18

Production of Enriched Biomass by Carotenogenic Yeasts - Application of Whole-Cell Yeast Biomass to Production of Pigments and Other Lipid Compounds

Ivana Marova[1], Milan Certik[2] and Emilia Breierova[3]
[1]*Brno University of Technology, Faculty of Chemistry, Centre for MaterialsResearch, Purkynova 118, 612 00 Brno,*
[2]*Slovak Technical University, Faculty of Chemical and Food Technology, Bratislava,*
[3]*Institute of Chemistry, Slovak Academy of Sciences, Bratislava,*
[1]*Czech Republic*
[2,3]*Slovak Republic*

1. Introduction

Yeasts are easily grown unicellular eukaryotes. They are ubiquitous microorganisms, occuring in soil, fresh and marine water, animals, on plants and also in foods. The environment presents for yeast a source of nutrients and forms space for their growth and metabolism. On the other hand, yeast cells are continuously exposed to a myriad of changes in environmental conditions. These conditions determine the metabolic activity, growth and survival of yeasts. Basic knowledge of the effect of environmental factors on yeast is important for understanding the ecology and biodiversity of yeasts as well as for control the yeast physiology in order to enhance the exploitation of yeasts or to inhibit or stop their harmful and deleterious activity.

The overproduction of some metabolites as part of cell stress response can be of interest to the biotechnology. For instance carotenogenic yeasts are well known producers of biotechnologically significant carotenoid pigments - astaxanthin, β-carotene, torulen, torularhodin and under stress conditions this carotenoid accumulation was reported to be increased. Knowledge of molecular mechanism of the carotenoid production stimulation can then lead to improvement of such biotechnological process. Red yeasts are able to accumulate not only carotenoids, but also ergosterol, unsaturated fatty acids, Coenzyme Q10 and other, which can contribute to the biomass enrichment. The use of this stressed biomass in feed industry could have positive effect not only in animal and fish feeds because of high content of physiologically active substances, but it could influence nutritional value and organoleptic properties of final products for human nutrition.

Yeast biomass, mainly in the form of *Saccharomyces cerevisiae*, represents the largest bulk production of any single-celled microorganism throughout the world. In addition to use of

live yeast biomass for the leavening of bread dough, many other applications of yeast cells and yeast cell extracts have emerged. Most yeast biomass for industrial use is derived from *Saccharomyces cerevisiae*, but other yeasts have specific uses and may be grown on a range of substrates unavailable to *S.cerevisiae*. Some yeast strains are usable to industrial single-cell protein production from lignocellulose materials, methanol, n-alkanes, starch, oils and also other cheap carbon sources. Except compresses baker´s yeasts for baking, brewing, winemaking and distilling also other whole-cell yeast products are industrially used as animal feed, human and animal probiotics, as biosorbents for heavy metal sequestration and, also as nutritional trace element sources. Yeasts are rich sources of proteins, nucleic acids, vitamins and minerals but mostly with negligible levels of triglycerides.
Pigmented yeasts are used as feed and food colorants and, come of them, also as single cell oil producers. This chapter will be focused on controlled production of biomass and some interesting lipid metabolites of several non-traditional non-*Saccharomyces* yeast species. Growing interest in yeast applications in various fields coupled with significance of carotenoids, sterols and other provitamins in health and dietary requirements has encouraged "hunting" for more suitable sources of these compounds.

2. Production of enriched biomass by carotenoid-forming yeasts

2.1 Characterization of red (carotenogenic) yeasts

2.1.1 Taxonomy

Yeasts belong to the kingdom Fungi (Mycota) - a large group of eukaryotic organisms that includes microorganisms such as yeasts and moulds. Some species grow as single-celled yeasts that reproduce by budding or binary fission. Dimorphic fungi can switch between a yeast phase and a hyphal phase in response to environmental conditions. The fungal cell wall is composed of glucans and chitin. Another characteristic shared with plants includes a biosynthetic pathway for producing terpenes that uses mevalonic acid and pyrophosphate as chemical building blocks (Keller et al., 2005). Fungi produce several secondary metabolites that are similar or identical in structure to those made by plants. Fungi have a worldwide distribution, and grow in a wide range of habitats, including extreme environments such as deserts or areas with high salt concentrations or ionizing radiation, as well as in deep sea sediments. Some can survive the intense UV and cosmic radiation.
Around 100,000 species of fungi have been formally described by taxonomists, but the global biodiversity of the fungus kingdom is not fully understood. There is no unique generally accepted system at the higher taxonomic levels and there are frequent name changes at every level, from species upwards. Fungal species can also have multiple scientific names depending on their life cycle and mode (sexual or asexual) of reproduction. The 2007 classification of Kingdom Fungi is the result of a large-scale collaborative research. It recognizes seven phyla, two of which—the *Ascomycota* and the *Basidiomycota*—are contained within a branch representing subkingdom Dikarya (Hibbett, 2007).
The Ascomycota constitute the largest taxonomic group within the *Eumycota*. These fungi form meiotic spores called ascospores, which are enclosed in a special sac-like structure called an ascus. This phylum includes single-celled yeasts (e.g., of the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, and *Candida*), and many filamentous fungi living as saprotrophs, parasites, and mutualistic symbionts.
Some yeast species accumulate carotenoid pigments, such as β-carotene, torulene, and thorularodin which cause their yellow, orange and red colours and are therefore called red

yeasts. Carotenogenic yeasts are a diverse group of unrelated organisms (mostly *Basidiomycota*) and the majority of the known species are distributed in four taxonomic groups: the *Sporidiobolales* and *Erythrobasidium* clade of the class *Urediniomycetes*, and *Cystofilobasidiales* and *Tremellales* of the class *Hymenomycetes* (Libkind et al., 2005). Along with the most known producer P*haffia rhodozyma*, there is evidence of the capacity for carotene formation by other well-known pigmented yeasts of the genus *Rhodotorula* (order *Sporidiobolales)*. The composition and amount of the carotenoid pigments in numerous natural isolates of the genera *Rhodotorula/ Rhodosporium* and *Sporobolomyces/Sporidiobolus* were studied in detail (Yurkov et al., 2008).

At this time the number of red yeasts species *Rhodotorula, Rhodosporidium, Sporidiobolus, Sporobolomyces, Cystofilobasidium, Kockovaella* and *Phaffia* are known as producers of carotene pigments. Many of these strains belong to oleaginous yeasts, some of them can effectively remove heavy metals from industrial effluents and detoxify certain pollutants. Studies with yeast mutants or carotenoid biosynthesis inhibitors have shown that carotenoid-deficient yeast strains are sensitive to free oxygen radicals or oxidizing environment, and that this sensitivity can be relieved by the addition of exogenous carotenoids (Davoli et al., 2004). The major yeast pigments are β-carotene, γ-carotene, torulene, torularhodin and astaxanthin (Dufosse, 2006).

2.1.2 Morphology and growth characteristics of main red yeast species

The genus *Rhodotorula* includes three active species; *Rhodotorula glutinis*, *Rhodotorula minuta* and *Rhodotorula mucilaginosa* (formerly known as *Rhodotorula rubra*) (Hoog et al., 2001). Colonies are rapid growing, smooth, glistening or dull, sometimes roughened, soft and mucoid (Figures 1 – 3). They are cream to pink, coral red, orange or yellow in color. Blastoconidia that are unicellular, and globose to elongate in shape are observed. These blastoconidia may be encapsulated. Pseudohyphae are absent or rudimentary. Hyphae are absent. *Rhodotorula glutinis* often called "pink yeast" is a free living, non-fermenting, unicellular yeast found commonly in nature. *Rhodotorula* is well known for its characteristic carotenoids "torulene, torularhodin and β-carotene. *Rhodotorula glutinis* is also reported to accumulate considerable amount of lipids (Perier et al., 1995).

The genus *Sporobolomyces* contains about 20 species. The most common one is *Sporobolomyces roseus* and *Sporobolomyces salmonicolor* (Hoog et al., 2001). *Sporobolomyces* colonies grow rapidly and mature in about 5 days. The optimal growth temperature is 25-30°C. The colonies are smooth, often wrinkled, and glistening to dull. The bright red to orange color of the colonies is typical and may resemble *Rhodotorula spp*. *Sporobolomyces* produces yeast-like cells, pseudohyphae, true hyphae, and ballistoconidia. The yeast-like cells (blastoconidia, 2-12 x 3-35 μm) are the most common type of conidia and are oval to elongate in shape. Pseudohyphae and true hyphae are often abundant and well-developed. Ballistoconidia are one-celled, usually reniform (kidney-shaped), and are forcibly discharged from denticles located on ovoid to elongate vegetative cells (Figures 4, 5) .

Among yeasts, *Rhodotorula* species is one of main carotenoid-forming microorganisms with predominant synthesis of β-carotene, torulene and torularhodin (Davoli et al., 2004; Libkind and van Broock, 2006; Maldonade et al., 2008). *Cystofilobasidium* (Figure 6) and *Dioszegia* were also found to synthesize these three pigments. Some of yeast carotenoids are modified with oxygen-containing functional groups. For example, astaxanthin is almost exclusively formed by *Phaffia rhodozyma* (*Xanthophyllomonas dendrorhous*; Frengova & Beshkova, 2009).

Nevertheless, although there are many strategies for stimulation of carotene biosynthetic machinery in yeasts, attention is still focused on unexplored yeast's habitats for selection of hyper-producing strains what is the important step towards the design and optimization of biotechnological process for pigment formation (Libkind & van Broock, 2006; Maldonade et al., 2008).

Studies on a number of fungi, including *Neurospora crassa*, *Blakeslea trispora*, *Mucor hiemalis, Mucor circinelloides* and *Phycomyces blakesleeanus* (oleaginous fungi with carotene-rich oil) have been published over the last twenty years (Dufosse, 2006). Fungal carotenoid content is relatively simple with dominat levels of β-carotene. Recent work with dimorphic fungal mutants *M. circinelloides* and *Blakeslea trispora* (Cerda-Olmedo, 2001) showed that these strains could be useful in a biotechnological production of carotenoids in usual fermentors.

In order to study yeast physiology under different conditions, it is important to know so called "reference parameters" which these yeasts possess under optimal condition. Red or carotenogenic yeasts are well known producers of valuable carotenoids. On agar plates they form characteristic yellow, orange and red coloured colonies. Red yeast can be of ellipsoidal or spherical shape (Figures 1 - 6). Under optimal conditions (28 °C, 100 rpm, permanent lightening) they are able to grow up in 5 to 7 days. The growth curve of *Rhodotorula glutinis* CCY 20-2-26 as well as other studied red yeast exhibited similarly typical two-phase character with prolonged stationary phase (Figures 7, 8) probably due to the ability of the yeast cells to utilize lipid storages formed during growth as additional energy source (Marova et al., 2010). The production of carotenoids during growth fluctuated and some local maxima and minima were observed. The maximum of beta-carotene production was obtained in all strains in stationary phase after about 80 hours of cultivation.

