruloides* CCT 7815 and Y4 are only two main examples of strains adapted to different undetoxified lignocellulosic hydrolysates by ALE work that show higher biomass, lipid and/or carotenoid production and promise to further increase industrial applications of these valuable low-cost renewable resources (Bonturi et al., 2017).

Despite progresses of adapted strains, metabolic engineering is still indispensable to make *R. toruloides* a competitive industrial chassis, because it offers more straightforward strategies to accurately regulate gene expression and achieve designed production. But synthetic biology of *R. toruloides* is still limited and not fully developed in comparison to *S. cerevisiae* or other popular species like *K. phaffii* or *Y. lypolytica*, but great efforts were made in the last decade to take the natural skills of this oleaginous red yeast to the next level using those engineering tools. Due to the high GC-content (about 61.5%) of the genome of *R. toruloides*

that induces more stable secondary structures and high melting temperature, polymerase chain reaction (PCR), primer design, sequencing and gene synthesis can be compromised, and additionally codon optimization of heterologous gene in *R. toruloides* must be considered for efficient expression (Bochman et al., 2012; Camiolo et al., 2019). However, many genetic elements are now available for *R. toruloides*, including constitutive, inducible and RNA polymerase III promoters, terminators, auxotrophic and antibiotic selection markers, and virus 2A sequences (Nora et al., 2019; Johns et al., 2016; Brink et al., 2023; Schultz et al., 2019; (Y. Liu et al., 2013); X. Lin et al., 2014; (Tully & Gilbert, 1985); Y. Liu et al., 2015; Jiao et al., 2018). Assembling these elements in a plasmid vector using Golden Gate assembly (GGA) method was recently described for an application in *R. toruloides* with the versatile and efficient Golden Gate Assembly System (RtGGA), an adaptation from the MoClo Yeast Toolkit (YTK) from *S. cerevisiae* (Bonturi et al., 2022; M. E. Lee et al., 2015). GGA can assemble more than 50 fragments (52) in one pot-reaction directly from a plasmid (Pryor et al., 2022). It employs T4 DNA ligase and the type IIS restriction enzyme BsaI, which targets the non-palindromic 5'-GGTCTC-3' recognition site that flanks inserts and cloning vector and cut distally, leaving a 4 nucleotide 5′-overhang one base adjacent to the eliminated recognition site. These 4bp overhangs are functional

parts of the insert in a seamless assembly or harmless extra nucleotides, and they can be specifically designed to allow the freely interchange of elements from a DNA library. A simple representation on the GGA mechanisms is depicted in Fig. 3. Beside GGA, other assembly platforms are also popular, such as the long-overlap-based Gibson assembly (GA), sequence- and ligation-independent cloning (SLIC) and circular polymerase extension cloning (CPEC), the type I restriction endonuclease-mediated BioBrick standard, or the Gateway cloning, but they suffer from limited ability to form a library of interchangeable parts, requiring repeated PCRs, sequencing, and digestion steps, and/or a scar-based assembly (Casini et al., 2015).

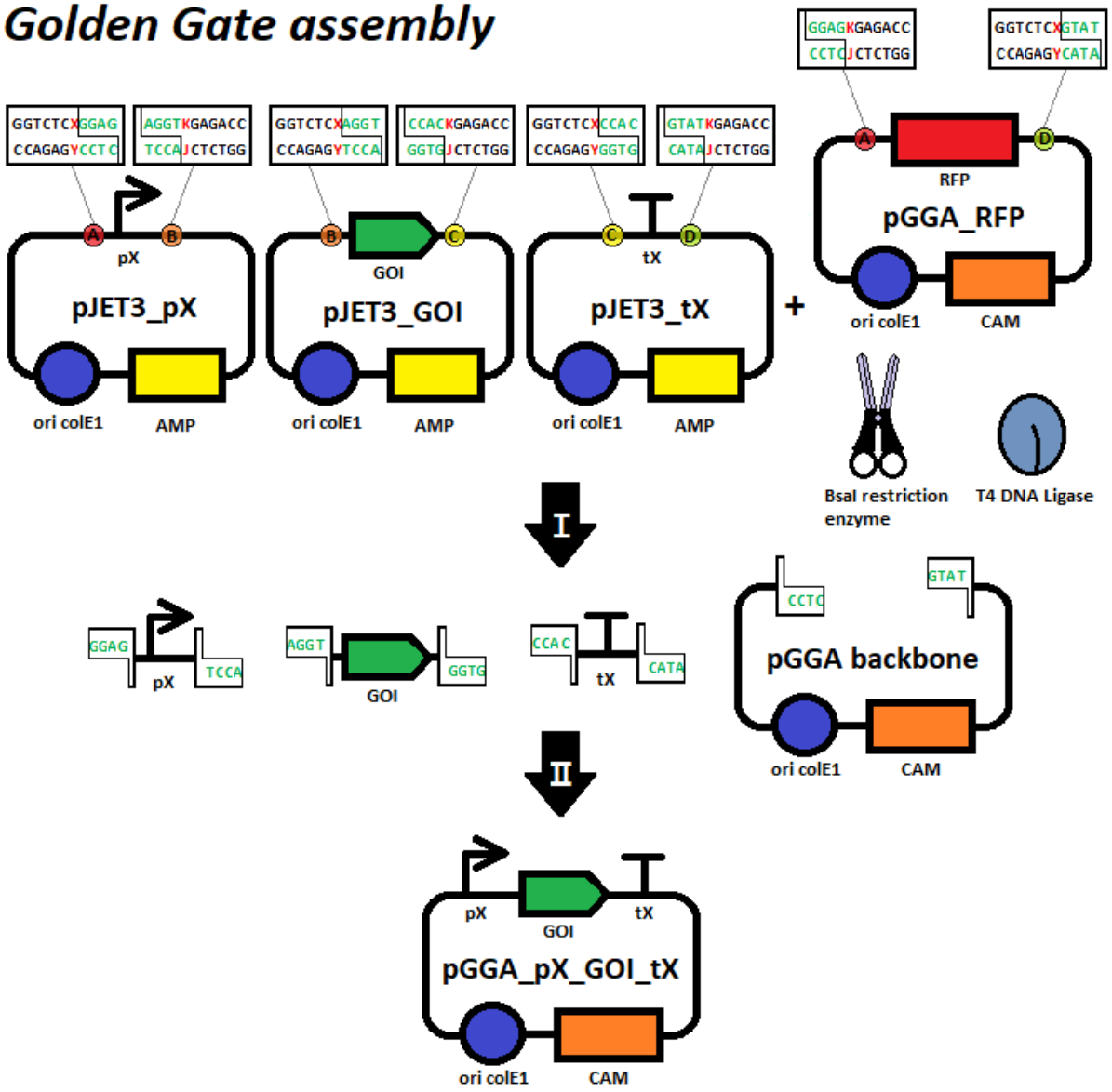

Figure 3. Golden Gate assembly workflow. The case involves a promoter (pX), a gene of interest (GOI), and a terminator (tX) inside distinct pJET3 plasmid vectors that assemble into a pGGA plasmid backbone after cleavage (I) by BsaI restriction enzyme and ligation (II) by T4 DNA ligase.

Introduction into *R. toruloides* of these recombinant plasmids as well as more effectively the linearized construct DNAs can be realized by different

transformation methodologies defined for this host, including Agrobacterium-mediated transformation (ATMT), spheroplast transformation, electroporation, and LiAc/SS-DNA/PEG-mediated chemical transformation (H. Liu et al., 2017; Tsai et al., 2017). The first two methods are long and not practical; electroporation is the most efficient (2×10^3 transformants/μg DNA), but it requires particular equipment; LiAc/SS-DNA/PEG protocol is the most common, but its efficiency for *R. toruloides* is not very high (25 transformants/μg DNA). Integration of the construct is required for *R. toruloides* because it lacks stable episomal plasmids, since no native autonomously replicating sequences (ARSs) have been identified yet and heterologous ARSs does not function (Schultz et al., 2021). Although an endogenous plasmid was recently reported, it was not later characterized (Martín-Hernández et al., 2021).

However, the genome of *R. toruloides* hardly integrates exogenous DNA into a target site because this process is mediated by homologous sequences in homology direct-repair (HDR) pathway, which is known to be disfavoured over non-homologous end joining (NHEJ) in the competition for DNA double-stand breaks (DSBs) repair, so random integration is the main destiny of transformed DNA in *R. toruloides* (Krappmann, 2007; Koh et al., 2014). Deletion of NHEJ-related genes such as Ku70/80, Nej1, and Dnl4 and overexpression of HDR-associated

genes like Rad51/52 and Sae2 are some of the possible strategies to enhance homologous recombination (HR) in *R. toruloides* (Shan et al., 2021). Furthermore, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, or CRISPR/Cas9, was applied to *R. toruloides* as a genome-editing tool for gene knockout with yet limited success when compared to *S. cerevisiae*, but it is presumed to increase integration of DNA constructs to alter gene function (Otoupal et al., 2019; Schultz et al., 2019; Jiao et al., 2019). Other useful genetic engineering tools implemented in *R. toruloides* for gene downregulation and removal are, respectively, RNA interference (RNAi) technique and site-specific recombinase (SSR) technology, including systems like Cre recombinase-loxP site (Cre-lox) and flippase recombinase (Flp)-Flp recognition target (Flp-FRT) (X. Liu et al., 2019; Sun et al., 2018).

All these advancements in manipulation of this yeast and understanding of its metabolism have accelerated the research and development of *R. toruloides* and broaden its applications. While manufacturing of recombinant products for pharmaceutical and biotech sector is already a well-established business with a global market value of 5.8 billion USD in 2021, the application of microorganisms to food industry for production of functional foods and dietary supplements is maybe the most urgent and dynamic biotechnology, representing a potential game

changer for current food system, and the number of start-up companies that has entered in this market is tremendously increased for the last few years (Allied Market Research, 2022; Graham & Ledesma-Amaro, 2023). The development of these novel food involves the most diverse microorganisms, and *R. toruloides* has the potential to be used as a chassis organism for industrial production of SCPs, SCOs, carotenoids, and sugar and fatty alcohols (Koukoumaki et al., 2023; Zhao et al., 2022; Jagtap et al., 2019; Schultz et al., 2022).