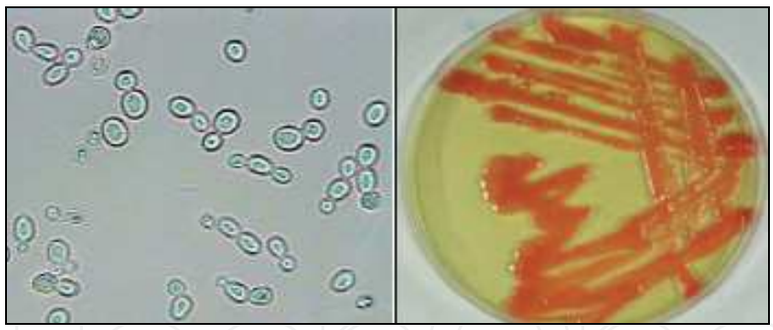

Fig. 1. Microscopic image and streak plate o*f Rhodotorula glutinis*

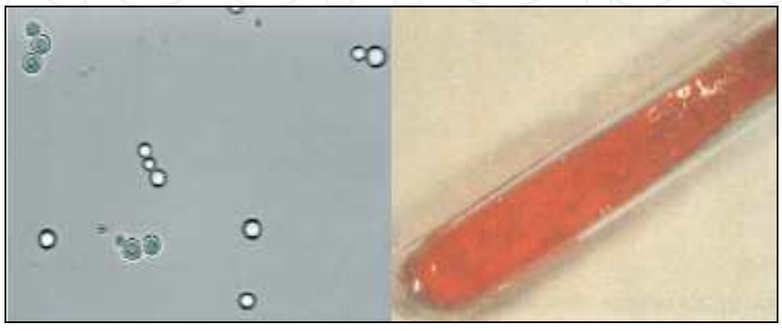

Fig. 2. Microscopic image and streak plate of *Rhodotorula rubra*

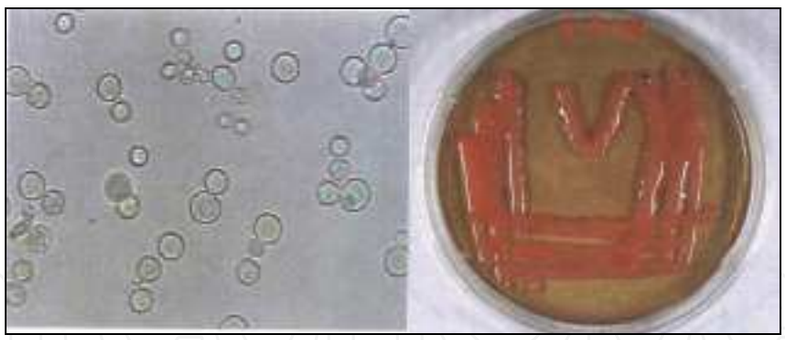

Fig. 3. Microscopic image of *Rhodotorula aurantiaca*

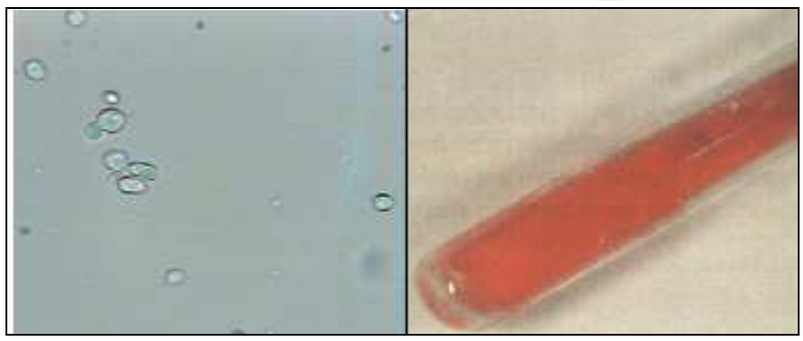

Fig. 4. Microscopic image and streak plate of *Sporobolomyces roseus*

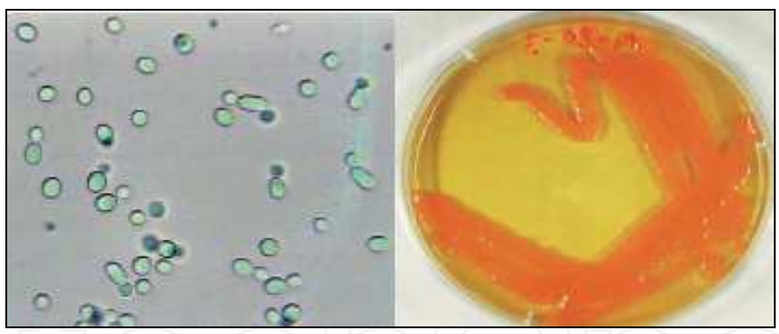

Fig. 5. Microscopic image and streak plate of *Sporobolomyces shibatanus*

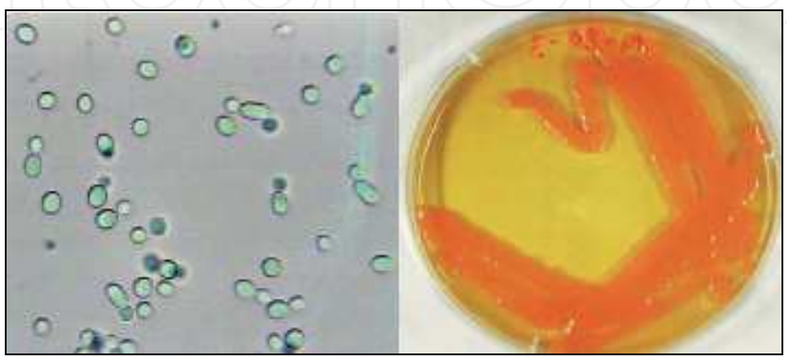

Fig. 6. Microscopic image and streak plate of *Cystofilobasidium capitatum*

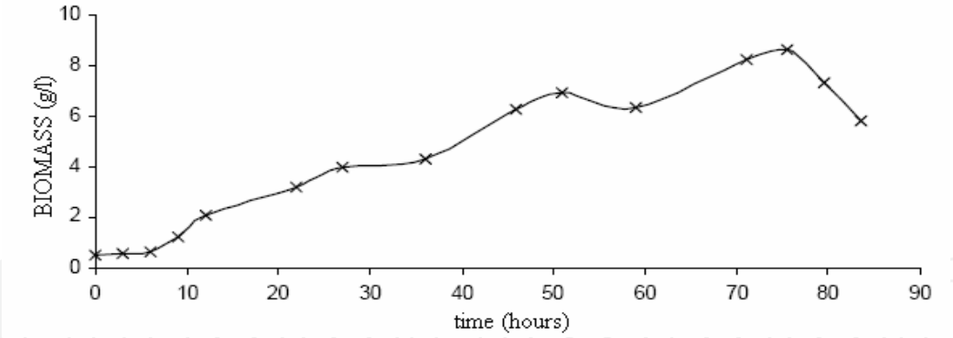

Fig. 7. Growth curve of *Rhodotorula glutinis*

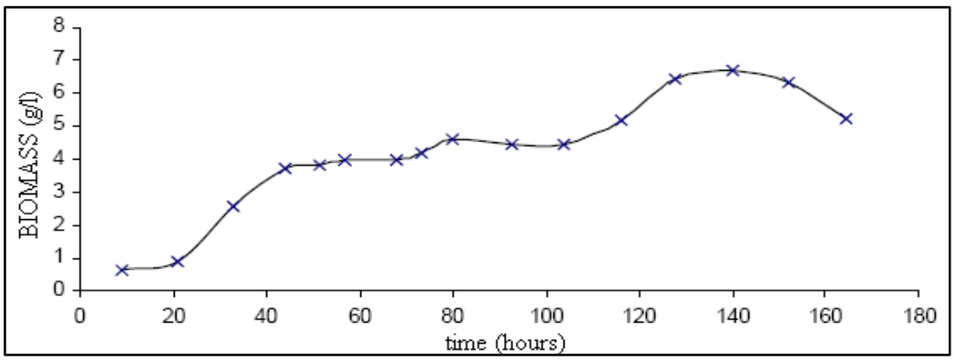

Fig. 8. Growth curve of *Sporobolomyces shibatanus*

Comparison of presented growth curves led to some partial conclusions about growth of red yeasts (Marova et al., 2010). All tested strains reached stationary phase after about 50 hours of cultivation. All strains also exhibited prolonged stationary phase with at minimum one, more often with several growth maxima. First growth maximum was observed in all strains after about 80 hours of growth. In strains followed for longer time than 100 hours additional growth maximum was observed after 105 – 140 hours. Carotenogenic yeasts probably utilize some endogenous substrates accumulated at the beginning of stationary phase. Growth maxima are mostly accompanied with carotenoid production maxima mainly in first 90 hours of cultivation. Cultivation in production media in presence of some stress factors or using waste substrates is recommended to carry out to first production maximum (about 80 – 90 hours) to eliminate potential growth inhibiton caused by nutrient starvation or toxic effect of stress. Longer cultivation can be also complicated by higher ratio of dead and living cells and in semi-large-scale and large-scale experiments also with higher production costs.

2.2 The main features of red yeast metabolism

Metabolism is the sum of cellular chemical and physical activities. It involves chemical changes to reactants and the release of products using well-established pathways regulated at many levels. Knowledge of such regulation in yeasts is crucial for exploitation of yeast cell physiology in biotechnology (Talaro & Talaro, 2001). At controlled cultivation conditions oleaginous red yeasts could be a good source (producer) of lipidic primary metabolites as neutral lipids, phospholipids and fatty acids and ergosterol, which is integrate part of yeast biomembranes.

Secondary metabolism is a term for pathways of metabolism that are not absolutely required for the survival of the organism. Examples of the products include antibiotics and pigments. The induction of secondary metabolism is linked to particular environmental conditions or developmental stages. When nutrients are depleted, microorganisms start producing an array of secondary metabolites in order to promote survival (Mann, 1990). Filamentous fungi and yeasts show a relatively low degree of cellular differentiation, but still they express a complex metabolism resulting in the production of a broad range of secondary metabolites and extracellular enzymes. This very high metabolic diversity has been actively exploited for many years. In terms of biotechnological application fungi and yeast have the advantage of being relatively easy to grow in fermenters and they are therefore well-suited for large-scale industrial production. Biomass enriched by suitable mixture of primary and secondary metabolites can be used too, mainly in feed and food applications (Mann, 1990, Walker 1998).
In general, biosynthesis of individual metabolites is governed by the levels and activities of enzymes employed to the total carbon flux through the metabolic system. Efficiency of that flow depends on the cooperation of individual pathways engaged in this process and which pathway is suppressed or activated varies with the growth medium composition, cultivation conditions, microbial species and their developmental stage. Because overall yield of metabolites is directly related to the total biomass yield, to keep both high growth rates and high flow carbon efficiency to carotenoids by optimal cultivation conditions is essential in order to achieve the maximal metabolite productivity (Certik et al., 2009).

2.2.1 The isoprenoid pathway

Isoprenoids occur in all eukaryotes. Despite the astonishing diversity of isprenoid molecules that are produced, there is a great deal of similarity in the mechanisms by which different species synthesize them. In fact, the initial phase of isoprenoid synthesis (the synthesis of isopentenyl pyrophosphate) appears to be identical in all of the species in which this process has been investigated. Thus, some early steps of isporenoid pathway could be used for genetic modification.
Starting with the simple compounds acetyl-CoA, glyceraldehyde-3-phopsphate, and pyruvate, which arise via the central pathawys of metabolism, the key intermediate isopentenyl diphosphate is formed by two independent routes. It is then converted by bacteria, fungi, plants and animals into thousands of different naturally occuring products. In fungi, carotenoids are derived by sequnce reactions via the mevalonate biosynthetic pathway. The main product 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is finaly reduced to the mevalonic acid. This two-step reduction of HMG-CoA to mevalonate is highly controlled and is also a major control factor of sterol synthesis (Metzler, 2003). From prenyl diphosphates of different chain lengths, specific routes branch off into various terpenoid end products (Figure 9).

2.2.2 Carotenoid biosynthesis

Carotenoids are synthesized in nature by plants and many microorganisms. In addition to very few bacterial carotenoids with 30, 45, or 50 carbon atoms, C40-carotenoids represent the majority of the more than 600 known structures. Two groups have been singled out as the most important: the carotenes which are composed of only carbon and hydrogen; and the xanthophylls, which are oxygenated derivatives (Frengova & Beshkova, 2009). In the

later, oxygen can be present as OH groups, or as oxy-groups or in a combination of both (as in astaxanthin). Hydroxy groups at the ionone ring may be glycosylated or carry a glycoside fatty acid ester moiety. Furthermore, carotenoids with aromatic rings or acyclic structures with different polyene chains and typically 1-methoxy groups can also be found. Typical fungal carotenoids possess 4-keto groups, may be monocyclic, or possess 13 conjugated double bonds (Britton et al., 1998).

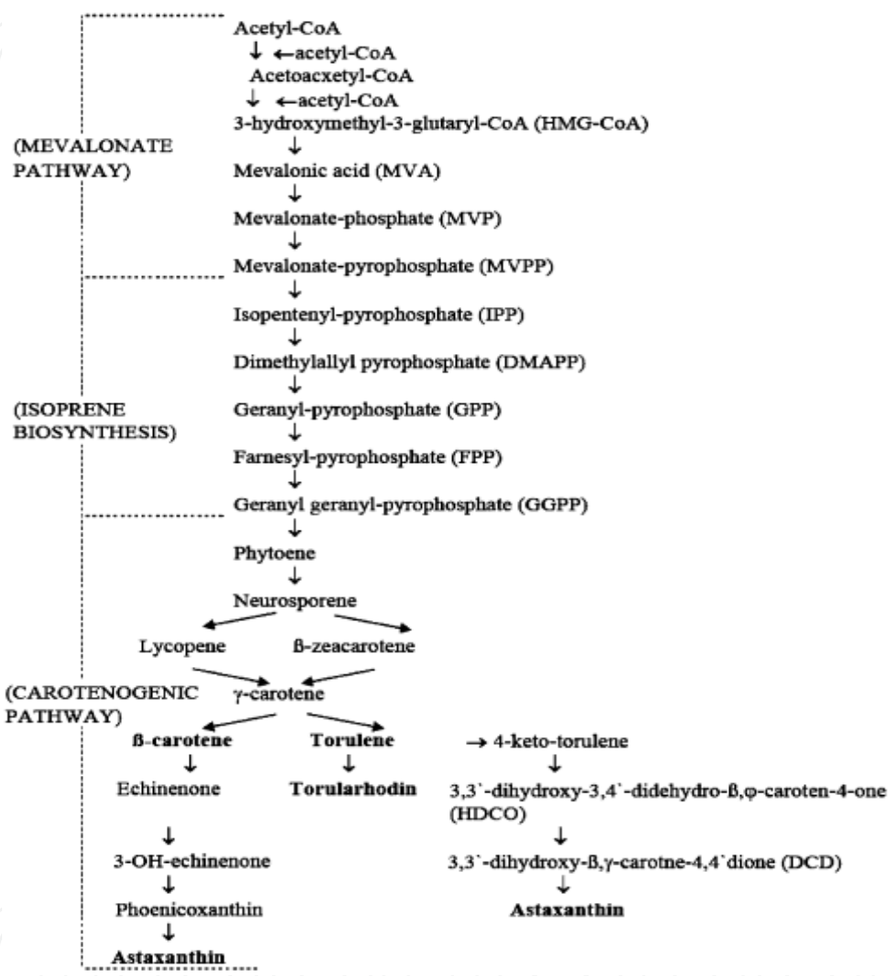

Fig. 9. Biosynthetic pathways from acetyl-CoA to β-carotene, torulene and torularhodin in *Rhodotorula* species and astaxanthin in *P. rhodozyma/X. dendrorhous* (Frengova & Beshkova, 2009)

All carotenoids are derived from the isoprenoid or terpenoid pathway. Carotenoids biosynthesis pathway commonly involves three steps: (i) formation of isopentenyl pyrophosphate (IPP), (ii) formation of phytoene and (iii) cyclization and other reactions of lycopene (Armstrong & Hearst, 1996). Before polyprenyl formation begins, one molecule of IPP must be isomerized to DMAPP. Condensation of one molecule of dimethylallyl diphosphate (DMADP) and three molecules of isopentenyl diphosphate (IDP) produces the diterpene geranylgeranyl diphosphate (GGDP) that forms one half of all C40 carotenoids. The head to head condensation of two GGDP molecules results in the first colorless

carotenoid, phytoene. As Figure 9 shows, phytoene synthesis is the first committed step in C40-carotenoid biosynthesis (Britton et al., 1998; Sandmann, 2001). Subsequent desaturation reactions lengthen the conjugated double bond system to produce neurosporene or lycopene (Schmidt-Dannert, 2000).
Following desaturation, carotenoid biosynthesis branches into routes for acyclic and cyclic carotenoids. In phototrophic bacteria acyclic xanthophylls spheroidene or spheroidenone and spirilloxanthin, respectively are formed (Figure 9). Synthesis of cyclic carotenoids involves cyclization of one or both end groups of lycopene or neurosporene. Typically, β-rings are introduced, but formation of ε-rings is common in higher plants and carotenoids with γ-rings are found, for example, in certain fungi. Most cyclic carotenoids contain at least one oxygen function at one of the ring carbon atoms. Cyclic carotenoids with keto-groups at C4(C4´) and/or hydroxy groups at C3(C3´) (e.g. zeaxanthin, astaxanthin, echinenone and lutein) are widespread in microorganisms and plants (Schmidt-Dannert, 2000).

2.2.3 Ergosterol biosynthesis

Ergosterol, one of the most important components in fungal membranes, is involved in numerous biological functions, such as membrane fluidity regulation, activity and distribution of integral proteins and control of the cellular cycle. Ergosterol pathway is fungal-specific; plasma membranes of other organisms are composed predominantly of other types of sterol. However, the pathway is not universally present in fungi; for example, *Pneumocystis carinii* plasma membranes lack ergosterol. In *S. cerevisiae*, some steps in the pathway are dispensible while others are essential for viability (Tan et al., 2003).
Biosynthesis of ergosterol similarly to carotenoids and other isoprenoid compounds (e.g. ubiquinone), is derived from acetyl-CoA in a three-stage synthehtic process (Metzler, 2003). Stage one is the synthesis of isopenthenyl pyrophosphate (IPP), an activated isoprene unit that is the key building block of ergosterol. This step is identical with mevalonate pathway (Figure 9). Stage two is the condensation of six molecules of IPP to form squalene. In the stage three, squalene cyclizes in an astounding reaction and the tetracyclic product is subsequently converted into ergosterol. In the ergosterol pathway, steps prior to squalene formation are important for pathway regulation and early intermediates are metabolized to produce other essential cellular components (Tan et al, 2003). It should be noted that isoprenoid pathway is of great importance in secondary metabolism. Combination of C5 IPP units to squalene exemplifies a fundamental mechanism for the assembly of carbon skeletons in biomolecules. A remarkable array of compounds is formed from IPP, the basic C5 building block. Several molecules contain isporenoid side chains, for example Coenzyme Q10 has a side chain made ud of 10 isporene units.

2.2.4 Gene regulation of isoprenoid pathway branches

The isoprenoid pathway in yeasts is important not only for sterol biosynthesis but also for the production of non-sterol molecules, deriving from farnesyl diphosphate (FPP), implicated in N-glycosylation and biosynthesis of heme and ubiquinones. FPP formed from mevalonate in a reaction catalyzed by FPP synthase (Erg20p). In order to investigate the regulation of Erg20p in *Saccharomyces cerevisiae*, a two-hybrid screen was used for its searching and five interacting proteins were identified. Subsequently it was showed that Yta7p is a membrane-associated protein localized both to the nucleus and to the endoplasmic reticulum. Deletion of Yta7 affected the enzymatic activity of cis-

prenyltransferase (the enzyme that utilizes FPP for dolichol biosynthesis) and the cellular levels of isoprenoid compounds. Additionally, it rendered cells hypersensitive to lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) that acts upstream of FPP synthase in the isoprenoid pathway. While HMGR is encoded by two genes, HMG1 and HMG2, only HMG2 overexpression was able to restore growth of the yta7- cells in the presence of lovastatin. Moreover, the expression level of the *S. cerevisiae* YTA7 gene was altered upon impairment of the isoprenoid pathway not only by lovastatin but also by zaragozic acid, an inhibitor of squalene synthase (Kuranda et al., 2009).

All enzymes involved in carotenoid biosynthesis are membrane-associated or integrated into membranes. Moreover, carotenoid biosynthesis requires the interaction of multiple gene products. At present more than 150 genes, encoding 24 different *crt* enzymes involved in carotenogenic branch of isoprenoid pathway, have been isolated from bacteria, plants, algae and fungi. The availability of a large number of carotenogenic genes makes it possible to modify and engineer the carotenoid biosynthetic pathways in microorganisms. A number of genetically modified microbes, *e.g. Candida utilis, Escherichia coli, Saccharomyces cerevisiae, Zymomonas mobilis*, etc. have been studied for carotenoid production (Wang et al. 2000; Schmidt-Dannert, 2000; Lee & Schmidt-Dannert, 2002; Sandmann 2001). However, lack of sufficient precursors (such as IDP, DMADP and GGDP) and limited carotenoid storage capability is the main task how to exploate these organisms as commercial carotenoid producers. Therefore, effort has been focuced on increasing the isoprenoid central flux and levels of carotenoid precursors. For example, overexpression of the IDP isomerase (*idi* - catalyzes the isomerization of IDP to DMAP) together with an archaebacterial multifunctional GGDP synthase (*gps* - converts IDP and DMADP directly to GGDP) resulted in a 50-fold increase of astaxanthin production in *E. coli* (Wang et al., 2000).

By combination of genes from different organisms with different carotenoid biosynthetic branches, novel carotenoids not found in any other pathway can be synthesized. Most *Mucor* species accumulate β-carotene as the main carotenoid. The crtW and crtZ astaxanthin biosynthesis genes from *Agrobacterium aurantiacum* were placed under the control of *Mucor circinelloides* expression signals. Transformants that exhibited altered carotene production were isolated and analyzed. Studies revealed the presence of new carotenoid compounds and intermediates among the transformants (Papp et al., 2006). *Fusarium sporotrichioides* was genetically modified for lycopen production by redirecting of the isoprenoid pathway toward the synthesis of carotenoids and introducing genes from the bacterium *Erwinia uredovora* (Leathers et al, 2004). Carotenoid biosynthetic pathway of astaxanthin producers of *Phaffia/Xanthophyllomyces* strains has also been engineered and several genes, such as phytoene desaturase, isopentenyl diphosphate isomerase and epoxide hydrolase were isolated and expressed in *E. coli* (Verdoes et al., 2003; Lukacz, 2006).

2.3 Some natural factors affecting growth and production of metabolites in red yeasts

2.3.1 Nutrition sources

Cellular organisms require specific internal conditions for optimal growth and function. The state of this internal milieu is strongly influenced by chemical, physical and biological factors in the growth environment. Understanding yeast requirements is important for successfull cultivation of yeast in the laboratory but also for optimalization of industrial fermentation process (Walker, 1998). Elemental composition of yeast cell gives a broad indication as to the nutritional reguirements of the yeast cell. Yeasts acquire essential elements from their growth environment from simple food sources which need to be

available at the macronutrient level (approx. 10^{-3} M) in the case of C, H, O, N, P, K, Mg and S or at the micronutrient level (approx. 10^{-6} M) in the case of trace elements. Yeasts are chemoorganotrophs as they use organic compounds as a source of carbon and energy. Yeasts can use a wide variety of substances as nutrient sources. Decreasing availability of one substrate can, in many instances, be compensated by the utilisation of another (Xiao, 2005).

When a single essential nutrient becomes limiting and eventually absent, the cellular proliferative machinery is efficiently shut down and a survival program is launched. In the absence of any one of the essential nutrients, yeast cells enter a specific, non-proliferative state known as stationary phase, with the ultimate aim of surviving the starvation period. In the presence of a poor carbon source, starvation for nitrogen induces sporulation and in the presence of a good carbon source stimulates pseudohyphal growth (Gasch & Werner-Washburne, 2002). Starvation is a complex, albeit common, stress for microorganisms. The nutrients for which a cell can be starved include carbon and nitrogen, with other elements such as phosphate, sulphur, and metals being less commonly evaluated.

The environment presents for yeasts a source of nutrients and forms space for their growth and metabolism. On the other hand, yeast cells are continuously exposed to a myriad of changes in environmental conditions (referred to as environmental stress). These conditions determine the metabolic activity, growth and survival of yeasts. Basic knowledge of the effect of environmental factors on yeast is important for understanding the ecology and biodiversity of yeasts as well as to control the environmental factors in order to enhance the exploitation of yeasts or to inhibit or stop their harmful and deleterious activity (Rosa & Peter, 2005).

In order to improve the yield of carotenoid pigments and subsequently decrease the cost of this biotechnological process, diverse studies have been performed by optimizing the culture conditions including nutritional and physical factors. Factors such as nature and concentration of carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light and stress have a major influence on cell growth and yield of carotenoids. Because carotenoid biosynthesis is governed by the levels and activities of enzymes employed to the total carbon flux through the carotenoid synthesizing system, the efficient formation of carotenoids can also be achieved by construction of hyperproducing strains with mutagenesis and genetic/metabolic engineering (Frengova & Beshkova, 2009).

The efficiency of the carbon source conversion into biomass and metabolites, and the optimization of the growth medium with respect to its availability and price has been subject of intensive studies. Numerous sources including pentoses and hexoses, various disaccharides, glycerol, ethanol, methanol, oils, n-alkanes, or wide variety of wastes derived from agricultural have been considered as potential carbon sources for biotechnological production of carotenoids.Carotenoid pigment accumulation in most yeasts starts in the late logarithmic phase and continues in the stationary phase (typically for secondary metabolites), and the presence of a suitable carbon source is important for carotenoid biosynthesis during the nongrowth phase. Yeasts can synthesize carotenoids when cultivated in synthetic medium, containing various simple carbon sources, such as glucose, xylose, cellobiose, sucrose, glycerol and sorbitol. Studies on carotenogenesis have led to a growing interest in using natural substrates and waste products from agriculture and food industry: grape juice, grape must, peat extract and peat hydrolysate, date juice, hydrolyzed mustard waste isolates, hemicellulosic hydrolysates (Parajo et al., 1998), hydrolyzed mung bean waste flour, sugar cane juice, sugar cane and sugar-beet molasses, corn syrup, corn

hydrolysate, milk whey. In recent years, raw materials and by-products of agro-industrial origin have been proposed as low-cost alternative carbohydrate sources for microbial metabolite production, with the view of also minimizing environmental and energetic problems related to their disposal (Frengova & Beshkova, 2009).
The chemical composition and concentration of nitrogen source in medium might also be means of physiological control and regulation of pigment metabolism in microorganisms. Several inorganic and organic nitorgen sources as well as flour extracts and protein hydrolysates have been studied for improvement of carotenoid production. However, it seems that variation in carotene content in yeasts with regard to N-source used in a medium and the rate of pigment production is influenced by the products of catabolism of the nitrogen source rather than being the results of direct stimulation by the nitrogen compound itself (Certik et al., 2009, Somashekar & Joseph, 2000).

2.3.2 Environmental stress

Single-celled organisms living freely in nature, such as yeasts, face large variations in their natural environment. Environmental conditions that threaten the survival of a cell, or at least prevent it from performing optimally, are commonly referred to as cell stress. These environmental changes may be of a physical or chemical nature: temperature, radiation, concentrations of solutes and water, presence of certain ions, toxic chemical agents, pH and nutrient availability. In nature, yeast cells often have to cope with fluctuations in more than one such growth parameter simultaneously (Hohman & Mager, 2003). In industry, yeast stress has several very important practical implications. In brewing, for example, if yeast is nutrient-starved during extended periods of storage, certain cell surface properties such as flocculation capability are deleteriously affected (Walker, 1998).
Carotenogenic yeasts are considered to be ubiquitous due to its world-wide distribution in terrestrial, freshwater and marine habitats, and to its ability to colonize a large variety of substrates. They can assimilate various carbon sources, including waste materials as cheap substrates. The red yeast is able to grow under a wide range of initial pH conditions from 2.5 to 9.5 and over a wide range of temperatures from 5 to 26°C (Libkind et al., 2008; Latha et al., 2005). The most important consenquence of environmental stress in red yeast is stimulation of carotenoid and other secondary (as well as primary) metabolite production. Changes of ergosterol production, lipid content, glycerol and trehalose as well as membrane remodeling are described as a response to stress (Hohman & Mader, 2003). Carotenoid pigments accumulation in most yeasts starts in the late logarithmic phase and continues in the stationary phase and is highly variable. Carotenoid production depends on differences between strains of the same species and is strongly influenced by the cultivation conditions. Addition of stress factors into cultivation medium led to different changes of growth according to the yeast species, type of stress factor or growth phase, in which stress factors were added (Marova et al., 2004).
Carotenogenesis in many organisms is regulated by light. However, the intensity and protocol of illumination varies with the microorganism. Temperature is another important factor affecting the performance of cells and product formation. The effect of temperature depends on the species specificity of the microorganism and often manifests itself in quantity variations of synthesized carotenoids. It was reported that lower temperatures (25°C) seemed to favor synthesis of β-carotene and torulene, whereas higher temperatures (35°C) positively influenced torularhodin synthesis by *R. glutinis* (Frengova & Beshkova,

2009). The effect of aeration is dependent on the species of the microorganism. The aeration influenced not only the amount of carotenoids produced, but also the composition of individual pigments making up the total carotenoids (Simova et al., 2004). At higher aeration, the concentration of total carotenoids increased relative to the biomass and fatty acids in *R. glutinis*, but the composition of carotenoids (torulene > β-carotene > γ-carotene > torularhodin) remained unaltered. In contrast, *S. roseus* responds to enhanced aeration by a shift from the predominant β-carotene to torulene and torularhodin (Davoli, 2004). Also other inducers of oxidative stress such as irradiation and free radical generators have a significant effect on the carotenoid production. By UV mutagenesis of the pink yeast *R. glutinis* the yellow colored mutant 32 was obtained which produced 24-fold more total carotenoids (2.9 mg/g dry cells) and 120-fold more β-carotene than the wild-type in a much shorter time (Bhosale & Gadre, 2001). Production of carotenoids by *Rhodotorula glutinis* cells grown under oxidative stress was about 5–6 times higher than in wild-type (Marova et al., 2004; Marova et al., 2010).

Tolerance to deleterious factors (e.g., low pH) refers to a microorganism's ability to survive a stress. This phenomenon is described as adaptive response, induced tolerance, habituation, acclimatization or stress hardening. Once cells have been challenged with a mild stress, they become more resistant to severe stress. Also exposure to one type of stress has been demonstrated to lead to tolerance to other types of stress as well (cross-protection) (Hohman & Mager, 2003). When cells are shifted to stress environments, they respond with changes in the expression of hundreds or thousands of genes, revealing the plasticity of genomic expression. Some of the expression changes are specific to each new environment, while others represent a common response to environmental stress. Comparative analysis of the genomic expression responses to diverse environmental changes revealed that the expression of roughly 900 genes (around 14% of the total number of yeast genes) is stereotypically altered following stressful environmental transitions. The functions of these gene products may protect critical aspects of the internal milieu, such as energy reserves, the balance of the internal osmolarity and oxidation-reduction potential, and the integrity of cellular structures. The protection of these features by the stress gene products likely contributes to the cross-resistance of yeast cells to multiple stresses, in which cells exposed to a mild dose of one stress become tolerant of an otherwise-lethal dose of a second stressful condition (Hohman & Mager, 2003; Gasch & Werner-Washburne, 2002; Gasch et al., 2000).