The SCOs market size is expected to reach more than USD 670 million by 2032 from the 60 of the 2022 (approx. +1000%), and most of its share would be destined to food chain (Global Market Insights, 2023). Maximizing lipid production and customizing FA composition are the targets of SCOs to seek economically sustainability and application niches. To my knowledge, *R. toruloides* Y4 recorded the best dry biomass (127.5 g/L), lipid titer (78.7 g/L) and lipid productivity (0.57 g/(L*h)) ever reported for a non-genetically modified *R. toruloides* strain, obtained in a 134 hours (h) fed-batch culture with glucose as sole carbon source (X. Zhao et al., 2011). Nevertheless, overexpression of a second copy of the native lipogenic genes SCD1 (stearoyl-CoA desaturase), ACC1 (acetyl-coenzyme A carboxylase) and DGAT1 (diacylglycerol acyltransferase) elevated lipid titer and productivity of *R. toruloides* IFO0880 to 89.4 g/L and 0.61 g/(L*h), respectively,

for similar cultivation conditions to those above-mentioned (S. Zhang et al., 2016). Unfortunately, lignocellulosic hydrolysates so far made *R. toruloides* able to register much lower values than the previous ones, but they can presumably bring about larger biomass and lipid production than traditional media after optimization of these low-cost substrates into the fermentation process (Saini et al., 2020). For example, *R. toruloides* CCT 7815 reached 6.6 g/L and 27.35 g/L of biomass and 3.9 g/L and 10.95 g/L of lipid titer in hemicellulosic hydrolysates of sugarcane bagasse and birch, respectively (Bonturi et al., 2017; Monteiro de Oliveira et al., 2021). To my knowledge, the best results for non-detoxified lignocellulosic hydrolysates were reported by the adapted strain *R. toruloides* CECT 13085, integrating an extra copy of SCD1 and DGAT1, which reached 64.5 g/L of dry biomass and 39.5 g/L of lipid titer in a fed-batch culture with non-detoxified wheat straw hydrolysate (Díaz et al., 2018).

Altering FA composition and achieving specific FA-rich lipid production are no less important task for SCOs industry. Depending on FA composition, provided from desaturation level, double bonds position and carbon chain length of their molecules, microbial lipids can be intended for different marketable products: SCOs with larger percentage of saturated FA, for example, can be considered to substitute coconut oil, milk oil, meat fat or cocoa butter; SCOs with greater amount

of MUFA can replace vegetable oils, including olive oil, rapeseed oil and others; SCOs with high PUFA are instead regarded to be fish oil equivalents (Papotti et al., 2021). *R. toruloides* has a FA composition that is comparable to vegetable oils, but at the same time it was proposed as potential producer of cocoa butter-like lipid (CBL), the main component of chocolate and probably the most expensive bulk oils and fats, with a predicted market value of more than USD 10 billion in 2033 (Wei et al., 2017; Ratledge, 1989; Future Market Insights, 2023). Harnessing *R. toruloides* to produce tailored lipids can be accomplished with optimization of culture conditions as well as metabolic engineering. For instance, xylose and arabinose (C5 sugars) as sole carbon, nitrogen starvation, supplementation of trace metals, increased oxygen availability, reduced temperature and light conditions are all settings for increasing PUFA content (Wiebe et al., 2012; Ye et al., 2021; Saini et al., 2023; Parreira et al., 2015; He et al., 2015; Pham et al., 2020). Overexpression of FA elongases (Elos) shows higher OA content, while FA desaturases (Fads) enhance OA, LA and ALA (Fillet et al., 2016; Tsai et al., 2019; Liu et al., 2019).

R. toruloides can also naturally produce sugar alcohols such as D-arabitol from xylose and galacticol from galactose, which can be particularly exploited as low-calorie sweetener in a multi-billion market, or it can be easily engineered to

synthesize fatty alcohols to produce detergents, lubricants, plastics, and cosmetics for the chemical industry (Jagtap & Rao, 2018; Fillet et al., 2015).

The global carotenoids market size was valued at USD 1.8 billion in 2021 and is expected to reach USD 2.7 billion by 2031 (+50%) (Allied Market Research, 2023). Carotenoids as food additive and dietary supplement together can have larger market share than the major animal feed application. So, their use as food colorants by animal feeding is the most common, but growing awareness of the health benefits of these molecules, ranging from antioxidant activity and provitamin A effect to cancer and cardiovascular prevention and antimicrobial action, is boosting their application in food and nutraceuticals, cosmetics, pharmaceuticals, and biomaterials (Franco et al., 2021; Gloria et al., 2014; Ungureanu & Ferdes, 2012). Actual production of carotenoids is essentially ascribed to chemical synthesis, but some studies showed carcinogenic activity of synthetic β-carotene, paving the way for the industry of microbial carotenoids (Black, 2004). *R. toruloides* is a natural producer of carotenoids and its cultivation conditions offer many advantages over other important carotenogenic species like Mucoral fungi and algae, but it is not very competitive with engineered strains of *S. cerevisiae* for production of single carotenoids owing to the long purification process. Moreover, *R. toruloides* stands out for higher accumulation of

torularhodin and torulene, which are less popular carotenoids but show better anti-oxidative, anti-microbial and anti-cancerous properties (Kot et al., 2018). Since methods and standards used to quantify carotenoids are different between studies of different groups, results are divergent and comparison between strains is not easy (Z. Liu, van den Berg, et al., 2021). To my knowledge, the WT strain *R. toruloides* ATCC 204091 recorded the best carotenoid production (62 mg/L) ever reported for the species, obtained in a 96 h food waste culture with C/N ratio of 30 (%/%) (the standard used for quantification was β-carotene) (Singh et al., 2018). A further improved *R. toruloides* CCT 7815 by ALE with H_2O_2 reported a carotenoid production of 44 mg/L in a 168 h batch culture with xylose as sole carbon source and under oxidative stress (light irradiation and H_2O_2), while its parental strain was able to produce 20.7 and 36.2 mg/L without and under light irradiation, respectively (Pinheiro et al., 2020). In another study, *R. toruloides* CCT 7815 produced 1.1 and 1.72 mg/L of total carotenoids in hemicellulosic hydrolysates of sugarcane bagasse and birch, respectively, whose lower values are in accordance with previous statement about the necessity for bioprocess optimization of these substrates (Bonturi et al., 2017; Monteiro de Oliveira et al., 2021). However, overexpression of a second copy of the native carotenogenic

genes CrtE, CrtI and CrtYB elevated carotenoid production of *R. toruloides* CCT 7815 by 41% (Bonturi et al., 2022).

Although carotenoids and oil are simultaneously extracted, for example, with a non-polar organic solvent like hexane, and could collectively serve the market as "red oil", the use of *R. toruloides* to produce whole foods out of its biomass in the application of SCPs is perhaps the most promising and economically viable microbial biotechnology when supported by rapid, inexpensive safety approvals. Microbes are normally characterized by high protein content and complete amino acids profile (Anupama & Ravindra, 2000). It was measured that *R. toruloides* CCT 7815 contained 0.636 and 0.438 g protein/gDCW during the exponential phase in high C/N ratio cultures with glucose and xylose, respectively, before making room for lipid accumulation in the nitrogen limited stage (Reķēna et al., 2023). In addition to high protein content, oils, and carotenoids, *R. toruloides* cells includes fibres, vitamins, minerals, and other functional compounds, which make it gaining the title of "superfood" and perhaps meeting the challenges of malnutrition, hunger, and climate change together with other microbes in the near future (Byrtusová et al., 2020).

Chapter second

OBJECTIVE OF THE THESIS

The objective of this Master's thesis was the overexpression of heterologous genes such as the truncated HMG-CoA reductase from *Y. lypolytica*, here t-ylHMG1, and phytoene dehydrogenase and lycopene cyclase/phytoene synthase from *Mucor circinelloides*, here mcCrtI and mcCrtYB, respectively, to increase production of carotenoids in *R. toruloides* CCT 7815. To my knowledge, their overexpression in multiple copies in an "obese" *Y. lypolytica* strain together with native geranylgeranyl diphosphate synthase (GSS1) enabled it to register the highest carotenoid production and yield for a microbial species described so far in the literature (Larroude et al., 2018).

In conformity to the iterative framework Design-Build-Test-Learn (DBTL) cycle that is routinely performed in synthetic biology to resolve bottlenecks in the development of microbial strains with desired functionalities, the first part of the thesis project was the "in silico" design of constructs, that included the above-mentioned genes together with genetic elements assigned to induce continuous expression of those genes, promote genome integration of the constructs, and allow selection of transformants (Lawson et al., 2019). The second step was the

actual manufacturing of the DNA constructs and its transfer in the yeast cell, and this employed genetic engineering methods such as plasmid isolation, restriction digestion, (colony-)PCR and electrophoresis, DNA purification, molecular cloning, Golden Gate assembly, and bacterial and yeast transformation. The third phase of the work was testing the yeast strains that acquired the assembled construct in shake-flask cultures for their enhanced carotenoid production. The last act of this study was the analysis of data resulted from the tested strains and their report in this Master's thesis. A new DBTL cycle started for those elements that did not make it till the end of the process.

At the same time, a new cycle is envisaged for those strains that has shown improved physiology to achieve the development of robust microbial cell factories capable of entering the market and establishing an industrial production of carotenoids and/or superior microbial food.

Chapter third

MATERIALS AND METHODS

3.1 Designing of constructs

Plasmid constructs were designed in the cloud-based platform Benchling (https://www.benchling.com), a molecular biology suite that allows to devise primers, run virtual restriction digest, visualize plasmid sequences and DNA fragments, assemble them with different cloning methods or edit via CRISPR-Cas9 system among others, and experiment workflow can be recorded in an electronic lab notebook. The genetic parts used in this study were either purchased from Twist (https://www.twistbioscience.com) in a synthetic plasmid form or amplified by PCR from *R. toruloides* genome and cloned into a plasmid, and then transformed to a competent *E. coli* strain for storing at -80°C. Primers were designed for these elements either to bear BsaI recognition site and a specific 4-nt overhang for GGA or to validate correct amplification or integration via PCR and DNA sequencing.