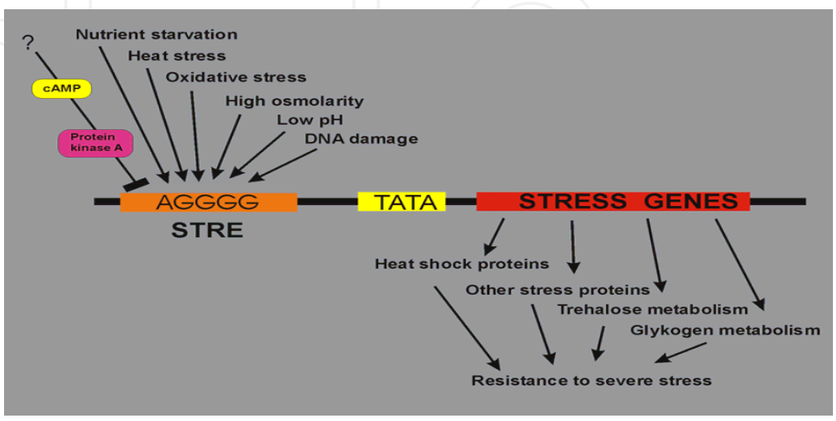

Fig. 14. Factors controlling stress response elements (STREs) and effects triggered by STRE activation in yeast (Walker, 1998)

A critical component of cell survival is maintaining a viable energy source. Glucose is the preferred carbon source in yeast, and upon stress, the cell induces a variety of genes that affect glucose metabolism. This includes genes encoding glucose transporters that serve to import external glucose into the cell and glucose kinases that activate the sugar for subsequent catabolism. In response to stressful environments, the fate of glucose is divided between trehalose synthesis, glycogen storage, ATP synthesis through glycolysis, and NADPH regeneration by the pentose phosphate shuttle (Hohman & Mager, 2003).

2.4 Strategies for improvement of carotenoid-synthesizing strains

2.4.1 Media compostion and cultivation mode

The production biotechnological process proceeds essentially in two stages: fermentation and product recovery. An important aspect of the fermentation process is the development of a suitable culture medium to obtain the maximum amount of desired product. In recent years, cheap raw materials and by-products of agro-industrial origin have been proposed as low-cost alternative carbohydrate sources for microbial metabolite production, with the view also of minimizing environmental and energetic problems related to residues and effluent disposal. For fermentation, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in carotene pigment and other additional metabolites, e.g. ergosterol, metal ions etc. In the whole-cell strategy product isolation is not necessary and, moreover, complex biotechnological product in the form of slightly modified biomass could be obtained.

The traditional batch production system has the disadvantage of inducing the Crabtree effect (characterized by the synthesis of ethanol and organic acids as fermentation products), due to high concentrations of initial sugars, diminishing pigment and biomass yield. The strategy for solving this problem is the fed-batch culture. Maximum astaxanthin production (23.81 mg/l) by *P. rhodozyma* was achieved in fed-batch fermentation with constant pH = 6.0, 4.8 times greater that the one obtained in a batch culture and the biomass concentration (39.0 g/l) was 5.3 times higher than that in the batch culture (Ramirez et al., 2006). The maximum astaxanthin concentration by *X. dendrorhous* at fed-batch fermentation with pH-shift control strategy reached 39.47 mg/l, and was higher by 20.2 and 9.0% than that of the batch and fed-batch fermentation, respectively, with constant pH = 5.0. However, the maximal cell density at fed-batch fermentation with pH-shift control was 17.42 g dry cells/l, and was lower by 2.0% than that of fed-batch fermentation with constant pH = 5.0. As a result of the two stage fed-batch culture *P. rhodozyma*, cell and astaxanthin concentrations reached 33.6 g/l and 16.0 mg/l, respectively, which were higher when compared with batch culture. The final specific astaxanthin concentration (mg/g dry wt of cells) in the second stage was ca. threefold higher than that in the first stage and 1.5-fold higher than that in the dissolved oxygen controlled batch culture, indicating that the astaxanthin production was enhanced mush more in the second stage than in the first stage (Hu et al., 2007).

The astaxanthin production was enhanced by a high initial C/N ratio in the medium (second stage), whereas a lower C/N ratio was suitable for cell growth (first stage). A significant increase (54.9%) in astaxanthin production by *X. dendrorhous* was achieved in pulse fed-batch process when compared with batch process. The astaxanthin concentration was 33.91 mg/l in pulse fed-batch when compared with 30.21 mg/l in constant glucose fed-batch and 21.89 mg/l in batch fermentation. In contrast with this strain producing high

yields of biomass and astaxanthin in pulse fed-batch process, another strain of *P. rhodozyma* demonstrated high astaxanthin-synthesizing activity during continuous fed-batch process (Hu et al., 2005). The utilization of continuous feeding showed to be the most efficient feeding method in fed-batch processes, as it did not lead to a reduction in the cellular astaxanthin concentration, as observed in the pulsed feeding. In the pulsed and continuous fed-batch processes, a cellular astaxanthin concentration of 0.303 mg/g biomass and 0.387 mg/g biomass, an astaxanthin concentration of 5.69 and 7.44 mg/l, a biomass concentration of 18.7 and 19.3 g/l were obtained, respectively.

Temperature was reported to control changes in enzyme activities that regulate metabolic activity in microorganisms. For example, *Rhodotorula glutinis* biosynthesized β-carotene more efficiently at lower temperature, whereas increased torulene formation was accompanied by higher temperature (Bhosale & Gadre, 2002). The reason might be found in γ-carotene that acts as the branch point of carotenoid synthesis. Subsequent dehydrogenation and decarboxylation leading to torulene synthesis is known to be temperature dependent since the respective enzymes are less active at lower temperature compared to the activity of β-carotene synthase. This is probable reason for an increase in the proportion of β-carotene at lower temperature in *Rhodotorula glutinis*. The moderately psychrophilic yeast *Xanthophyllomyces dendrorhous* also displayed a 50% increase in total carotenoids at low temperatures with elevated levels of astaxanthin (Ducrey Sanpietro & Kula, 1998).

Fed-batch co-cultures *R. glutinis–D. castellii* gave a volumetric production of 8.2 mg total carotenoid/l, about 150% of that observed in batch co-cultures and biomass concentration of 9.8 g/l which was about two times higher when compared with batch fermentation (Buzzini, 2001). The fedbatch technique maximized the specific growth rate of *R.glutinis*, resulted in higher biomass and minimized substrate inhibition of pigment formation. Molasses in the fed-batch mode led to increased biomass by 4.4- and 7-fold in double- and triple-strength feed, respectively when compared with 12.2 g/l biomass in batch fermentation. *R. glutinis* also produced a very high carotenoid concentration for double- and triple-strength feed supplement (71.0 and 185.0 mg/l, respectively), and was higher 2- and 3.7-fold of that observed in batch fermentation (Frengova & Beshkova, 2009).

2.4.2 Specific supplements and exogenous factors enhancing metabolic activity of red yeasts

There have been several reports on the enhancement of volumetric production (mg/l) as well as cellular accumulation (mg/g) of microbial carotenoid upon supplementation of metal ions (copper, zinc, ferrous, calcium, cobalt, alluminium) in yeasts and molds (Bhosale, 2004; Buzzini et al., 2005). Trace elements have been shown to exert a selective influence on the carotenoid profile in red yeasts. It may be explained by hypothesizing a possible activation or inhibition mechanism by selected metal ions on specific carotenogenic enzymes, in particular, on specific desaturases involved in carotenoid biosynthesis (Buzzini et al., 2005). The other explanation is based on observations that presence of heavy metals results in formation of various active oxygen radicals what, in a turn, induces generation of protective carotenoid metabolites that reduce negative behaviour of free radicals. Such strategy has been applied in several pigment-forming microorganisms to increase the yield of microbial pigments (Breierova et al., 2008; Rapta et al., 2005).

In order to achieve rapid carotenoid overproduction, various stimulants can be added to the culture broth. One group of such enhancers is based on intermediates of the tricarboxylic

acid cycle which play an important role in metabolic reactions under aerobic conditions, forming a carbon skeleton for carotenoid and lipid biosynthesis in microbes. Because pigment increase is paralleled by decreased protein synthesis, restriction of protein synthesis is an important way how to shift carbon flow to carotenoid synthesis (Flores-Cotera & Sanchez, 2001). It was also proposed that high respiratory and tricarboxylic acid cycle activity is associated with production of large quantities of reactive species and these are known to enhance carotenoid production (An, 2001). It should be emphasized that the degree of stimulation was dependent on the time of addition of the citric acid cycle intermediate to the culture medium. Some fungi showed that addition of organic acids to media elevated β-carotene content and concomitantly decrease γ-carotene level with complete disappearance of lycopene (Bhosale, 2004).

Chemical substances capable of inhibiting biosynthetic pathways have been applied to characterize metabolic pathways and elucidate reaction mechanisms. In general, compounds that inhibit biosynthesis can act through various mechanisms, such as inhibiting the active site directly by an allosteric effect (reversible or otherwise), altering the regulation of gene expression and blocking essential biochemical pathways or the availability of cofactors, among other possibilities. From this view, number of chemical compounds including terpenes, ionones, amines, alkaloids, antibiotics, pyridine, imidazole and methylheptenone have been studied for their effect on carotene synthesis (Bhosale, 2004). In order to obtain commercially interesting carotenoid profiles, the effect of supplementation with diphenylamine (DPA) and nicotine in the culture media of *Rhodotorula rubra* and *Rhodotorula glutinis* was investigated. DPA blocks the sequence of desaturation reactions by inhibiting phytoene synthase, leading to an accumulation of phytoene together with other saturated carotenoids and nicotine inhibits lycopene cyclase, and consequently the cyclization reactions (Squina & Mercadante, 2005). Cultivation of *Xanthophyllomyces dendrorhous* in the presence of diphenylamine and nicotine at 4°C was reported to trigger interconversion of β-carotene to astaxanthin (Ducrey Sanpietro & Kula, 1998).

The addition of solvents such as ethanol, methanol, isopropanol, and ethylene glycol to the culture medium also stimulate microbial carotenogenesis. It should be noted that while ethanol supplementation (2%, v/v) stimulated β-carotene and torulene formation in *Rhodotorula glutinis*, torularhodin formation was suppressed (Bhosale, 2004). It was proposed that ethanol-mediated inhibition of torulene oxidation must be accompanied by an increase in β-carotene content suggesting a shift in the metabolic pathway to favor ring closure. Detailed studies revealed that ethanol activates oxidative metabolism with induction of HMG-CoA reductase, which in turn enhances carotenoid production. However, stimulation of carotenoid accumulation by ethanol or H_2O_2 was more effective if stress factors were employed to the medium in exponential growth phase than from the beginning of cultivation (Marova et al, 2004).

2.4.3 Mutagenesis

Mutagenesis is an alternative to classical strain improvement in the optimization of carotenoid production. Mutagenic treatment with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG), UV light, antimycin, ethyl-methane sulfonate, *γ*–irradiation, high hydrostatic pressure have been used successfully to isolate various strains with enhanced carotenoid-producing activity. UV mutant *R.gracilis* has shown 1.8 times higher carotenoid synthesizing activity than that of the parent strain and the relative share of β-carotene in the total carotenoids was 60%. The yellow colored mutant 32 was also obtained by UV

mutagenesis of the pink yeast *R. glutinis* and produced a large quantity of total carotenoids (2.9 mg/g dry cells), which was 24-fold higher accumulation of total carotenoids compared with the wild-type. Mutant 32 produced 120-fold more beta-carotene (2.05 mg/g dry cells) than the parent culture in a much shorter time (36 h), which was 82% (w/w) of the total carotenoid content. Later, after the treatments of five repeated cycles by high hydrostatic pressure of 300 MPa, the mutant *R. glutinis* RG6p was obtained, beta-carotene production of which reached 10.01 mg/l, increased by 57.89% compared with 6.34 mg/l from parent strain (Frengova & Beshkova, 2009).

A fivefold increase in beta-carotene accumulation was reported for yellow mutant *P. rhodozyma* 2-171-1 which was obtained after ethyl-methane sulfonate mutagenesis of dark red strain *P. rhodozyma*. This mutant is likely to be blocked in the oxidase step and therefore unable to perform the conversion of beta-carotene to echinenone and latter to astaxanthin. The UV-mutant *P. rhodozyma* PG 104 produced 46-fold more β-carotene (92% of total carotenoids) than the parent culture (2% of total carotenoids) and maximum beta-carotene yields were 1.08 mg/g dry cells and 9.95 mg/l. Using NTG mutagenesis two different strains of carotenoid accumulating *X. dendrourhous* mutants JH1 and JH2 were also isolated. Astaxanthin-overproducing mutant JH1 produced 4.03 mg astaxanthin/g dry cells, and this value was about 15-fold higher than that of wild-type. Mutant JH2 produced 0.27 mg beta-carotene/g dry cells, and this was fourfolds increase from that of wild-type and the mutant *X. dendrourhous* JH1 produced maximum astaxanthin concentration of 36.06 mg/l and 5.7 mg/g dry cells under optimized cultivation conditions (Kim et al., 2005).

To isolate a carotenoid-hyperproducing yeast, *P.rhodozyma* 2A2 N was treated by low-dose gamma irradiation below 10 kGy and mutant 3A4-8 was obtained. It produced 3.3 mg carotenoids/g dry cells, 50% higher carotenoid content than that of the unirradiated strain (antimycin NTG-induced mutant 2A2 N). Gamma irradiation produces oxygen radicals generated by radiolysis of water and could induce mutation of *P. rhodozyma* through a chromosomal rearrangement. A primary function of carotenoids in *P. rhodozyma* is to protect cells against singlet oxygen and these compounds have been demonstrated to quench singlet oxygen. Oxygen radicals have been known to cause changes in the molecular properties of proteins as well as enzyme activities. Thus, oxygen radicals generated by gamma irradiation might modify the pathway in astaxanthin biosynthesis of *P. rhodozyma* and cause an increase in carotenoid production of the mutant 3A4-8 isolated by gamma irradiation (Frengova & Beshkova, 2009).