Four constructs named “HGA”, “PGA”, “mut-PGA” and “PGA2.0” were in-silico designed, and their functional parts are depicted in the Fig. 4.

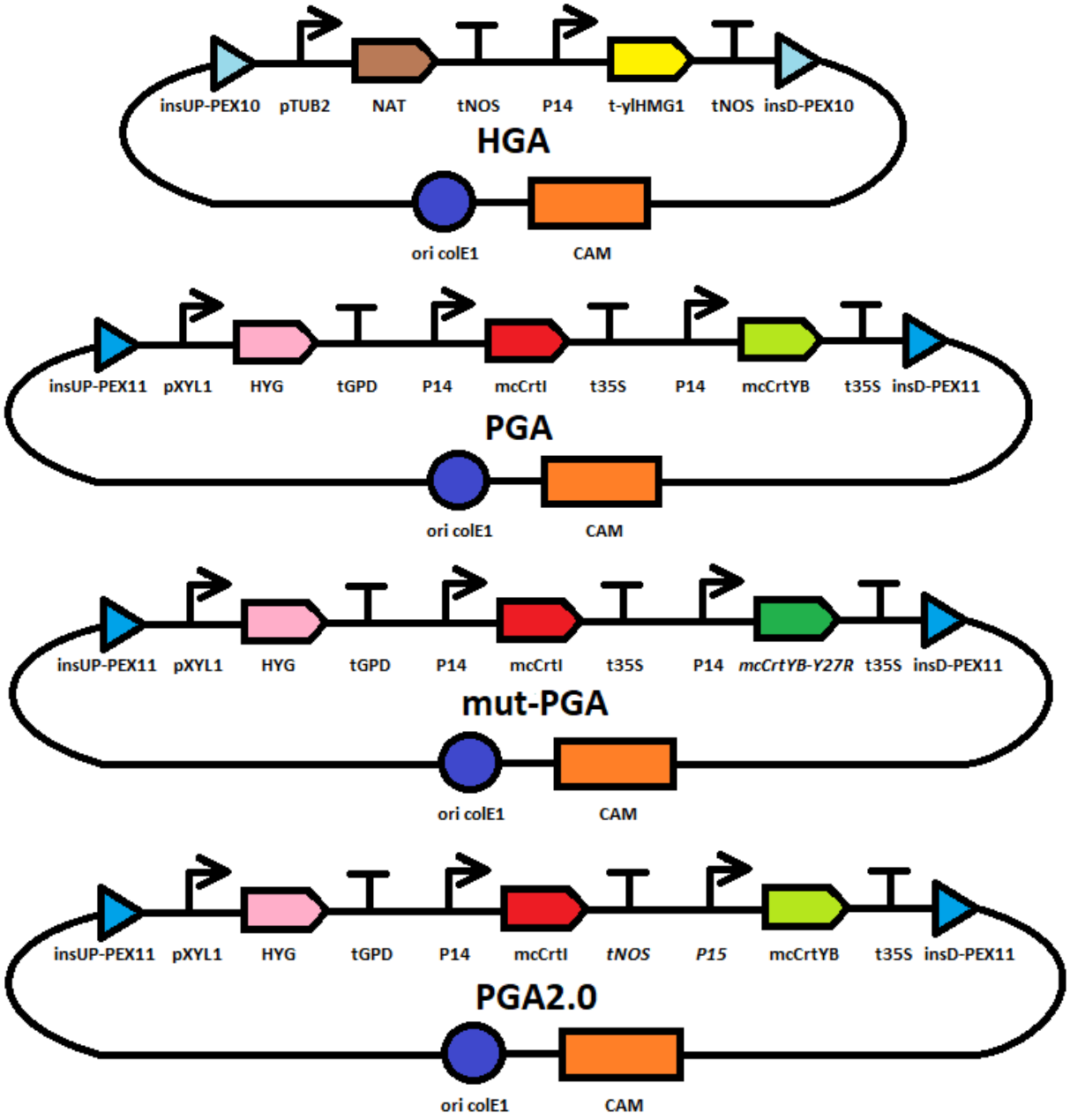

Figure 4. Graphical illustration of the HGA, PGA, mut-PGA, and PGA2.0 constructs. Parts are distinguished by different pattern whose design was inspired by the standard symbols proposed by the Synthetic Biology Open Language. Italicized names of the elements in mut-PGA and PGA2.0 highlight the difference with the original construct PGA.

The parts involved in these genetic constructs included:

- **Insertional regions**: insUP-PEX10, insD-PEX10, insUP-PEX11 and insD-PEX11 were obtained by amplification of five hundred base pairs fragments upstream and downstream of the peroxisomal biogenesis protein 10 (PEX10, RHTO_05319) and 11 (PEX11, RHTO_03603) genes from the genome of the adapted strain *R. toruloides* CCT 7815 (Coleção de Culturas Tropicais, Fundação André Tosello, Campinas, Brazil), herein called SBY29.
- **Promoters**: P14 and P15 are, respectively, the constitutive promoters of translation elongation factor 1-alpha (TEF1) and the mitochondrial ATP/ADP carrier SLC25A4S of *R. toruloides*, and they were acquired from the promoter library made by Nora et al., 2019 for *R. toruloides* IFO0880, where they are described as the strongest within the group of promoters considered in the study. pTUB2 and pXYL1 are, respectively, the promoter of the *R. toruloides* essential gene β-tubulin (TUB2), obtained from a synthetic construct made by Otoupal et al., 2019, and the promoter of xylose reductase (XYL1, RHTO_03963), amplified from the genome of *R. toruloides* CCT7815 strain.

- **Genes of interest (GOI)**: mcCrtI and mcCrtYB are, respectively, the CDSs of the *M. circinelloides* genes phytoene dehydrogenase (carB, GenBank: AJ238028.1) and lycopene cyclase/phytoene synthase (carRP, GenBank: AJ250827.1); named after homology with *R. toruloides* CrtI and CrtYB genes, these sequences were ordered and chemically synthesized after codon optimization for *R. toruloides* using Benchling. t-ylHMG1 is the codon-optimized truncated gene of *Y. lypolytica* 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase (YALI0_E04807g, Gene ID: 2912214); annotated as follows due to the homology with *S. cerevisiae* HMG1 gene, this gene was synthesized in a truncated version to remove the ER membrane-spanning domain in its N-terminal region that was linked to the induction of ER membrane stacks ("karmellae") and translational control of early sterol intermediates, resulting in an increase of the activity of this enzyme (Polakowski et al., 1998).
- **Terminators**: t35S and tNOS are, respectively, the terminators of 35S gene (GenBank: MF116010) of *Cauliflower Mosaic Virus* (CaMV) and nopaline synthase (GenBank: MF116010.1) of *Agrobacterium tumefaciens*, while the terminator of glyceraldehyde-3-phosphate dehydrogenase (GPD1,

RHTO_03746), tGPD, was amplified from the genome of *R. toruloides* CCT7815.

- **Antibiotic resistance markers**: the antibiotic resistance cassette pTUB2_NAT_tNOS was obtained by joining pTUB2 and the nourseothricin acetyltransferase (NAT) gene of the synthetic construct from Otoupal et al., 2019 with tNOS. The other cassette pXYL1_HYG_tGPD was assembled by fusing together pXYL1 and tGPD with the codon-optimized *E. coli* resistance gene hygromycin phosphotransferase hpt-3 (GenBank: KX377638.1), here HYG, from Y. Liu et al., 2013.

Several destination plasmids were used during the experiments, such as:

- **pJET1.2**

  (https://www.snapgene.com/plasmids/basic_cloning_vectors/pJET1.2): developed by Thermo Fisher, this plasmid backbone was used for cloning the single parts (insertional regions, promoters, genes of interest (GOI), terminators, and markers) via EcoRV restriction and ligation reaction; it carries the high-copy colE1 origin of replication and the ampicillin resistance (AMP) gene, and relies on the restriction endonuclease Eco47I for the selection of recombinant plasmid.

- **pJET3**: this in-house plasmid derives from pJET1.2 and was used alike to clone single parts; in contrast to the precursor, pJET3 depends on the lethal gene ccdB for selection against WT plasmids and lacks the BsaI recognition site, reducing generation of artifacts in the following GGA step.
- **pCR-XL-2-TOPO** (https://www.snapgene.com/plasmids/basic_cloning_vectors/pCR-XL-2-TOPO): designed by Thermo Fisher, this plasmid vector was used in place of pJET1.2 and pJET3 when the insert to clone held an EcoRV restriction site in a blunt-end topoisomerase-based cloning; it includes colE1 ori, AMP and kanamycin resistance (KAN) genes, and selection of recombinants is based on the ccdB cytotoxic system.
- **pGGAselect** (https://www.snapgene.com/plasmids/basic_cloning_vectors/pGGAselect) : developed by New England Biolabs, this destination plasmid was employed for cloning the assembled constructs via BsaI restriction and ligation reaction, namely Golden Gate assembly (GGA); it contains colE1 ori and chloramphenicol resistance (CAM) gene.
- **pGGA_RFP**: this in-house plasmid originated from pGGAselect was used in the same way to clone assembled constructs by GGA; compared to the

precursor, pGGA_RFP incorporates a red fluorescent protein (RFP, http://parts.igem.org/Part:BBa_E1010) that helps in the visual screening for positive colonies before colony PCR.

3.2 Liquid bacterial culture and plasmid isolation

From -80°C freezer, bacterial samples containing the parts of interest were inoculated in a 15-mL tube with 3 mL of lysogeny broth (LB) medium (tryptone, 10 g/L; sodium chloride (NaCl), 10g/L; yeast extract, 5g/L) and 3 µL of the corresponding antibiotics (50 µg/mL), and then incubated overnight in a shaker at 37°C.

Plasmids were then isolated from the cultures by FavorPrep™ - Plasmid Extraction Mini Kit according to the manufacturer's specifications. Ultimately, NanoDrop 1000 Spectrophotometer (Thermo Fisher) was used to measure plasmid concentration (ng/µL) and purity (mainly $OD_{260/280}$ and $OD_{260/230}$) of the samples.

3.3 PCR to add specific overhangs for GGA

To add the specific overhangs via designed primers, the parts were amplified by PCR into the Eppendorf™ Mastercycler™ Nexus Thermal Cycler with the engineered proofreading DNA polymerase Invitrogen™ Platinum™ SuperFi™

PCR Master Mix (Thermo Fisher) following the settings provided by the manufacturer's manual.

After PCR, amplicons were stained with 6X TriTrack DNA Loading Dye (Thermo Fisher) and run on a 1% agarose electrophoresis gel at a voltage of 120 V with GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher).

Subsequently, PCR fragments with proper size were cut out of gel and purified with FavorPrep™ GEL/PCR Purification Kit (Favorgen) according to manufacturer's instructions, and concentrations and purity were evaluated with NanoDrop 1000 Spectrophotometer (Thermo Fisher).

3.4 Cloning parts in pJET and assembling them with GGA

Purified PCR samples of every single part were cloned into pJET1.2 or pJET3 plasmid vector. pJET cloning reaction was prepared with components and their quantities described in Tab. 1.

Table 1. Setup of a typical reaction mixture used for pJET cloning

Reagent	**Volume (µL)**
pJET plasmid	*100 ng*
PCR sample	*2-fold molar excess of pJET plasmid*
50% w/v PEG_{4000}	1
Tango Buffer, 10X (Thermo Fisher)	1
T4 DNA Ligase (Thermo Fisher)	0.25
EcoRV (Eco32I) (Thermo Fisher)	0.25
diH2O	up to 10

The mixture was incubated at room temperature for 30 minutes and 10% volume/volume of TOP10 E. coli cells (Thermo Fisher) was transformed.

Inserts now stored in pJET vector were assembled by GGA in the final constructs using the NEB Golden Gate Assembly Mix (New England Biolabs), which contain pGGAselect plasmid vector, T4 DNA Ligase Buffer (New England Biolabs), and an optimized mix of BsaI restriction enzyme and T4 DNA Ligase contained in the NEB GG Enzyme Mix (New England Biolabs). When pGGAselect was not used, pGGA_RFP was taken its place as destination vector.

Overhangs of non-adjacent inserts were too similar in PGA and PGA2.0 constructs, so the pJET plasmids were divided in sub-group reactions with (PGA) or without (PGA2.0) pGGA, and later these sub-groups of inserts were joined together within pGGA vector in the so-called "2-step reaction" GGA. After first step of PGA construction, inserts were cloned inside pGGA and required PCR to change overhangs for the second step of GGA, while for PGA2.0 sub-groups were not cloned and avoided PCR step. GGAs with *n* inserts were generally set up as specified in Tab. 2.

Table 2. General setting used for a Golden Gate reaction

Reagent	**Volume (µL)**
pGGA plasmid (plasmid with the insert) × *n* inserts	*50-75 fmol*
T4 DNA Ligase Buffer, 10X (New England Biolabs)	2
NEB GG Enzyme Mix (New England Biolabs)	1
diH2O	up to 20

The volume of pGGA plasmid added to the mixture was calculated according to the formulae (1-2). From (1), 660 g/mol is the average molar mass of a single

nucleotide, while the number of moles (mol) set for all plasmids to use in the reaction was around 50 to 75 fmol (Tab. 2).

$$Mass\ of\ the\ plasmid\ (ng) = \left\{ moles\ of\ the\ plasmid\ (mol) \times \left[lenght\ of\ the\ plasmid\ (bp) \times\ 660\ \frac{g}{mol} \right] \right\} \times\ 10^{-6} \quad (1)$$

$$Volume\ of\ the\ plasmid\ (uL) = \frac{mass\ of\ the\ plasmid\ (ng)}{plasmid\ concentration\ (\frac{ng}{uL})} \quad (2)$$

GGA reactions were run in the thermocycler (Eppendorf) with conditions (Tab. 3) that varies according to the type of GGA (1- or 2-step reaction GGA) used and the step (first or second step of the 2-step reaction) considered.

Table 3. Conditions set for a regular Golden Gate assembly reaction

Temperature (°C)	Time (min)	Times
37	3-5	30-50
16	2-5	
55	5	-
80	5	-
15	∞	-

Like pJET cloning, 10% volume of the GGA mix over volume of TOP10 E. coli cells (Thermo Fisher) was transformed.

3.5 Bacterial transformation and screening

Bacterial transformation with both pJET- and pGGA-recombinant plasmids employed the chemically competent cells Escherichia coli TOP10 (Thermo Fisher) according to a variant of the Hanahan method (Green & Sambrook, 2018). 100 µL of TOP10 cells (Thermo Fisher) were taken out from -80°C to left thawing on ice for 15 min. 10 µL of plasmids (10% v/v) were transferred inside the tube and mixed by steering. After holding for 30 min on ice, the mixture was heat-shocked at 42°C for 1 min and returned on ice. 300 µL of LB were then added. According to the antibiotics mechanism, cells transformed with pJET plasmid (AMP) were first incubated at 37°C for 1 hour, while cells with pGGA plasmid (CAM) were directly plated on LB agar plates with the corresponding antibiotics and incubated overnight at 37°C. Concentration of the selection antibiotics inside plates was either 100 µg/mL of ampicillin or 25 µg/mL of chloramphenicol.

In the colony PCR method, colonies were spread in grid patches of a second plate and simultaneously screened for the presence of construct by PCR with predefined set of primers, specifically designed for each plasmid backbone, using the Taq DNA polymerase DreamTaq Green PCR Master Mix 2X (Thermo Fisher) according to the manufacturer's manual. Colonies with correct constructs were cultured overnight using 3 mL of LB and the corresponding selection antibiotics.

750 µL of each culture was stored with 15% (v/v) glycerol at -80°C. The rest of the volume was used for plasmid isolation using FavorPrep™ Plasmid DNA Extraction Mini Kit (Favorgen). Concentration and purity of extracted plasmids was assessed with NanoDrop 1000 Spectrophotometer (Thermo Fisher), and correctness of sequence and construct assembling was also confirmed by Sanger sequencing.

3.6 Yeast transformation and screening

Yeast transformation with pGGA-recombinant plasmids involved the *R. toruloides* strains SBY29 and SBY92 (Tab. 5) according to a procedure inspired by Nora et al., 2019. These yeasts were pre-inoculated within a 250 mL-Erlenmeyer flask with vented cap in 50 mL of Yeast Extract–Peptone–Dextrose (YPD) medium (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L) and incubated overnight with 200 rpm-stirring speed at 30°C.

Cultures were then used as inoculum by dilution to an OD_{600} of 0.2 in 50 mL of YPD, which was incubated at 30°C on a shaker until an OD600 of 0.8 (approximately 4 hours are needed because duplication time of *R. toruloides* is about 2 hours in these conditions). Consequently, cells were harvested by repeated centrifugation and washing with sterile diH_2O and resuspended in 1 mL per 8 OD

of lithium acetate/Tris-EDTA (LiOAc/TE) solution (100 mM LiOAc, 10 mM Tris-HCl, 1 mM EDTA; pH 7.5). 500 µL of suspension was transferred to 1.5 mL-microfuge tube for each transformation and pelleted at 3000 g for 30 seconds to remove supernatant. At this stage, a transformation mix (Tab. 4) prepared in advanced was added to the pelleted cells.

Table 4. Components of the transformation mixture and their quantities

Reagent	**Volume (µL)**
PEG_{4000}	240
1 M LiAc	36
Sterile water	24
Salmon Sperm DNA (boiled)	10
Transforming DNA (digested to free constructs)	*2-3 µg* in 50 µL

After incubation at 30°C for 30 minutes, 34 µL of DMSO was included and the mixture was heat-shocked at 42°C for 15 minutes. Supernatant was removed by double centrifugation at 3000 g for 30 seconds each, and cells were resuspended in 14 mL-snap cap tubes with 2 mL of YPD for overnight incubation on a shaker at 30°C. Plating was done onto YPD agar plates with corresponding selection antibiotics and following incubation was set for 2 or 3 days at 30°C. Concentration

of the selection antibiotics inside plates was either 50 µg/mL of hygromycin or 100 µg/mL of nourseothricin.

Prior to yeast colony PCR, genome of yeast colonies was extracted according to the "Potassium hydroxide-Boiling-Centrifugation" (PBC) method proposed by Y. Liu et al., 2022. Analysis for the integration of the constructs by PCR using DreamTaq Green PCR Master Mix 2X (Thermo Fisher) or Invitrogen™ Platinum™ SuperFi™ PCR Master Mix (Thermo Fisher) involved sets of primers designed on either the regions near the target site or the insertional arms of the construct (ectopic integration). Colonies with integrated constructs were cultured overnight using 3 mL of YPD and 750 µL of each culture was successively stored with 15% (v/v) glycerol at -80°C.

The yeast strains that were transformed with the assembled constructs together with the new strains obtained after transformation are described in Tab. 5.

Table 5. List of the *R. toruloides* strains involved in the study

Strain code	Description
SBY29	*R. toruloides* CCT 7815 (Bonturi et al., 2017)
SBY92	SBY29Δku70::CrtE+CrtI+CrtYB (Bonturi et al., 2022)
SBY161	SBY29Δpex10::tHMG1 (this work)
SBY162	SBY92Δpex10::tHMG1 (this work)

3.7 Strain characterization and analytics

Selected strains were characterized in terms of growth, sugar consumption, biomass production, carotenoid production and profile, and FA composition by analysis of samples run in triplicates. Cells were inoculated into 250 mL-vented cap flasks with 50 mL of YPD (60 g/L of glucose) and 0.2 M potassium chloride (KCl) as cultivation medium and then incubated at 30°C and 200 rpm-shaking speed for 92 hours. Sampling for cell density measurement and determination of glucose consumption rate occurred every 24 hours, while other analyses were based only on samples taken at the end of the fermentation.