2.4.4 Use of recombinant strains

One possibility for the improvement of the metabolic productivity of an organism is genetic modification. This strategy can be successful when an increase of the flux through a pathway is achieved by, e.g., the overproduction of the rate-limiting enzyme, an increase of precursors, or the modification of the regulatory properties of enzymes. In the carotenogenic yeasts, mevalonate synthesis, which is an early step in terpenoid biosynthesis, is a key point of regulation of the carotenoid biosynthetic pathway. In fact, addition of mevalonate to a culture of *X. dendrourhous* stimulated both astaxanthin and total carotenoid biosynthesis four times (from 0.18 to 0.76 mg/g and from 0.27 to 1.1 mg/g dry cells, respectively). This indicates that the conversion of HMG-CoA to mevalonate by HMG-CoA reductase is a potential bottleneck on the road to modified strains with higher astaxanthin content (Verdoes et al., 2003).

Like carotenoids, ergosterol is an isoprenoid and it is biosynthetically related to them by common prenyl lipid precursor, FPP. Astaxanthin production by *P. rhodozyma* strain was enhanced (1.3-fold) when sgualene synthase phenoxypropylamine-type inhibitor for sterol biosynthesis was added to the medium. The isolation and characteristic of the carotenogenic genes of yeasts facilitates the study of the effect of their overexpression on carotenoid biosynthesis. Use of recombinant DNA technology for metabolic engineering of the astaxanthin biosynthetic pathway in *X. dendrourhous* was described too. In several transformants containing multiple copies of the phytoene synthase-lycopene cyclase-encoding gene (*crtYB*), the total carotenoid content was higher (with 82%) than in the control strain. This increase was mainly due to an increase of the beta-carotene and echinenone content (with 270%), whereas the total content of astaxanthin was unaffected or even lower.

Alternatively, in recent years, several food-grade non-pigmented yeasts (*Saccharomyces cerevisiae*, *Candida utilis*) have been engineered in order to obtain strains possessing the ability to produce selected carotenoids (Verwaal et al., 2007). Identification of genes of enzymes from the astaxanthin biosynthetic pathway and their expression in a non-carotenogenic heterologous host have led to the overproduction of beta-carotene. The possibility of the use of *S. cerevisiae*as a host for efficient beta-carotene production by successive transformation with carotenogenic genes (*crtYB* which encodes a bifunctional phytoene synthase and lycopene cyclase; *crtI*, phytoene desaturase; *crtE*, heterologous GGPP synthase; *tHMGI*, HMG-CoA reductase) from *X. dendrorhous* was studied. Like *X. dendrorhous*, *S. cerevisiae* is able to produce FPP and converts it into GGPP, the basic building block of carotenoids. *S. cerevisiae*, the industrially important conventional yeast, cannot produce any carotenoid, while it synthesizes ergosterol from FPP by a sterol biosynthetic pathway. Conversion of FPP into GGPP is catalyzed by GGPP synthase encoded by *BTS1* gene in *S. cerevisiae*. Construction of a strain, producing a high level of beta-carotene (5.9 mg/g dry cells) was succesful. Oleaginous yeasts are also suitable host strains for the production of lipophilic compounds due to their high lipid storage capacity. Recently, the carotenoid-producing *Yarrowia lipolytica* has been generated by metabolic engineering. Acording to these results entire biosynthetic pathways can be introduced into new host cells through recombinant DNA technology and carotenoids can be produced in organisms that do not normally produce carotenoids.

2.5 Application of whole-cell yeast biomass to production of pigments and other lipid compounds

2.5.1 Carotenoid and ergosterol enriched biomass

Red yeasts are used predominantly as carotenoid producers and, thus, carotenoid-enriched biomass is the most frequently produced. The growing scientific evidence that carotenoid pigments may have potential benefits in human and animal health has increased commercial attention on the search for alternative natural sources. Comparative success in microbial pigment production has led to a flourishing interest in the development of fermentation processes and has enabled several processes to attain commercial production levels. An important aspect of the fermentation process is the development of a suitable culture medium to obtain the maximum amount of desired product. In recent years, cheap raw materials and by-products of agro-industrial origin have been proposed as low-cost alternative carbohydrate sources for microbial metabolite production, with the view also of minimizing environmental and energetic problems related to residues and effluent disposal.

During the produt recovery process, the biomass is isolated and transformed into a form suitable for isolating carotene, which can be further isolated from the biomass with appropriate solvent, suitably purified and concentrated. Using whole biomass as final product, isolation of metabolites is not necessary and other cell active components can be utilized. Nevertheless, cell disruption is recommended for better bioavailability of the most of lipid-soluble substance (Frengova & Beshkova, 2009). Several types of microbes have been reported to produce carotenoids and carotenoid-rich biomass; but only a few of them have been exploited commercially (Bhosale, 2004).

Among the few astaxanthin producing microorganisms, *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) is one of the best candidate for commercial production of pigment as well as enriched biomass. Therefore, many academic laboratories and several companies have developed processes which could reach an industrial level. *Phaffia/ Xanthophyllomyces* has some advantageous properties that make it attractive for commercial astaxanthin production: (i) it synthesizes natural form astaxanthin (3*S*,3'*S* configuration) as a principal carotenoid, (ii) it does not require light for its growth and pigmentation, and (iii) it can utilize many types of carbon and nitrogen sources (Lukacs et al, 2006; Dufosse, 2006). Studies on physiological regulation of astaxanthin in flasks cultivations was verified in bioreactors and the ataxanthin amount reached 8.1 mg/L (Dufosse, 2006). Enhanced production of the pigment was achieved during fed-batch fermentation with regulated additions of glucose and optimized fermentation condition finally yielded up to 20 mg astaxanthin/L (Certik et al., 2009). High carbon/nitrogen ratio induced amout of astaxanthin and C/N-regulated fed-batch fermentation of *P. rhodozyma* led to 16 mg astaxanthin/L. Thus, this strain can be considered as a potential producer of astaxanthin. In addition, to avoid isolation of astaxanthin from cells, two-stage batch fermentation technique was used (Fang & Wang, 2002), where *Bacillus circulans* with a high cell wall lytic activity was added to the fermentation tank after the accumulation of astaxanthin in *P. rhodozyma* was completed. Astaxanthin is the principal colorant in crustaceans, salmonids and flamingos. There is current interest in using *P.rhodozyma* biomass in aquaculture to impart desired red pigmentation in farmed salmon and shrimps.

Biotechnological production of β-carotene by several strains of the yeast *Rhodotorula* is currently used industrially. This yeast is convenient for large-scale fermentation because of its unicellular nature and high growth rate. Because *Rhodotorula glutinis* synthesizes β-carotene, torulene and torularhodin, the rate of production of the individual carotenoid depends upon the incubation conditions. Specially prepared mutants of *Rhodotorula* not only rapidly increased formation of torulene or thorularhodin, but amount of β-carotene reached the level of 70 mg/L (Sakaki *et al*., 2000). Better strategy than isolation of individual pigments seems to be use of the whole enriched biomass to feed and food industry.

In our recent work exogenous stress factors were used to obtain higher production of carotenoids in *R. glutinis* CCY 20-2-26 strain. Physical and chemical stress factors were applied as single and in combination. Adaptation to stress was used in inoculum II. Short-term UV irradiation of the production medium led to minimal changes in biomass production. The production of carotenoids in *R. glutinis* cells was stimulated in all samples of exponentially growing cells when compared with control cultivation. In stationary phase, the production of carotenoids was induced only by 35-min irradiation. Ergosterol production exhibited very similar changes as β-carotene production both under temperature and UV stress. Our results are in good agreement with recent findings of the effect of weak white light irradiation on carotenoid production by a mutant of *R. glutinis* (Sakaki et al., 2000).

Using chemical stress, the influence of osmotic (2-10 % NaCl) stress, oxidative (2-10 mM H2O2) stress and combined effects of these stress factors on the morphology, growth and production of biomass, carotenoids and ergosterol by *R. glutinis* CCY 20-2-26 cells were studied (Marova et al., 2010). First, *R. glutinis* cells were exposed to higher concentration of stress factors added into the production medium. Further, low concentrations of NaCl and H_2O_2 were added to the inoculum medium or to both inoculum and production media. Exposition of red yeast cells to all tested stress factors resulted in higher production of carotenoids as well as ergosterol, while biomass production was changed only slightly. Under high stress 2-3 times increase of β-carotene was observed. The addition of low salt or peroxide concentration into the inoculation media led to about 2-fold increase of carotenoid production. In Erlenmeyer flasks the best effect on the carotenoid and ergosterol production (3- to 4-fold increase) was exhibited by the combined stress: the addition of low amount of NaCl (2 mM) into the inoculum medium, followed by the addition of H_2O_2 (5 mM) into the production medium. The production of ergosterol in most cases increased simultaneously with the production of carotenoids.

Cultivation of *R. glutinis* carried out in a 2-litre laboratory fermentor was as follows: under optimal conditions about 37 g/L of yeast biomass were obtained containing approx. 26.30 mg/L of total carotenoids and 7.8 mg/L of ergosterol. After preincubation with a mild stress factor, the yield of biomass as well as the production of carotenoids and ergosterol substantially increased. The best production of enriched biomass was obtained in the presence of peroxide in the inoculation medium (52.7 g/L of biomass enriched with 34 mg/L of carotenoids) and also in combined salt/peroxide and salt/salt stress (about 30–50 g/L of biomass enriched with 15–54 mg/L of total carotenoids and about 13-70 mg/L of ergosterol). *Rhodotorula glutinis* CCY 20-2-26 strain could be a suitable candidate for biotechnological applications in the area of carotenoid rich biomass production. Preliminary cultivation in a 2-litre laboratory fermentor after preincubation with stress factors in well-ballanced experiments led to the yield of about 40-50 g per litre of biomass enriched by 20-40 mg of β-carotene+lycopene sum (approximately 30–50 mg of total carotenoids per litre) and about 70 mg of ergosterol per litre. Addition of simple cheap stress factor substantially increased metabolite production without biomass loss. Therefore, this strain takes advantage of the utilization of the whole biomass (complete nutrition source), which is efficiently enriched for carotenoids (provitamin A, antioxidants) and also ergosterol (provitamin D). Such a product could serve as an additional natural source of significant nutrition factors in feed and food industry (Marova et al, 2010).

Our further work was focused on possiblity to use carotenogenic yeasts cultivated on alternative nutrition sources combined with stress factors (Marova et al., 2011). Both physiological and nutrition stress can be used for enhanced pigment production. Three red yeast strains (*Sporobolomyces roseus, Rhodotorula glutinis, Rhodotorula mucilaginosa*) were studied in a comparative screening study. To increase the yield of these pigments at improved biomass production, combined effect of medium with modified carbon and nitrogen sources (waste materials - whey, potato extract) and peroxide and salt stress was tested. The production of carotene-enriched biomass was carried out in flasks as well as in laboratory fermentor. The best production of biomass was obtained in inorganic medium with yeast extract. In optimal conditions tested strains differ only slightly in biomass production. Nevertheless, all strains were able to use most of waste substrates. Biomass and pigment production was more different according to substrate type. It was observed that addition of non-processed or processed whey or potato extract to media can increase beta-

carotene production, while biomass production changed relatively slightly (Marova et al, 2011).

In *Rhodotorula glutinis* addition of whey substrate into production medium led to 3.5x increased production of beta-carotene without substantial changes in biomass. Non-processed whey or potato extract added to production media led to about 3x increase of beta-carotene production accompanied by biomass loss. The highest yield was reached after addition of lyophillized non-processed whey to INO II as well as to production media. Also potato extract added into INO II led to increased beta-carotene production while biomass yield was lower. *Sporobolomyces roseus* exhibited significant changes in biomass:carotene ratio dependent on whey substrate addition. Substantial biomass decrease in presence of lyophilized whey in INO II (under 5 g/L) was accompanied by very high beta-carotene yield (2.54 – 2.75 mg/g d.w.). Potato extract addition into production medium led to about 11-times increase of β-carotene production, while production of biomass was lower than in control. Preincubation of *S.roseus* cells with potato extract and following cultivation in production medium with 5% hydrogen peroxide led to about 20-times higher β-carotene production as in control, in this cultivation conditions biomass decreased only slightly. In general, total production of biomass by *S.roseus* was about 2-x lower as in *R.glutinis.* So, this is the reason why *S.roseus* CCY 19-4-8 cells is less suitable to enriched biomass production. *Rhodotorula mucilaginosa* CCY 20-7-31 seems to be relatively poor producer of carotenoids when compared with the other two strains. Production of biomass in this strain was more similar to *R.glutinis* (about 8 g/L). However, addition of potato extract into INO II combined with salt stress in production medium enabled to reach the highest biomass as well as β-carotene production observed in this strain yet (1.56 mg/g d.w.). It seems that this strain needs for optimal pigment/biomass production some additional nutrition factors which are no present in simple (but cheap) inorganic medium, but can be obtained from different waste substrates (also cheap).

In laboratory fermentor better producers of enriched biomass were both *Rhodotorula* strains. In experiments with *Rhodotorula glutinis* the production of yeast biomass in a laboratory fermentor was in most types of cultivation more than 30 grams per litre (about 3-times higher yield than in Erlenmeyer flasks; Table 1). The balance of cultivation in a fermentor in optimum conditions is as follows: we obtained about 37.1 g/l of biomass containing 17.19 mg per litre of β-carotene (see Table 1). The production of β-carotene was induced in most types of media combinations. High total yield of β-carotene was obtained in whey production medium (44.56 g/L of biomass; 45.68 mg of β-carotene per litre of culture). The highest total yield of β-carotene was obtained using combined whey/whey medium (51.22 mg/L); this cultivation was accompanied also with relatively high biomass production (34.60 mg/L). In experiments with *Sporobolomyces roseus* CCY 19-4-8 substantially higher production of biomass was obtained in fermentor when compared with cultivation in flasks. Mainly in whey medium about 3-times biomass increase (about 12 g/L) was reached and production of beta-carotene was mostly higher than in *R.glutinis*. Because of low biomass production, total yields were in *S.roseus* mostly lower than in *R.glutinis* cells. Yeast strain *Rhodotorula mucilaginosa* CCY 20-7-31 exhibited in most cases similar biomass production characteristics as *R.glutninis*, while pigment production was substantially lower (see Table 4). As the only substrate suitable for β-carotene production was found potato extract in INO II combined with 5% salt in production medium. Under these conditions 55.91 mg/L of β-carotene was produced in 30.12 g of cells per litre of medium (Marova et al, 2011).