Growth of selected strains was monitored by measuring absorbance (AU) of a 1 mL-sample with Ultrospec 10 Cell Density Meter (Biochrom) at a wavelength of 600 nm. Consumption of glucose was determined using the high-performance liquid chromatography (HPLC) Shimadzu LC-2050C (Shimadzu, Japan) equipped with Aminex HPX-87H column (Bio-Rad) and RID-20A refractive index detector (Shimadzu), at 45°C and 5 mM H_2SO_4 as mobile phase with isocratic elution at 0.6 mL/min.

To evaluate production of biomass, carotenoids and FAs, cells were first freeze-dried after removing supernatant. Biomass production (g/L) was calculated by the

ratio between dry cell weight (DCW) and volume of culture. For carotenoid analysis, which included carotenoid production (mg/L), yield (mg/gDCW) and profile, carotenoids were extracted and quantified according to a method adapted from Matthäus et al., 2014. 100 mg of freeze-dried cells were resuspended in 1 mL of acetone and lysed with acid-washed glass beads (400–650 μm) using FastPrep homogeniser (MP Biomedicals) for three cycles at 4 m/s for 20 seconds. The acetone solution containing carotenoids was separated from cells debris by centrifugation at 15,000 g for 5 minutes and collected in 1.5 mL-amber coloured tubes. These steps were repeated one more time to maximize carotenoid extraction from cells. Absorbance curves were constructed by analysis of the extract with Cary 50 Bio UV-Visible Spectrophotometer (Varian) at a range of wavelength between 400 to 600 nm. Carotenoid production (4) and yield (3) were calculated using the average value of absorbances at 485 nm, 455 nm, and 430 nm, that are the wavelengths around which torulene, torularhodin, β-carotene, and γ-carotene have their maximum absorption and most peaks reside. From (2), 0.03 is a factor employed to convert the average absorbance to the concentration of carotenoids in the solvent (acetone). From (3), 0.002 L (or 2 mL) and 0.1 gDCW (or 100 mgDCW) are, respectively, the amount of solvent and biomass used for the extraction.

$$Measured\ carotenoids\ \left(\frac{mg}{L\ of\ solvent}\right) = \frac{\bar{x}(Abs\ 485\ nm, Abs\ 455\ nm, Abs\ 430\ nm)}{0.03} \quad (3)$$

$$Carotenoid\ yield\ \left(\frac{mg}{g\ DCW}\right) = \frac{measured\ carotenoids\ \left(\frac{mg}{L}\right) \times 0.002\ L}{0.1\ g\ DCW} \quad (4)$$

$$Carotenoid\ production\ \left(\frac{mg}{L}\right) = \frac{carotenoid\ yield\ (\frac{mg}{g\ DCW})}{biomass\ production\ (\frac{g\ DCW}{L})} \quad (5)$$

Carotenoid profiles were calculated from the titer of single carotenoids resulted from the analysis with the Shimadzu LC-2050C HPLC (Shimadzu, Japan) equipped with Kinetex Core-Shell C18 column (Phenomenex) and LC-2050 UV detector (Shimadzu), at 40°C and a gradient of water and acetone as mobile phase at 0.8 mL/min. Detection was performed at 450 nm and all identified peaks were quantified using the β-carotene standard (Alfa Aesar, United States) according to the known carotenoid retention time profile (Weber et al., 2007).

Ultimately, FAs composition of the samples was determined by quantitative gas chromatography–mass spectrometry (GC-MS) analysis with an internal standard method adapted from Tammekivi et al., 2019.

Chapter forth

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Assembling HGA and PGAs constructs

The aim of this study was to enhance carotenoid production using *R. toruloides* by overexpression of the heterologous genes t-ylHMG1, mcCrtI and mcCrtYB, which proved to bring improved carotenoid phenotype to other yeasts in previous works. To achieve the goal, these genes were originally envisaged to take part in two genetic constructs, HGA and PGA (Fig. 4), that would integrate the *R. toruloides* genome and increase metabolic flux toward carotenoid synthesis (Fig. 2).

The constitutive promoters P14 and P15 were considered strong promoters to drive the consistent expression of these genes, while the universal terminators t35S and tNOS could complete the cassettes by adequately ending transcription. PEX10 and PEX11 genes were described earlier in the literature to be suitable loci for integration of exogenous DNA by homologous recombination in similar or same host species (Schultz et al., 2022; Monteiro de Oliveira et al., 2023). NAT and HYG resistance markers with pTUB2 and pXYL1 promoters, respectively, were both assumed to correctly select transformants in plates.

The DNA sequences of these elements were deduced from NCBI database, codon-optimized for *R. toruloides* and chemically synthesized into plasmids, which transformed the competent TOP10 *E. coli* cells (Thermo Fischer) and colony PCR-positive clones were deposited in the lab repository at -80°C.

The specific overhangs necessary for the assembly were not originally included in the synthetic fragments but were added by amplification with designed primers using high-fidelity polymerase. Gel-purified PCR fragments with BsaI recognition site and corresponding overhangs were cloned in pJET1.2 plasmid that transformed competent cells. These recombinant plasmids were sent for DNA sequencing before further steps. If mutations emerged, another clone was tested; when sequence was resulting correct, related colony was grown and stored at -80°C.

The HGA and PGA constructs were then built by assembling these parts together within pGGAselect plasmid (NEB GGA kit) via the Golden Gate assembly (GGA) method. GGA relies on the type IIS cleavage of BsaI restriction enzyme to expose the 4-nt overhangs and the activity of T4 DNA ligase for their junction after natural hydrogen bonding. HGA was assembled by GGA according to the classic one-pot assembly, while PGA required switching to a two-step methodology to produce a successful Golden Gate reaction because of the similarities between some of the

overhangs. In the first step of GGA, 3 or 4 parts were joined together and cloned in pJET1.2, enriching the repository; in the second stage, PCR amplification of these combined parts using primers designed to add new BsaI recognition sites and overhangs drove PGA assembly.

Additionally, a new construct, mut-PGA, was developed by introducing a point mutation, Y27R, in mcCrtYB gene of PGA construct, which was demonstrated by Ma et al., 2022 to remove substrate inhibition, improving enzyme selectivity and β-carotene titer. This mutation was generated by site-directed mutagenesis in a single PCR round with PGA as template. Since the primers contained the desired mutation and a BsaI site, circularization of the PCR product was achieved by incubation with BsaI restriction enzyme and T4 DNA ligase.

HGA, PGA and mut-PGA constructs were verified by DNA sequencing and used to transform *R. toruloides* SBY29 and SBY92; selected transformants from one construct were also expected to be integrated by the other. HGA successfully transformed both strains, and analogous candidates were validated and deposited as SBY161 and SBY162, respectively. On the other hand, PGA and mut-PGA never resulted in confirmed, lively clones producing more carotenoids. The transformation process was repeated many times, and different strategies such as higher amount of transforming DNA, shorter heat-shock time, or lower antibiotic

concentration in plates were tested inconclusively. Therefore, it was decided to make a new construct, PGA2.0, that did not bear mcCrtI and mcCrtYB CDSs under same promoter and terminator to exclude intramolecular recombination as the cause for transformation failure.

PGA2.0 was built in the same manner as PGA with the only difference being pJET3 and pGGA_RFP as the destination vectors of, respectively, the single parts and the final construct. Just like PGA, transformation of SBY29, -92, -161 and -162 with PGA2.0 did not originate colonies for 50 μg/mL of hygromycin nor confirmed clones using half of the concentration of the selective antibiotics. Moreover, when these non-confirmed clones obtained from transformation of SBY29 with PGA2.0 were grown in shake-flasks, they reported a much lower carotenoid production and growth than parental strain.

In conclusion, SBY161 strain was characterized for improved carotenoid production along with the parental SBY29 and SBY92 used as reference strains.

4.1.2 Characterization of SBY161

SBY29, -92 and -161 were cultivated in shake-flasks with YPD (60 g/L of glucose) and 0.2 M KCl at 30°C for 92 hours. Cultures were grown in triplicates, so the values obtained for each strain at the set times (0-, 26-, 45-, 69- and 92-hours)

were the result of arithmetic average. Additionally, the significance level used to assess statistical significance was set at 0.10.

From daily sampling conducted for HPLC analysis of glucose and cell density measurements, significant difference between the metabolically engineered strains and the parental SBY29 was observed in sugar consumption (Fig. 5) and growth (Fig. 6). At the end of the cultivation (92-h), both SBY161 and SBY92 assimilated about 45 g/L of glucose from a starting concentration of 56 g/L measured at 0-h, while consumption of SBY29 reached 42 g/L. At the same time, SBY29 showed a smaller final absorbance, which was very reduced compared to the absorbance measured at 69-h as opposed to the other two yeasts, whose exponential and stationary phases were extended and their death stage did not occur after 92 hours. Absorbance at 92-h of SBY161 was about 100, while for SBY92 it was more than 13 AU higher. Nonetheless, the maximum specific growth rate (μmax) calculated for SBY29 (0.151 1/h) was similar to those of SBY161 (0.148 1/h) and SBY92 (0.146 1/h) (Fig. 7).

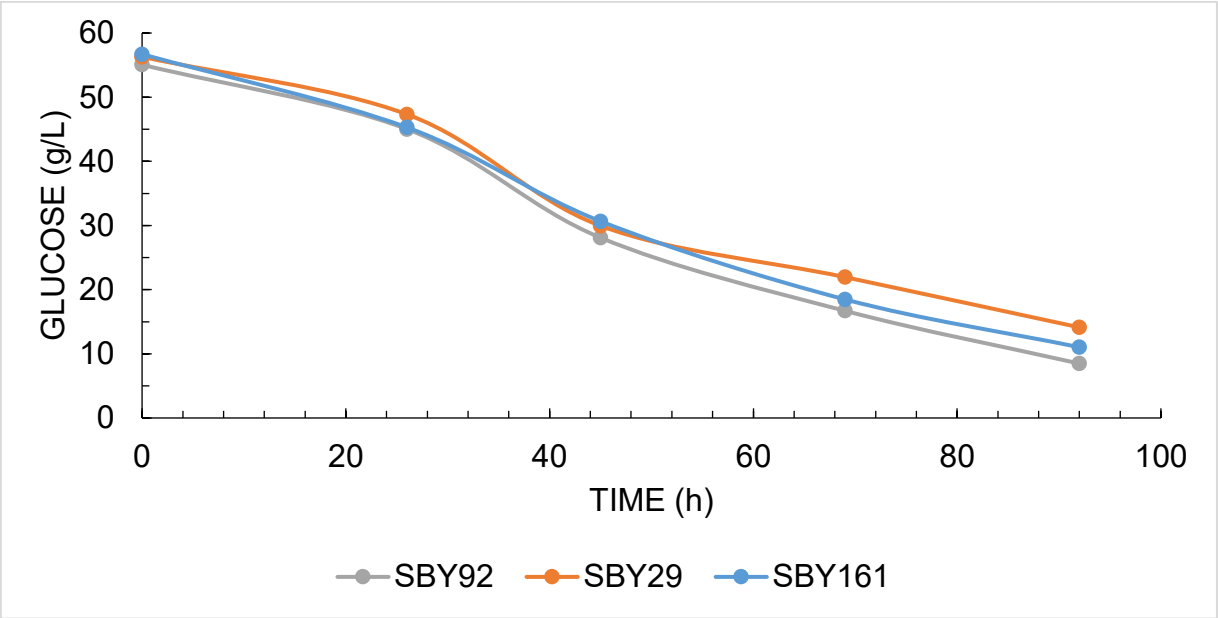

Figure 5. Glucose consumption of the three strains during the 92-h cultivation. SBY29 was statistically different than both SBY92 and SBY161, but not between SBY92 and SBY161.

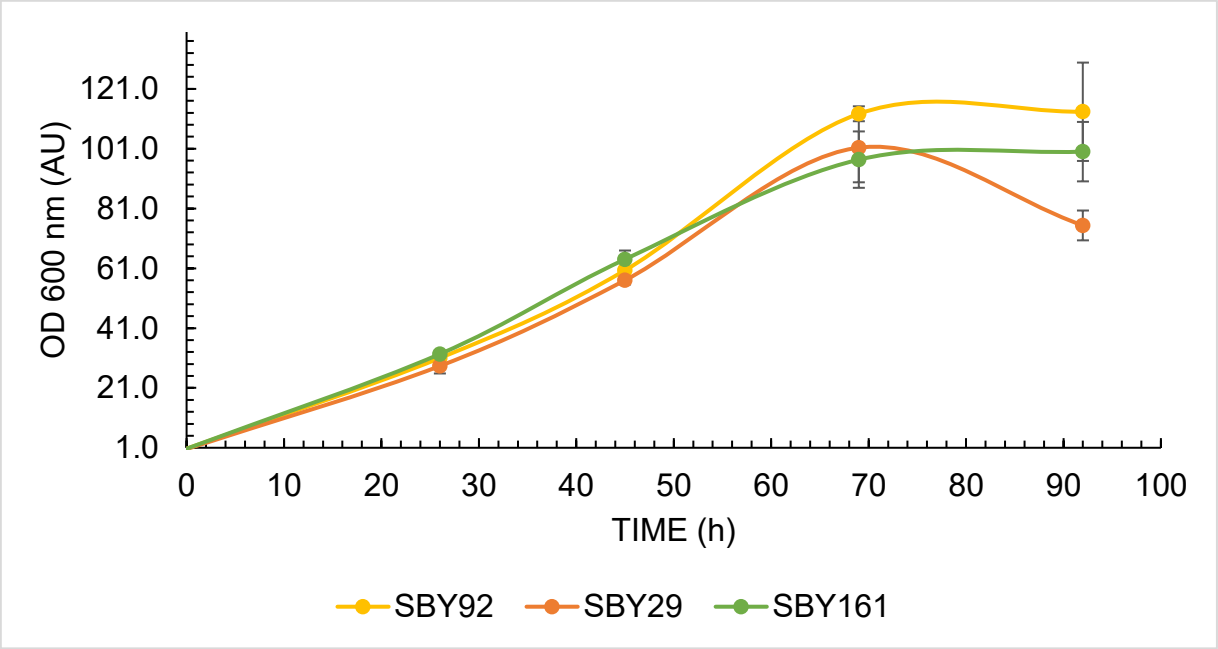

Figure 6. The characteristic growth curves of SBY92, -29, and -161 during the fermentation. SBY29 showed all the four growth stages before 92-h, while SBY92 and SBY161 arrested their growth before entering the last declining phase.

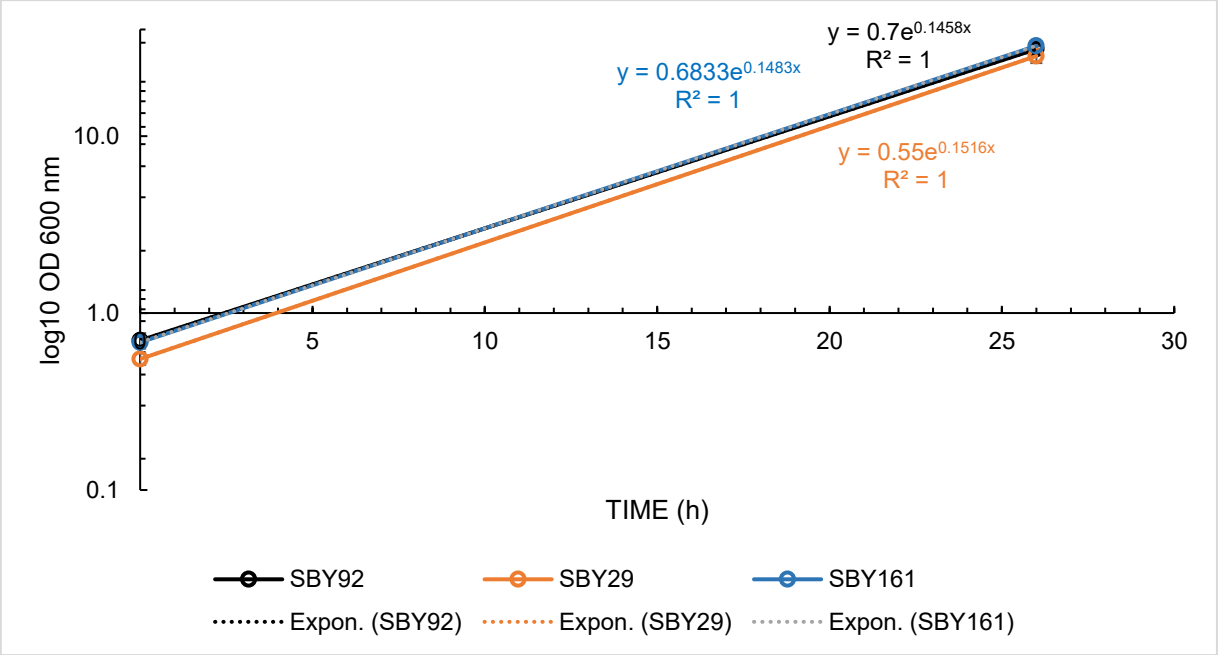

Figure 7. Graphical representation of the maximum specific growth rate (μmax) of three strains. μmax of SBY29 was similar although slightly less compared to those of SBY92 and SBY161.

Analysis of biomass, carotenoids and lipids were performed on samples drawn when fermentation terminated. Although SBY92 reported the highest absorbance at 92-h, SBY161 exhibited the highest biomass production, 21 (0.6) gDCW/L, and SBY92 the lowest, but they were not statistically different just because of the large error in SBY92 (Fig. 8). The concentration of the final biomass of SBY161 was 8% and 3% more than those produced by SBY92 and SBY29, respectively. The decrease in biomass production of SBY92 compared to parental strain was in line with the results obtained by Bonturi et al., 2022.