The aim of all preliminary experiments carried out in laboratory fermentor was to obtain basic information about potential biotechnological use of the tested strains to the industrial production of β-carotene/ergosterol enriched biomass. The results of both *Rhodotorula* strains are very promising. The yield of *R.glutinis* CCY 20-2-26 biomass (37 – 44.5 g/L) produced in minimal cultivation medium was similar to the maximal biomass yield obtained in fed-batch cultivation of *Phaffia rhodozyma* (36 g/L), which is widely used as an industrial producer of astaxanthin (Lukacs et al., 2006). The maximal production of total carotenoids by used *P. rhodozyma* mutant strain was 40 mg/L, which is also similar to the yields obtained in *R. glutinis* CCY 20-2-26 cells grown in whey medium. The highest yields of pigments were obtained in *Rhodotorula glutinis* CCY 20-2-26 cells cultivated on whey medium (cca 45 g per liter of biomass enriched by 46 mg/L of beta-carotene) and in *Rhodotorula mucilaginosa* CCY 20-7-31 grown on potato medium and 5% salt (cca 30 g per liter of biomass enriched by 56 mg/L of beta-carotene). Such dried carotenoid-enriched red yeast biomass could be directly used in feed industry as nutrition supplement (Marova et al., 2011).

	Biomass			**Production of β-carotene**		
Substrate/stress factor	**R.g.** (g/l)	**S.r.** (g/l)	**R.m.** (g/l)	**β -carotene** (mg/l)	**β -carotene** (mg/l)	**β -carotene** (mg/l)
Control 0/0	37.14	17.00	26.55	17.93	3.25	4.31
0/whey deprot.*	44.56	9.59	27.06	45.68	23.36	8.80
0/potato	28.12	10.80	38.50	25.45	17.50	26.18
Whey*/ salt	40.86	8.16	18.35	28.00	14.23	10.81
Whey*/ whey	34.60	10.15	29.82	51.22	29.40	11.33
potato/salt	26.10	7.14	30.12	22.23	7.55	55.91
Potato/potato	18.56	6.28	28.48	22.48	6.13	27.23

Table 1. Production of beta-carotene enriched biomass in 2 L laboratory fermentor (Marova et al., 2011)

An alternative for utilization of some natural substrates for production of carotenoids by *Rhodotorula* species is the method of cocultivation. A widespread natural substrate is milk whey containing lactose as a carbon source. Carotenoid synthesis by lactose-negative yeasts (*R. glutinis*, *R. rubra* strains) in whey ultrafiltrate can be accomplished: by enzymatic hydrolysis of lactose to assimilable carbon sources (glucose, galactose) thus providing the method of co-cultivation with lactose-positive yeasts (*Kluyveromyces lactis*), producers of galactosidase or by creating conditions under which lactose is transformed into carbon sources (glucose, galactose, lactic acid) easily assimilated by the yeast when they were grown in association with homofermentative lactic acid bacteria or yogurt starter culture (Frengova & Beshkova, 2009). The maximum carotenoid yields for the microbial associations [*R. rubra* + *K.lactis*; *R. glutinis* + *Lactobacillus helveticus*; *R. rubra* + *L.casei*; *R. rubra* + (*L. bulgaricus* + *Streptococcus thermophillus*)] were as follows: 10.20, 8.10, 12.12, 13.09 mg/l, respectively. These yields are about five times higher than that of a lactose-positive strain *R. lactosa* cultivated in whey reported in literature (Frengova et al., 2004). *R. glutinis*–*Debaryomyces castellii* co-cultures was produced (5.4 mg carotenoids/l) about three times the

amount of total carotenoids formed by the red yeast cultured alone in low hydrolyzed corn syrup (Buzzini, 2001) The author concluded that oligosaccharides and dextrins of syrup could be utilized for pigment production by *R. glutinis* after hydrolysis to maltose and glucose by the extracellular amylolytic enzymes produced by *D. castellii* DBVPC 3503 in co-cultures.

Rhodotorula species	Carbon source	Cultivation process	Cell mass (g/l)	Carotenes (mg/g dry cells)	Carotenes (mg/l culture)	References
R. glutinis	WLA 2	batch	8.12	8.20	66.32	Marova et al., 2011
R. glutinis	pastes + enzymes	batch	11.68	3,60	40.10	Marova et al., 2010
R. glutinis ATCC 26085	glucose	batch				Davoli et al., 2004
R. glutinis 32	glucose	batch	23.90	5.40	129.00	Bhosale & Gadre, 2001
R. glutinis 32	sugar cane molasses	fed-batch	78.00	2.36	183.00	Bhosale & Gadre, 2001
R. glutinis DBVPG 3853 *D. castellii* DBVPG 3503	corn syrup	fed-batch	15.30	0.54	8.20	Buzzini, 2001
R. glutinis TISTR	hydrolyzed mung bean waste flour	batch	10.35	0.35	3.48	Tinoi et al., 2005
R. glutinis 22P *L. helveticus* 12A	whey ultrafiltrate	batch	30.20	0.27	8.10	Frengova & Beshkova, 2009
R. mucilaginosa NRRL-2502	sugar-beet molasses	batch	4.20	21.20	89.0	Aksu & Eren, 2005
R. mucilaginosa NRRR-2502	whey	batch	2.40	29.20	70.0	Aksu & Eren, 2005

Table 2. Comparison of carotenoid production by *Rhodotorula* species cultivated on different waste substrates

As mentioned above, waste substrates and alternative nutrition sources were used to production of astaxanthin-enriched biomas sof *Xanthophyllomonas dendrorhous* sources (Lukacs et al, 2006; Dufosse, 2006). Batch culture kinetics of this yeast revealed reduction in biomass with glucose and lower intracellular carotenoid content with fructose. Figures were different when compared to sucrose. In contrast, specific growth rate constant stayed between 0.094 - 0.098 h−1, irrespective of the carbon sources employed. Although the uptake rate of glucose was found to be 2.9-fold faster than that of fructose, sucrose was found to be a more suitable carbon source for the production of carotenoids by the studied strain. When sugar cane molasses was used, both the specific growth rate constant and the intracellular carotenoid content decreased by 27 and 17%, respectively. Compared with the batch culture

using 28 g/L sugar cane molasses, fed-batch culture with the same strain resulted in a 1.45-fold higher cell yield together with a similar level of carotenoid content in *X. dendrorhous* SKKU 0107 (Park et al, 2008).

Phaffia rhodozyma NRRL Y-17268 cells were proliferated in xylose-containing media made from Eucalyptus wood. Wood samples were subjected to acid hydrolysis under mild operational conditions, and hydrolysates were neutralized with lime. Neutralized hydrolysates were treated with charcoal for removing inhibitors and then supplemented with nutrients to obtain culture media useful for proliferation of the red yeast *P.rhodozyma.* Biomass was highly pigmented and volumetric carotenoid concentrations up to 5.8 mg carotenoids/L (with 4.6 mg astaxanthin/L) were reached. Further experiments in batch fermentors using concentrated hydrolysates (initial xylose concentrations within 16.6 and 40.8 g/L) led to good biomass concentrations (up to 23.2 g cells/L) with increased pigment concentration (up to 12.9 mg total carotenoids/L, with 10.4 mg astaxanthin/L) and high volumetric rates of carotenoid production (up to 0.079 mg/L/h (Parajo et al., 1998).

In the future, other types of waste materials (for instance from winemarket) are intended to be tested as carbon sources for carotenogenesis in red yeasts (Table 2). Moreover application of an environmental stress in combination with waste materials can lead to overproduction of carotenoids and lipids and decrease cost of their production. Such strategies could result into production of yeast biomass rich not only in carotenoids and other provitamins, but also in other nutrition components (proteins, PUFA, metal ions etc.) that originate both from yeast cells and from cultivation substrates. This is the way to production of complex food additives based on naturally enriched yeast biomass.

2.5.2 Single-oil cell processes and lipid production by red yeasts

A number of microorganisms belonging to the genera of algae, yeast, bacteria, and fungi have ability to accumulate neutral lipids under specific cultivation conditions. The microbial lipids contain high fractions of polyunsaturated fatty acids and have the potential to serve as a source of significant quantities of transportation fuels (Subramaniam et al., 2010). Microorganisms possess the ability to produce and accumulate a large fraction of their dry mass as lipids. Those with lipid content in excess of 20% are classified as 'oleaginous' (Ratlege and Wynn, 2002).

Oleaginous yeasts have a fast growth rate and high oil content, and their triacylglycerol (TAG) fraction is similar to that of plant oils. These organisms can grow on a multitude of carbon sources (see above). Most oleaginous yeasts can accumulate lipids at levels of more than 40% of their dry weight and as much as 70% under nutrient-limiting conditions (Beopoulos et al., 2009). However, the lipid content and fatty acid profile differ between species. Some of the yeasts with high oil content are *Rhodotorula glutinis*, *Cryptococcus albidus*, *Lipomyces starkeyi*, and *Candida curvata* (Subramaniam et al., 2010). Newly, lipid production by the oleaginous yeast strain *Trichosporon capitatum* was described too (Wu et al, 2011). The main requirement for high lipid production is a medium with an excess of carbon source and other limiting nutrients, mostly nitrogen. Hence, production of lipids is strongly influenced by the C/N ratio, aeration, inorganic salts, pH, and temperature.

Yeasts are able to utilize several different carbon sources for the production of cell mass and lipids. In all cases, accumulation of lipids takes place under conditions of limitations caused by a nutrient other than carbon. Recently, production of lipids by the yeast *R. glutinis* on different carbon sources (dextrose, xylose, glycerol, mixtures of dextrose and xylose, xylose

and glycerol, and dextrose and glycerol) was explored (Easterling et al., 2009). The highest lipid production of 34% TAG on a dry weight basis was measured with a mixture of dextrose and glycerol as carbon source. The fraction of unsaturated fatty acids in the TAGs was dependent on carbon source, with the highest value of 53% on glycerol and lowest value of 25% on xylose. With whey permeate for production of lipids by different yeast strains, *L. starkeyi* ATCC 12659 was found to have the highest potential of accumulating lipids among *Apiotrichum curvatum* ATCC 10567, *Cryptococcus albidus* ATCC 56297, *L. starkeyi* ATCC 12659, and *Rhodosporidium toruloides* ATCC. The yeast *L. starkeyi* is unique in that it is known not to reutilize the lipids produced by it and it produces extracellular carbohydrolases. Effect of C/N ratio on production of lipids by *L. starkeyi* and conditions favoring accumulation of lipids result in reduced growth of cells were confirmed. The cells could consume liquefied starch in batch culture and produced cells containing 40% lipids at a cell yield of 0.41 g dry weight per g starch. The yield on starch was higher than when glucose was used as carbon source (Subramaniam et al., 2010).

Culture temperature and pH influence the total cell number and lipid content in yeast cells. In minimal medium with glucose as carbon source, the yeast *L. starkeyi* accumulates large fractions of dry weight as lipids with a high yield in the pH range of 5.0–6.5. At higher temperatures, the cellular lipid content, the glucose conversion efficiency, and the specific lipid production rates in *L. starkeyi* were high, but the degree of fatty acid unsaturation was low (Subramaniam et al., 2010). Fastest growth of *L. starkeyi* cells occurred at 28°C (specific growth rate 0.158 h-1), and the lipid fraction in cells under these conditions was 55%. However, the fraction of oleic acid in the lipids increased from 52 to 60% of lipids when the accumulation phase temperature was reduced from growth temperature of 28–15°C. High lipid accumulation in cells of oleaginous yeast is obtained under limiting nitrogen concentration conditions. The oleaginous yeast *L. starkeyi* delivered lipid content of 68% at a C/N ratio of 150 compared to 40% in the presence of a C/N ratio of 60 while growing on digested sewage sludge (Subramaniam et al., 2010). The key fatty acids produced were C16:0, C16:1, C18:0, and C18:1. Accumulation of lipids by *Cryptococcus curvatus* cells also required a high C/N ratio of 50 in batch and fed-batch cultures (Hassan et al., 1996); the fatty acids produced were mainly oleic (C18:1), palmitic (C16:0), and stearic (C18:0). The highest fraction of stearic acid (18:0) in batch cultures was 14 and 19% in fed-batch culture.

Under optimal fermentation conditions in a batch reactor (100 g/L glucose as carbon source, 8 g/L yeast extract, and 3 g/L peptone as nitrogen sources, initial pH of 5.0, inoculation volume of 5%, 28°C temperature, and 180 rpm agitation in a 5-l bioreactor), *Rhodotorula glutinis* can accumulate lipids up to 49% of cell dry weight and 14.7 g/L lipid. In continuous culture, the cell biomass, lipid content, and lipid yield increase with decreasing growth rate. The yield 60.7% lipids in cells and 23.4 g l-1 lipid production in a continuous mode of operation was obtained (Subramaniam et al., 2010). In *R. toruloides* cultivated in fed-batch mode, oleic, palmitic, stearic, and linoleic acids were the main fatty acids (Li et al., 2007). Also in *R. mucilaginosa* TJY15a, 85.8% long-chain fatty acids were composed of palmitic, palmitoleic, stearic, oleic, and linolenic acids (Li et al., 2010). Under continuous culture conditions, nitrogen-limited medium and a dilution rate of about one-third of the maximum is recommended to achieve the maximum content of lipids in a microorganism (Dai et al., 2007). Mix cultivation of microalgae (*Spirulina platensis*) and yeast (*Rhodotorula glutinis*) for lipid production was studied (Xue et al., 2010). Mixing cultivation of the two microorganisms significantly increased the accumulation of total biomass and total lipid yield.