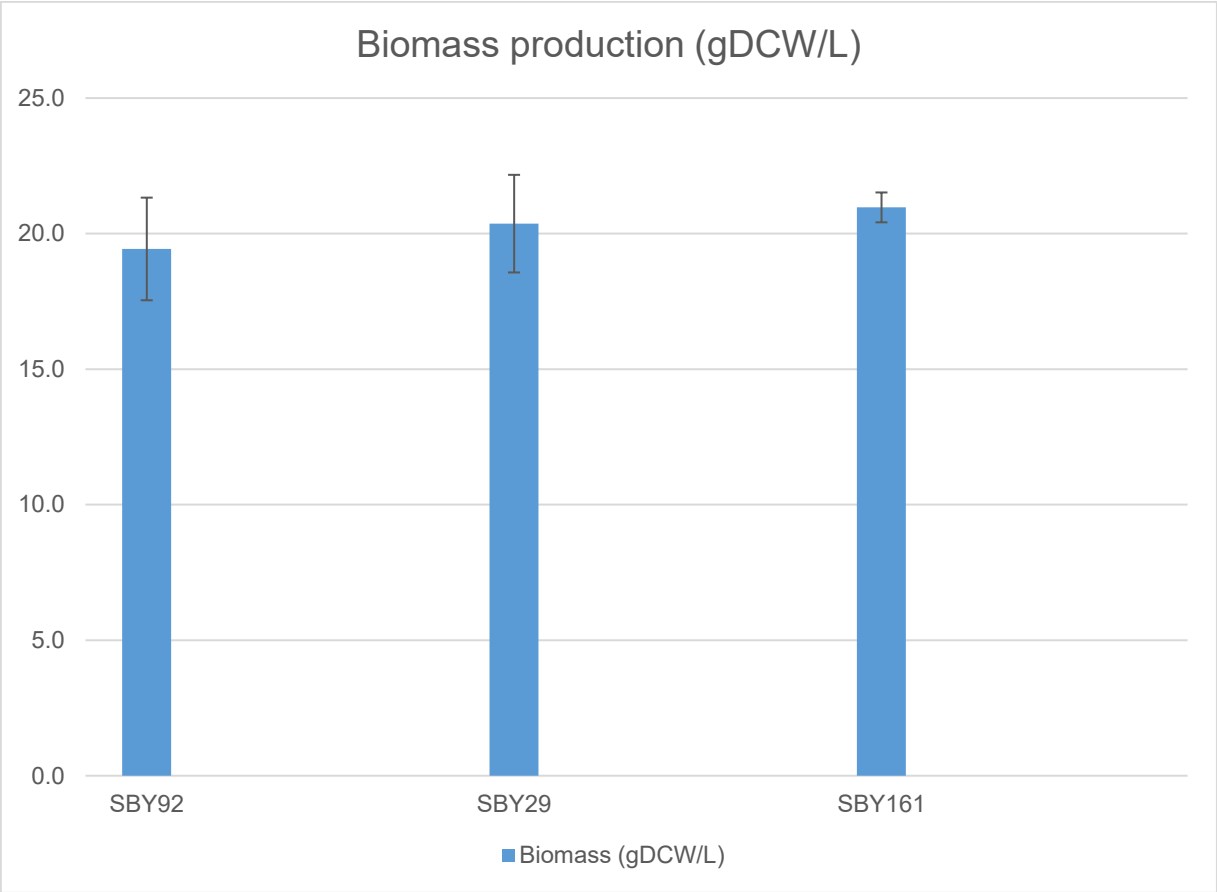

Figure 8. Biomass production by SBY92, -29, and -161 at the end of the cultivation. SBY161 reported the largest biomass, just slightly higher than SBY29. Instead, SBY92 produced the least but was characterized by a larger error.

According to the UV spectrophotometer analysis, the carotenoid production recorded by SBY161 was the highest and reached about 8.8 (0.3) mg/L, which was significantly different than SBY92 but not from SBY29 just because of the large error (Fig. 9). Based on these data, SBY161 revealed an improvement in the production of carotenoids of 9% and 20%, respectively, in comparison with SBY92 and SBY29. These results were divergent from the quantification

performed with the HPLC method, where SBY92 showed larger carotenoid production in terms of torulene, torularhodin, β- and γ-carotene, than the other two strains. As seen in previous publication, the reason for this discrepancy could be attributed to the failure of the used HPLC method to detect carotenoid intermediates, which were presumed to be present to a greater extent in SBY161 than in the others because of the integrated t-ylHMG1 gene that redirects the metabolic flux to the MVA pathway providing precursors for carotenoid synthesis.

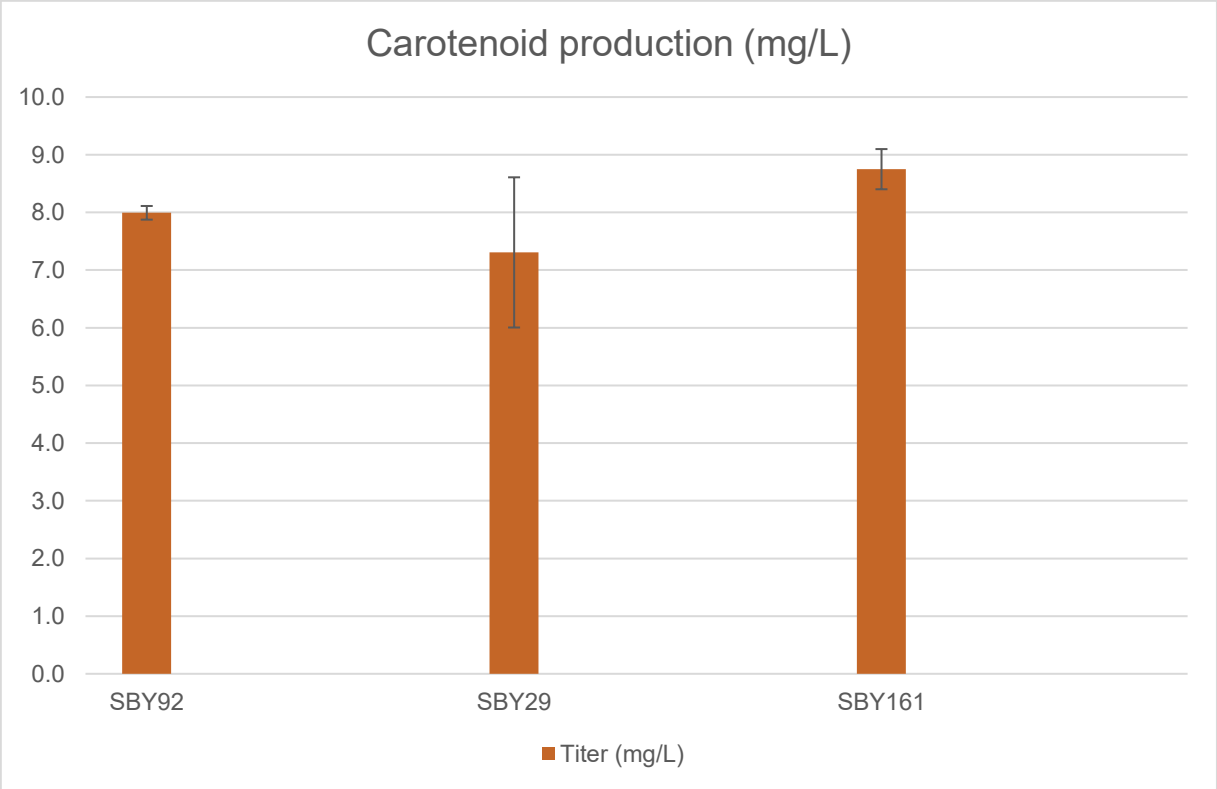

Figure 9. Carotenoids production by the three strains after 92-h fermentation. SBY161 remarkably improved carotenoid titer to respect of the parental SBY29 rather than SBY92 but was not significantly different because of the larger error unlike SBY92.

Nevertheless, carotenoid composition of the three strains was determined using the results of HPLC analysis (Fig. 10). SBY161 was statistically different from SBY92 for γ- and β-carotene but not from SBY29, whose profile was almost identical. When considered as all total carotenoids, β-carotene was the most accumulated (~58%) by SBY161, followed by torulene (~23%) and torularhodin (~16.5%), while γ-carotene was the least (~2.5%). SBY92 increased β- and γ-carotene percentage content of more than 10% and 3%, respectively, compared to SBY161, while torulene and torularhodin diminished in the opposite way. This trend was observed also in the UV absorption spectra.

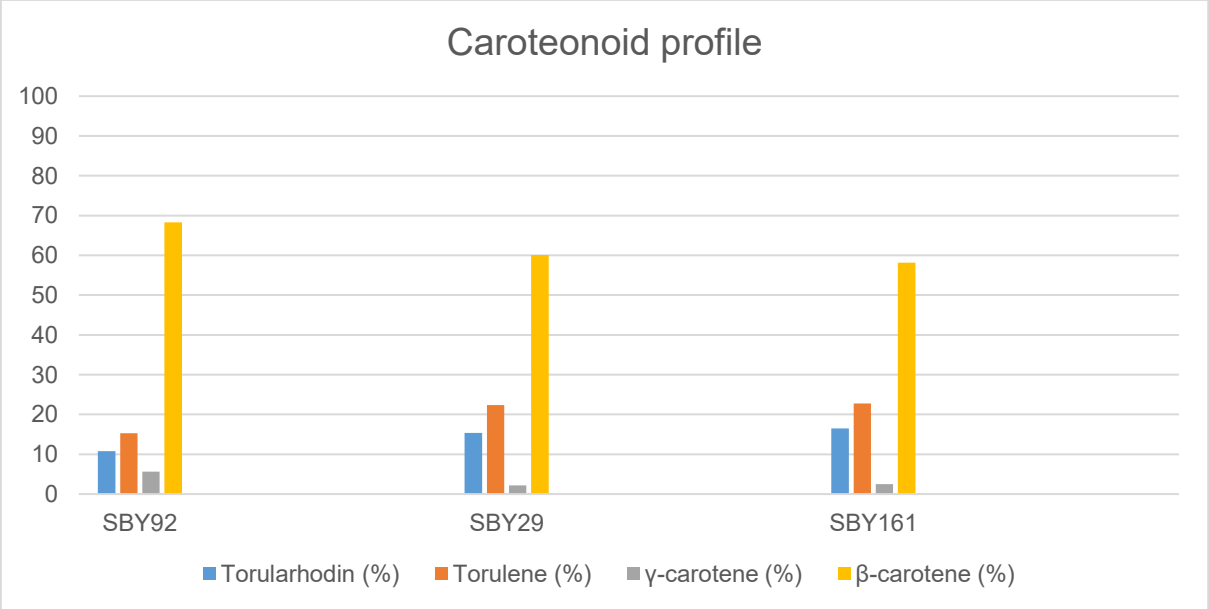

Figure 10. Percent carotenoid composition of the three strains when considering torularhodin, torulene, γ- and β-carotene. SBY161 and SBY29 showed same composition, while SBY92 produced higher β- and γ-carotene.

The carotenoid yield of SBY161 was significantly improved than SBY29 (Fig. 11). However, it was still comparable with SBY92 because the increase of carotenoid production of SBY161 was followed by the increment of its biomass. The carotenoid yield calculated for SBY161 was around 0.42 (0.01) mg/gDCW, which was almost 17% higher than that obtained by SBY29.

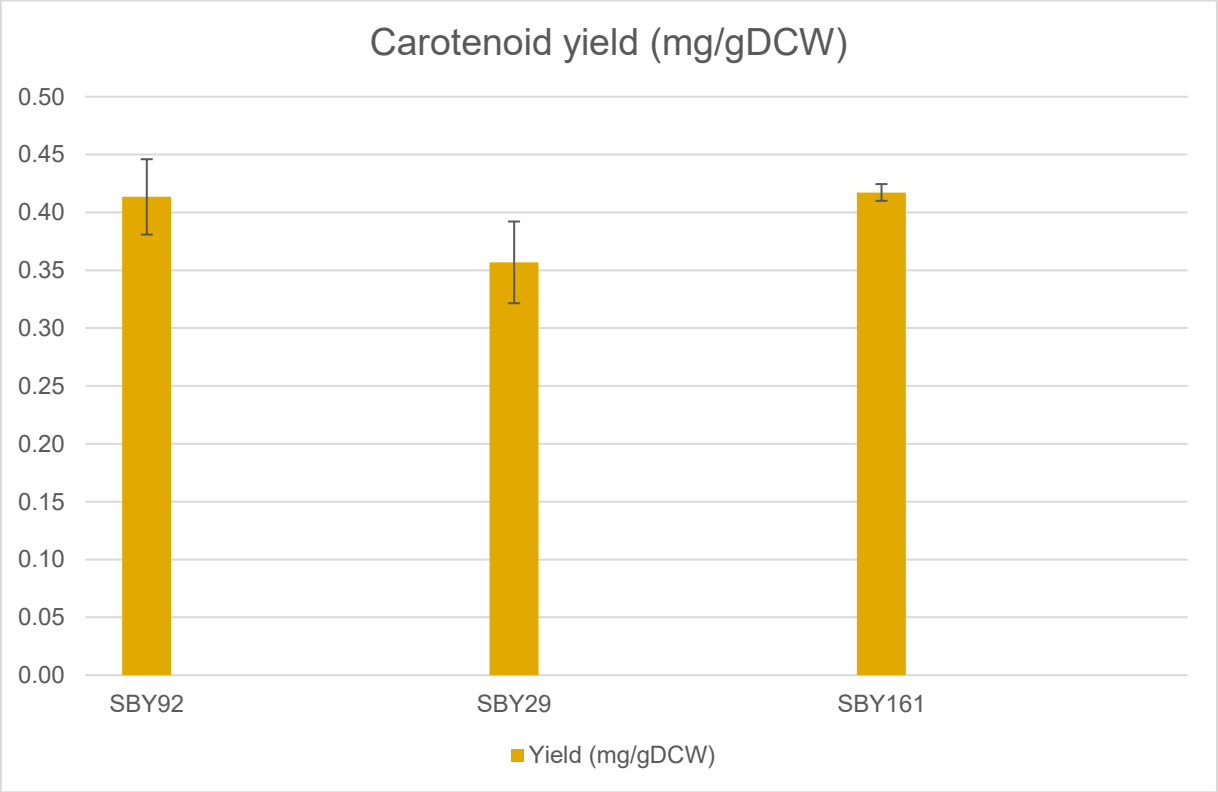

Figure 11. Carotenoid yield calculated for SBY161 was the same as SBY92 because of the increase of both carotenoid production and biomass but was much higher than SBY29.

4.2 Discussion

Overall, SBY161 strain showed 3% more biomass production, 20% more carotenoid production and 17% more carotenoid yield compared to the parental SBY29. With respect to SBY92, SBY161 exhibited 8% and 9% more biomass and carotenoid production, respectively. These results demonstrated that truncated HMG1 gene from *Y. lypolytica* was first correctly assembled into the HGA genetic construct, integrated in the genome of the adapted *R. toruloides* SBY29 strain, and sufficiently expressed to grant improved phenotype to the yeast.

The titer of carotenoids produced in this study by SBY161 was above or within the range of concentrations obtained in not fully optimized strains and culture conditions. However, to be considered for industrial production of carotenoids as the *Y. lypolytica* ob-CHC^{TEF}C^{TEF} strain that was set in this study as model due to its outstanding records of carotenoid production and yield (454.36 mg/L and 61.1 mg/gDCW of β-carotene in flask culture; 6.5 g/L and 90 mg/gDCW in fed-batch fermentation), it is necessary for SBY161 to simultaneously overexpress multiple copies of endogenous and/or heterologous tHMG1, CrtE, CrtI and CrtYB (or CrtYB-Y27R) genes under a well-studied combination of strong promoters that maximize carotenoid synthesis (Larroude et al., 2018).

HMG-CoA reductase is a rate-limiting enzyme of MVA pathway, and truncation of its gene is positively associated with increased catalytic activity in the conversion of HMG-CoA to MVA. Overexpression of t-ylHMG1 increases the yield of precursors of both the downstream ergosterol and carotenoid biosynthetic pathways (Verwaal et al., 2007). Accordingly, the larger carotenoid production of SBY161 calculated after the analysis with UV spectrophotometer and not supported by the HPLC quantification was attributed to the higher carotenoid intermediates, which could not be detected by the HPLC method and neither affected the carotenoid composition considered when compared to the parental SBY29.

Conversely, geranylgeranyl diphosphate synthase (CrtE), phytoene dehydrogenase (CrtI), and phytoene synthase/lycopene cyclase (CrtYB) are endogenous genes of *R. toruloides* whose enzymes are involved in the carotenoid pathway, so their overexpression in SBY92 correctly led to a variation in the carotenoid profile as well as in their production. Introduction of heterologous CrtI and CrtYB into *R. toruloides* genome by transformation of the PGAs constructs was not achieved. This shortcoming could be attributed to the knockout of PEX11 gene, although this was already described appropriate in similar or same host, and/or to the defective expression of the heterologous genes, but a more plausible

explanation could be found in pXYL1 promoter of hygromycin resistance marker. pXYL1 is the original promoter of xylose reductase, whose protein abundance was measured by Reķēna et al., 2023 to be about 8 times higher when xylose was the only carbon source, probably not guaranteeing an adequate expression of HYG when glucose was used for selection in YPD plates with antibiotics. Developing a construct with a stronger promoter for constitutive expression of the resistance gene like pTUB2 for NAT in HGA construct could solve the problem.

X. K. Zhang et al., 2020 described how integration of multiple copies of mcCrtI and mcCrtYB, and simultaneous overexpression of endogenous genes belonged to the MVA pathway, including HMG-CoA reductase, made a *Y. lypolytica* strain to accumulate 408 mg/L of β-carotene.

Besides t-ylHMG1, the Y27R mutation in mcCrtYB to remove substrate inhibition is another example of how engineering of enzymes is essential to fine-tune production of target molecules. Although never validated in this work, this mutation was applied by Ma et al., 2022 to the *M. circinelloides* CrtYB by structure-guided protein engineering, and introduction of this gene in *Y. lypolytica* showed significantly increased of β-carotene selectivity as well as improved of its production because of the complete loss of enzyme inhibition due to lycopene accumulation. In the same research, concomitant overexpression of mcCrtI,

conventional, truncated, and mutated mcCrtYB, heterologous lycopene cyclases and several endogenous genes of the MVA pathway, including HMG-CoA reductase, induce that strain to generate 3.43 g/L of β-carotene.

In any case, to compare different strains and devise strategies for improving carotenoid production, it is essential to develop a standard methodology for the analysis of carotenoids. For instance, Bonturi et al., 2022 and Rusadze, 2020 reported different carotenoid profiles for same strains and identical cultivation conditions. In this study, SBY29 and SBY92 were both cultured in shake flasks with a medium at C/N ratio of 80 (mol/mol) containing 30 g/L of xylose as sole carbon source, 0.8 g/L of ammonium sulphate ($(NH_4)_2SO_4$), 3 g/L of monopotassium phosphate (KH_2PO_4), 0.5 g/L of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 1 mL of vitamins solution, and 1 mL of trace metal solutions (Lahtvee et al., 2017). Bonturi et al., 2022 described γ-carotene as the most abundant carotenoid for both strains, which was even higher for SBY92 at the expense of β-carotene along with torularhodin. For Rusadze, 2020, β-carotene was the preeminent carotenoid for SBY29, while torulene predominated in SBY92. When SBY29 was instead characterized by Pinheiro et al., 2020 in 1-L bioreactor with the same medium composition but higher concentration of xylose (70 g/L) and $(NH_4)_2SO_4$ (1.95 g/L), torularhodin accumulated the most, and torulene had

the second place, both under light irradiation and not. In the same work, it was shown the fluctuation of carotenoid titer (%) during the progress of cultivation. β-carotene, together with torularhodin, took a higher place at the beginning of the cultivation but it descended in favor of torulene by the end. Furthermore, under light irradiation torularhodin was 50% higher during nitrogen starvation probably owing to its stronger antioxidant activity.

Chapter fifth

CONCLUSIONS

This Master's thesis was intended to improve carotenoid production of *R. toruloides* CCT 7815 (SBY29) by overexpression of heterologous genes related to the metabolic pathway of carotenoid biosynthesis.

Both HGA construct with t-ylHMG1 and PGAs with mcCrtI and mcCrtYB were successfully in silico designed and correctly assembled by the popular GGA cloning, and together with their parts enriched the laboratory repository.

The *R. toruloides* SBY161 strain, resulted from transformation of SBY29 with HGA, showed increased biomass production (21 gDCW/L), carotenoid titer (8.8 mg/L) and yield (0.42 mg/gDCW) with respect to the parental and the engineered SBY92 strain after characterization in shake-flask.

Unfortunately, SBY162 strain obtained from transforming SBY92 with HGA was not characterized for enhanced carotenoid phenotype due to limited time.

At the same time, transformation of the PGA construct and their derivates, mut-PGA and PGA2.0, was not attained in this work despite considerable efforts.

In conclusion, to transform *R. toruloides* into a competitive microbial cell factory for industrial-scale production of carotenoids or value added-SCP and -SCO, it is

crucial to first select high lipid overproducer strains, refine expression of genes from MVA and carotenoid biosynthesis pathways, minimize inhibition of their enzymes, increase cofactors, disrupt competing pathways, expand carotenoid storage, and adjust cultivation conditions (C. Li et al., 2020).

Chapter sixth

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