Oils and fats are primarily composed of triacylglycerols (TAGs). TAGs serve as a primary storage form of carbon and energy in microorganisms; their fatty acid composition is also superior to that of other cellular lipids (phospholipids and glycolipids) for biodiesel production (Subramaniam et al., 2010). Although fatty acids in microbial lipids range from lauric acid (C12:0) to docosahexaenoic acid (C22:6), palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids constitute the largest fraction. Of these, palmitic and oleic acids are the most abundant. Considering the saturated and unsaturated acid components, approximately 25–45% are saturated fatty acids, and 50–55% are unsaturated. Thus, the ratio of unsaturated to saturated fatty acids in microbial oils ranges between 1 and 2, which is somewhat similar to that in plant oils (such as palm). When cultivated under appropriately optimized conditions, microorganisms are capable of producing significant quantities of γ-linoleic (C18:2) and arachidonic (C20:4) acids. These fatty acids have high nutraceutical value, and microbial oils are generally marketed as extracted oils as health food. Technologically, the production of these high value compounds is accompanied by production of significant quantities of other neutral lipids. Hence, separation of non-nutraceutical fatty acids from the PUFA needs to be explored (Subramaniam et al., 2010).

Production of microbial lipids to biofuel production is limited by cost; economically viable biofuels should be cost competitive with petroleum fuels. The single-cell oil production cost depends mainly upon the species chosen for cultivation, lipid concentration within cells, and the concentration of cells produced. The cost of feed stock or carbon source required for the production of microbial lipids accounts for 60 to 75% of the total costs of the biodiesel. Thus, the cost of lipid production was influenced strongly by the cost of medium nutrients (50%) needed for cultivation of cells and the cost of solvent (25%) for the extraction of lipids from biomass. Hence, the economics of single-cell oil production can be improved by using carbon in wastes such as wastewater, municipal, and other carbonaceous industrial wastes and CO2 in flue gases from boilers and power plants. Economic analyses have indicated the need to minimize costs of medium components and for further research dealing with microbial systems capable of producing lipids at relatively high productivities in minimal media (Subramaniam et al., 2010).

Lipid production in *Rhodotorula* cells occurs over a broad range of temperatures and it can be considered an interesting genus for the production of single cell oils. The extent of the carbon excess had positive effects on triacylglycerols production, that was maximum with 120 g/L glucose, in terms of lipid concentration (19 g/L), lipid/biomass (68%) and lipid/glucose yields (16%). Both glucose concentration and growth temperature influenced the composition of fatty acids, whose unsaturation degree decreased when the temperature or glucose excess increased. Fatty acid profiles were studied in six carotenoid-producing yeast species isolated from temperate aquatic environments in Patagonia. The proportion of each FA varied markedly depending on the taxonomic affiliation of the yeast species and on the culture media used. The high percentage of polyunsaturated fatty acids (PUFAs) found in Patagonian yeasts, in comparison to other yeasts, is indicative of their cold-adapted metabolism (Libkind et al., 2004). The hydrolysis of triacylglycerols to free FA and glycerol by lipases from oleaginous yeasts as *R.glutinis* or *Yarrowia lipolytica* can have many prospective industial applications e.g. digestive acids, flavour modifications, interesterification of oils etc.

Growth and lipid modifications of pigment-forming yeasts of genus *Rhodotorula* and *Sporobolomyces* growing under presence of selenium recently were studied (Breierova et al.,

2008). Because some of the red yeasts also produce enough quantity of lipids, such selenized red yeasts might be considered as a valuable source of both carotene pigments and useful lipids. However, until date there has not been any available data dealing with effect of selenium on fatty acid alternations in microorganisms. Therefore the aim of our further study was to describe modification in fatty acid profile in various lipid structures of red yeasts grown under selenium addition to the cultivation medium. Sensitivities of all cultures to selenium were similar and yeasts commonly accepted up to 0.12 mM selenium ions. It should also be noted that addition of selenium to the media prolonged lag-phase of yeasts significantly probably as a consequence of adaptation on selenium presence (Certik M., unpublished data).

Total lipids, neutral lipids and the main membrane lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol) of investigated yeasts consisted of mainly palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C 18:1), linoleic (C18:2) and linolenic (C18:3) acids. Oleic acid was the main fatty acid almost in all investigated lipid structures, palmitic and stearic acids were also abundant in PE and in PS+PI fractions. Neutral lipids did not show such intensive changes in fatty acid composition as their polar counterparts. On the other hand, phosphatidylcholine displayed remarkable high amounts of C18:2 and C18:3 fatty acids in all investigated yeasts. Because conversion of oleic acid to its C18 di- and three-unsaturated metabolites is catalyzed by membrane-bound Δ^{12} and Δ^{15} fatty acid desaturases (Certik et al., 1998), it is tempting to speculate that biosynthesis of C18 unsaturated fatty acids in *Rhodotorula* and *Sporobolomyces* species is associated with phosphatidylcholine moieties. Microsomal PC was also found as the predominant site for fatty acid desaturations in other yeasts and fungi (Jackson et al., 1998).

Selenium in the medium without any doubt triggers a set of various mechanisms affecting overall metabolisms of yeasts. It is known that phospholipids as the basic structural elements of the membranes are sensitive to the environment alterations. Since fatty acids are the major constituents of the membrane lipids, modulation of number and position of double bonds in acyl chains by individual fatty acid desaturases play crucial role in preserving of suitable dynamic state of the bilayer. Preliminary results in *R. glutinis* demonstrate that selenium stimulates biosynthesis of C18 fatty acids as well as it promotes distribution unsaturated C18 fatty acids in the membrane lipids. These findings might be very useful for preparation of selenized red yeasts containing carotenoid pigments with enhanced accumulation of linoleic and linolenic acids. (Breierova et al 2008, Certik et al., 2009).

2.5.3 Production of red yeast biomass with accumulated metals

Heavy metals are natural components of the Earth´s crust. As trace elements, some heavy metals (e.g., copper, selenium, zinc) are essential to maintain the metabolism of the human body. However, at higher concentrations they can lead to poisoning. A special case of antioxidant/prooxidant behavior of carotenoids emerge in the presence of metals (e.g. metal-induced lipid peroxidation). In this case metal ions (Fe^{2+} or Cu^{2+}) react with hydroperoxides, via a Fenton-type reaction, to initiate free radical chain processes. There are several studies which indicate that β-carotene offers protection against metal-induced lipid oxidation. Presence of carotenoid in the reaction system not only decreases the free radical concentration, but also the reduction of Fe^{3+} to Fe^{2+} by carotenoids may occur. Recently free radical scavenging and antioxidant activities of metabolites produced by carotenogenic

yeasts of *Rhodotorula* sp. and *Sporobolomyces* sp. grown under heavy metal presence were studied using various EPR experiments (Rapta et al., 2005). Since carotenogenic yeast differ each to other in resistance against the heavy metals due to their individual protective system, quenching properties and antioxidant activities of carotenoids yeasts were modulated by metal ions variously. Thus, activated biosynthesis of carotenoides by yeasts exposed to heavy metal presence could be in part explained by their scavenger characters (Rapta et al., 2005) as a protection against the harmful effect of the environment.

Several divalent cations (Ba, Fe, Mg, Ca, Zn and Co) have been demonstrated to act as stimulants for growth of *R. glutinis. T*race elements have been shown to exert a selective influence on the carotenoid profile in *R. graminis*—Al3+ and Zn2+ had a stimulatory effect on beta-carotene synthesis, while Zn2+ and Mn2+ had a inhibitory effect on torulene and torularhodin synthesis (Buzzini et al, 2005). The observed effect of trace elements on the biosynthesis of specific carotenoids in red yeasts may be explained by hypothesizing a possible activation or inhibition mechanism by selected metal ions on specific carotenogenic enzymes, in particular, on specific desaturases involved in carotenoid biosynthesis. In a recent study, calcium, zink and ferrous salts were shown to have a stimulatory effect on volumetric production as well as cellular accumulation of carotenoids from the yeast *R. glutinis (*Bhosale & Gadre, 2001*)*. Divalent cation salts increased the total carotenoid content (mg/L) about two times. It can be assumed that this positive response was due to a stimulatory effect of cations on carotenoid-synthesizing enzymes, or to the generation of active oxygen radicalcals in the culture broth. In contrast, the addition of manganese salt in the presence of generators of oxygen radicals had an inhibitory effect on carotenoid formation in *X. dendrorhous* since manganese acts as a scavenger; however, this effect could be concentration dependent as manganese is also known to act as a cofactor for enzymes involved in carotenoid biosynthesis and thus enhances carotenoid accumulation at certain concentrations (Frengova & Beshkova, 2009).

Astaxanthin content was decreased significantly at >1 mg/L FeCl3 and growth of *P.rhodozyma* was poor at an FeCl3 concentration of <0.1–1.0 mg/L (An et al., 2001). Carotenoid production decreased in yeast with increasing Mn2+ concentration (0–10 mg/l) when succinate was used as the sole C source, but not when growth took place in the presence of glucose. The week oxygen radical scavengers Zn2+ and Cu2+ had no effect on carotenoid production by *P. rhodozyma*, whereas Cu2+ below 3.2 μM increased the astaxanthin content of cells *P. rhodozyma* but at the expense of a slightly decreased growth. In yeast, there are at least two intracellular enzyme systems requiring copper: cytochrome-*c*-oxidase and superoxide dismutase. These enzymes are probably related to the increased astaxanthin production seen in concentrations of Cu2+ below 3.2 μM. Copper deficit decreases the activity of antioxidant enzyme Cu,Zn-superoxide dismutase, as reported previously and may induce oxidative stress and astaxanthin synthesis because of diminished antioxidant defences. In contrast, iron below 1 μM decreased both the growth and astaxanthin content of cells *P. rhodozyma* (Flores-Cotera & Sanchez, 2001).

Selenium (Se) is a key trace element required in small amounts in humans and animals for the function of a number of Se-dependent enzymes; however, this element can also be toxic in larger doses. Se is incorporated into proteins to provide selenoproteins, which are important antioxidant enzymes; other selenoproteins participate in the regulation of thyroid function and play a role in the immune systém (Wang & Xu, 2008). Organically bound Se is considered as more bioavailable and suitable for dietary application than sodium selenite or podium selenate, the two inorganic forms of Se commonly used in the feed industry. Yeasts

naturally incorporate Se into the biomass where it is stored as selenomethionine. The organic form of Se produced in yeasts is of the similar type as that obtained from food. Recently preparation of antioxidant formula based on carotenoid forming yeasts *Rhodotorula glutinis* and *Sporobolomyces roseus* that also efficiently accumulated selenium from the growth medium was reported (Breierova et al., 2008).

In the presence of Se, carotenogenic yeast strains produced less carotene pigments. The results obtained indicate that the most dramatic change was observed in the significantly lowered levels of β-carotene, while torularhodin and torulene contents decreased to a lesser extent (Breierova et al., 2008). Previously, it has been shown that Cd, Ni, and Zn induce the opposite effect and stimulate production of β-carotene. It was found that direct incorporation of Se into yeast cells during cultivation in Se-rich medium can not be used for preparation Se-enriched yeast biomass. Instead, cultivation of the yeasts and a subsequent treatment with sodium selenite during 24h should be applied. A non-lethal and simultaneously maximum tolerated concentration of Se was determined based on the growth curves of the individual strains. A 60-ppm concentration was used with all strains, and the distribution of Se in the cells, on the surface of cells, and in the exopolymers was analyzed. The maximum Se sorption was observed with the cells of species *Rhodotorula glutinis* CCY 20-2-26 (17 mg/g dry weight), while its exopolymers accumulated only 7% of the total adsorbed Se. The remaining Se was sorbed onto the fibrillar part of the cell wall and into the cells. Similarly, two other studied strains, CCY 19-6-4 and CCY 20-2-33, sorbed Se primarily into cells (63–74%) and the fibrillar part of cell wall (2–22%), whereas exopolymers bound only 12–32% of the total sorbed amount. The yeasts with high content of the carotenoid pigments and selenium may be used for the preparation of a new type of antioxidant formula that could be directly applied for various human and animal diets. Such a formula can only be produced by separate processes of the cultivation of red yeasts and a subsequent sorption of selenium into the cells (Breierova et al., 2008).

In general, there have been several reports on the enhancement of volumetric production (mg/l) as well as cellular accumulation (mg/g) of microbial carotenoid upon supplementation of metal ions (copper, zinc, ferrous, calcium, cobalt, alluminium) in yeasts and molds (Bhosale, 2004; Buzzini et al., 2005). Trace elements have been shown to exert a selective influence on the carotenoid profile in red yeasts. It may be explained by hypothesizing a possible activation or inhibition mechanism by selected metal ions on specific carotenogenic enzymes, in particular, on specific desaturases involved in carotenoid biosynthesis, in agreement with previous studies reporting activation or inhibition by metal ions in microbial desaturases (Buzzini et al., 2005). The other explanation is based on observations that presence of heavy metals results in formation of various active oxygen radicals what, in a turn, induces generation of protective carotenoid metabolites that reduce negative behaviour of free radicals. Such strategy has been applied in several pigment-forming microorganisms to increase the yield of microbial pigments (Rapta et al., 2005; Breierova et al., 2008).

2.5.4 Enrichment of red yeast biomass by specific isoprenoid compounds – ergosterol and Coenzyme Q10

In previous text main groups of biotechnologically important metabolites used for enrichment of red yeast biomass were described. Mainly carotenoids, ergosterol, lipids and metal accumulation in red yeast cells makes them attractive for industrial applications.

Ergosterol is provitamin D, part of was followed partly as the additional parameter of biomass quality and also to monitor the competition of two specialized branches of isoprenoid pathway, which is used for the biosynthesis of both carotenoids and sterols. The production of ergosterol was very similar to the production of β-carotene, even if these metabolites were formed in competitive branches of isoprenoid metabolic pathway (Marova et al., 2010). Practically simultaneous oscillation in carotenoid and ergosterol production under optimal conditions could be caused by the role of both metabolites in *R. glutinis* stress response. Carotenoids act as antioxidants and may prevent cells or cell membranes against negative effects of increased oxidative stress. Ergosterol is an integral component of yeast cell membranes, which are very sensitive to external stress. Recently it has been found that the major changes in intact cells of red yeast *Rhodotorula minuta* irradiated by UV-B were interpreted as combination of changes observed in the cell wall and membrane, the changes observed in the membrane preparations were attributed to ergosterol (Tan et al., 2003).

Ergosterol is a precursor of Vitamin D2 and it is also used for the production of cortisone (Metzler 2003). Now ergosterol as single product is commercially produced by yeast fermentation using *Saccharomyces cerevisiae* strains. The popular means to improve the ergosterol fermentation are optimization of the culture medium, screening of the high ergosterol producing strains. Different carbon sources, nitrogen sources and other nutrient materials had different influences on cell growth and accumulation of ergosterol in yeast biomass. A new yeast strain, obtained by way of protoplast fusion, increased the biomass to 2.45 g/100 ml (dry cell weight) and the ergosterol content to 3.07% (Frengova a Beshkova, 2009). It was reported that the synthesis of ergosterol was not determined by cell growth but by the oxygen consumption rate. Ethanol was formed in yeast fermentation and it had an obvious influence on the growth of yeast. In yeast culture process, glucose is preferred and when the glucose concentration reaches a low value, the cell growth is confined. Then after a short period of adaption, cells continue to grow by consuming the ethanol produced in the first phase as the carbon source. The whole process appeared to be a two-phase process. The ergosterol content increased when the specific growth rate decreased. The environmental and physiological parameters such as the dissolved oxygen, oxygen uptake rate of yeast cells culture had direct or indirect influences on the accumulation of ergosterol and the growth of yeast cells. The interaction relation might help to optimize the ergosterol fermentation. But until now little work has been reported on this relation (Tan et al., 2003).

Carotenoids are important natural pigments that play an essential role as accessory light-harvesting pigments and, especially, in protection against damage by photosensitized oxidation. Several yeast genera—*Rhodotorula, Sporobolomyces, Rhodosporidium,* and *Cryptococcus*—produce also coenzyme Q10 (CoQ10; Dimitrova et al., 2010). CoQ10 has a similar isoprenoid chain in its structure. It is also an interesting product for biotechnology. CoQ10 is present in all cells and membranes, and in addition to being a member of the mitochondrial respiratory chain, it also has several other functions of great importance for the cellular metabolism, such as participation in the extra-mitochondrial electron transport (plasma membranes and lysosomes), regulation of the mitochondrial permeability of transition pores, and regulation of the physicochemical properties of membranes. CoQ10, especially, is widely used as an essential component of ATP generation in the oxidative phosphorylation process and as an antioxidant preventing lipid peroxidation and scavenging superoxide. It has been proved that yeast CoQ10 is much better absorbed by the skin than the synthetic CoQ10. Peroxide reduction in the stratus corneum is considerably more pronounced after yeast CoQ10

application. Therefore, research efforts on the production of CoQ10 by microorganisms focus on the development of potent strains by conventional mutagenesis and metabolic engineering, analysis and modification of the key metabolic pathways, and optimization of fermentation strategies. Various microorganisms, including bacteria (e.g., *Agrobacterium, Rhodobacter*) and yeasts (e.g., *Candida, Rhodotorula*, and *Saitoella*), are reported as CoQ10 producers in patented laid-open applications purposely applied in pharmaceutical and cosmetic industry (Dimitrova et al., 2010, Yurkov et al., 2008).

Strains of basidiomycetous yeasts isolated from different sources were studied in order to determine the content of carotenoid pigments and ubiquinone Q10 for subsequent selection work to obtain producers of these substances. The high specific productivity of carotenoids (600–700 mg/g) was revealed in the representatives of the following species: *Cystofilobasidium capitatum, Rhodosporidium diobovatum, R. sphaerocarpum, Rhodotorula glutinis, Rhodotorula minuta,* and *Sporobolomyces roseus.* The ratio of the major pigments (torulene, torularhodine and β-carotene) in the representatives of different species was studied. Certain specific features of pigment formation in relation to the taxonomic position of the yeasts were determined. Eurybiont species with substantial ecological lability are the most active producers of carotenoids and ubiquinone Q10 among the epiphytes. It is the first time a comparative analysis of the coenzyme Q10 content in different taxa has been performed using several strains of the same species. The maximal coenzyme Q10 production (1.84 mg/g of dry biomass) was found in the yeast species *R. sphaerocarpum* (Yurkov et al., 2008).

2.5.5 Carotenoid-synthesizing yeasts—directions for their use

Because of the biological role of the carotenoids as vitamin A precursors in humans and animals and owing to their antioxidant properties and suspected activity in preventing some forms of cancer as well, carotenoid pigments represent a group of most valuable molecules for industrial applications of red yeasts. The pharmaceutical, chemical, feed and food industries have shown increased interest in the use of carotenoids, mainly as provitamin A, but also as natural food and feed colorants. Accordingly, the red yeast *P. rhodozyma* is currently used for the production of astaxanthin, an important carotenoid pigment that can be exploite in aquaculture to give an appealing pink color to the fresh of farmed salmonid fish, and it also helps to impart a desirable golden color to the egg yolk and fresh of poultry. Salmon farming is an industry that is growing and gradually replacing the world's wild salmon fisheries. The most expensive ingredient in salmonid feeds is astaxanthin, and though the actual revenues are privately held, it has been estimated that the market for astaxanthin in >US $100 milion per year (Frengova & Beshkova, 2009).

Similarly to *Xanthophyllomonas*, also other red yeast strains could be used for industrial puropses to pruduction of carotenoids – beta-carotene, torulene, lycopene, as well as further lipid metabolites produced in cells. In many works mostly *Rhodotorula glutinis* sems to be perspective strain. Combined enrichment of *Rhodotorula* biomass by provitamin A (carotenes) and provitamin D (ergosterol) could be used in food and feed supplements (Marova et al., 2010), aditional enrichment by Coenzyme Q10 is suitable product for cosmetics and could be used also in food and feed (Dimitrova et al., 2010). Formulas based on selenium-enriched red yeast biomass with enhanced carotenoid content could be used as nutrition suplement too (Breierova et al, 2008). There is also posibility to use oleaginous red yeasts to single cell oil production; in this case production of other lipid metabolites could be reduced and the main flow of acetylCoA will be directed to fatty acid and lipid biosynthesis (Dai et al., 2007).

One limitation impacting the industrial utility of *P. rhodozyma*/ *X. dendrourhous* or *Rhodotorula* species has been hindered absorption of carotenoids, due to the yeast`s thick cell wall. Because of presence of other specific biologically active compounds as well as high level of nutritionally sigificant yeast cell components (proteins, unsaturated fat, vitamins…) the best strategy is to disrupt cells and to use the whole biomass without isolation of individual compounds. The biotechnology industry has developed different means of active compounds liberation by the yeast including optimization of drying conditions, mechanical breakage, microwave treatment and enzyme treatment, as described below (Frengova & Beshkova, 2009).

When disrupted cells *P. rhodozyma*, without cell walls are added to the diets of animals, astaxanthin is readily absorbed from the gut; it effectively colors the fresh of penreared salmonids, and also helps impart a desirable golden color to the egg yolk and fresh of poultry. Astaxanthin in yeast (*X. dendrorhous*) prepared by spray drying and Xat-roller milling was well absorbed by laying hens and was successfully used as a pigmentation agent in animals (An, 2005). Specifically, when spray-dried and milled yeast was supplied in the feed (40 mg astaxanthin/kg feed), astaxanthin was successfully absorbed (1,500 ng/ml blood and 1,100 ng/g skin) by laying hens. Extrusion temperature did not affect utilization of dietary astaxanthin or rainbow trout fresh color significantly, but cell wall disruption of red yeast cells was critical to optimize carotenoid utilization. Increasing the degree of enzymatic cell wall disruption increased fresh astaxanthin concentrations from 2.2 to 6.7 mg/kg, redness values from 5.5 to 10.7, yellowness values from 11.7 to 16.7 and astaxanthin retentions in the muscle from 3.7 to 17.4%. A formulation of *P. rhodozyma* cells blended with ethoxyquin, lecithin and oil prior to drying also increased astaxanthin deposition in salmonid fish fresh and rainbow trout fresh when supplied in feed as an additive. Absorption and accumulation of biological astaxanthin were higher thah those of chemical astaxanthin, probably because of the high contents of lipids in the yeast (17%). Lipid peroxide formation in skin was significantly decreased by astaxanthin. The peroxide production in chickens fed chemical astaxanthin was markedly lowered compared to biological astaxanthin (Frengova & Beshkova, 2009) .

The levels of serum transaminase activities and of lipid peroxides in fish fed oxidized oil were significantly higher that those of the control fish fed non-oxidized oil. However, the supply of freeze-dried red yeast preparation considerably decreased both enzyme activities and lipid peroxides level. Furthermore, the serum lipid (triglycerides, total cholesterol and phospholipids) concentrations were also significantly decreased. Especially, the serum triglyceride level of fish fed the red yeast was as low as that of the control. Recently was found that Zn2+ ions induced changes in yeasts (*R. glutinis* and *R. rubra*) leading to more efficient scavenging and antioxidant capacities compared with Ni2+ ions, and antioxidants (carotenoids) present in yeast's walls showed higher ability to scavenge free radicals than those from inside the cells (Rapta et al., 2005). Later, the in vivo antioxidant and protective effects of astaxanthin isolated from *X. dendrorhous* against ethanol-induced gastric mucosal injury were established in animal models, especially rats (Kim et al., 2005). Oral administration of astaxanthin showed significant protection against ethanol-induced gastric lesion and inhibited elevation of the lipid peroxide levels in gastric mucosa. A histologic examination clearly indicated that the acute gastric mucosal lesion induced by ethanol nearly disappeared after pretreatment with astaxanthin (Frengova & Beshkova, 2009).

Chemopreventive and anticarcinogenic effects of carotenoids by *Rhodotorula* on the development of preneoplastic lesions during *N*-nitrosodiethylamine (DEN)-induced

hepatocarcinogenesis in female Wistar strain rats were also studied (Bhosale et al., 2002). Spray-dried yeast *R. glutinis* (containing carotenoid pigments torulene, torularhodin and beta-carotene in proportion 58:33:2) showed significant effect on the prevention of liver tumor development. However, *R. glutinis* effects were relatively more significant in groups where *R. glutinis* was administered after DEN treatment, suggesting that *R. glutinis* is quite effective in the prevention of liver tumor development especially when administered after DEN treatment, indicating possible protective effects at the promotional stages.

3. Conclusions

Yeast is, due to its physiological properties, widely used in the food, feed, chemical and pharmaceutical industries for production of various valuable compounds. Red yeast is well known producer of carotenoids which are significant because of their activity as vitamin A precursors, colorants, antioxidants and possible tumor-inhibiting agents. Biological sources of carotenoids receive major focus nowadays because of the stringent rules and regulations applied to chemically synthesized/purified pigments. Compared with the extraction from vegetables, the microbial production of carotenoids is of paramount interest, mainly because of the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin. Moreover, red yeast is a rich source of other specific compounds – ergosterol, Coenzyme Q10, as well as unsaturated fatty acids, fats, proteins and vitamins and can be incorporated in feeds to enhance the nutritional value of yeast biomass. One limitation impacting the industrial utility of carotenogenic yeast has been complicated liberation and bioavailability of carotenoids and other active compounds, due to the yeast's thick cell wall.The biotechnological industry has developed different means of pigment liberation by the yeast including optimization of drying conditions, mechanical breakage, microwave treatment and enzyme treatment.

The other very important limitation involved in the practical exploitation of yeasts is the high cost of microbial production. The production cost could be reduced by increasing yields of product, as well as using less expensive substrates. There is a need to improve fermentation strategies. Biomass and metabolites production by red yeast is highly variable and can be influenced by cultivation conditions (light, temperature, pH, aeration etc.). Different approaches for improving the production properties of the yeast strains, such as environmental stress, mutagenesis or genetic modification, have been studied and optimized. The other possibility for production cost reduction is using various low-cost materials as carbon or nitrogen source. The potential of several waste materials (whey, potato mass, apple mass and various cereals) as substrates for carotenoid and ergosterol production by some yeast strains belonging to the genus *Rhodotorula* and *Sporobolomyces* were succesfully examined. Mild nutrition stress cause by several waste substrates was found to be the suitable induction factor for higher carotenogenesis and ergosterol production in red yeasts.

Environmental stress was reported to induce carotenoid, ergosterol and lipid production as part of red yeast stress response. Under stress cells posses altered phenotype biotechnologically significant and/or undesirable in a dose-dependent manner. Phenotypic profiling of the environmental stress responses demonstrates genetic susceptibility of yeast to environmental stress. Low concentrations of oxidative and osmotic stress, which can under specific conditions induce carotenogenesis, have no significant effect on yeast growth. Red yeast cultivated under osmotic and oxidative stress or on various waste substrates

shows no significant differences in cell morphology when compared with yeast cultivated in conventional glucose medium under optimal conditions. Thus, low environmental stress can be used for induction of carotenogenesis and use of non-toxic stress factors (salt, metals) can enable utilization o whole cell biomass to industrial use. Simple and cheap stress factor in relatively low concentration can substantially enhance biotechnologically significant metabolite production.

Growing interest in pigment and other metabolite applications in various fields coupled with their significance in health and dietary requirements has encouraged "hunting" for more suitable sources of these compounds. Due to restrictions, there is no possibility to apply carotenoids prepared by chemical synthesis for food, pharmaceutical and medical purposes. However, the success of microbial pigments, metabolites and single cell oils depends upon their acceptability in the market, regulatory approval, and the size of the capital investment required to bring the product to market. Therefore, the focus of biotechnology on highly valuable yeast biomass requires knowledge how microorganisms control and regulate the biosynthetic machinery in order to obtain metabolites and enriched biomass in high yield and at low price. From this view, attempts have been directed at the development and improvement of biotechnological processes for the utilization of red yeasts on an industrial scale. Current successes using mutation methods and molecular engineering techniques carried out over recent years have not only answered some fundamental questions related to pigment formation but has also enabled the construction of new microbial varieties that can synthesize unusual carotene metabolites. Elucidation of these mechanisms represents a challenging and potentially rewarding subject for the further research and may finally allow us to move from empirical technology to predictable carotenoid and/or isoprenoid metabolite design. Thus, the manipulation and regulation of red yeast metabolism open a large number of possibilities for academic research, demonstrates the enormous potential in its application and creates new economic competitiveness and market of microbial lipid compounds.

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5. References

Aksu, Z. & Eren, A. T. (2005). Carotenoids production by the yeast Rhodotorula mucilaginosa: Use of agriculture wastesas a carbon source. *Process Biochemistry* Vol. 40, No.9, pp. 2985-2991, ISSN 1359-5113

An, G.H. (2001). Improved growth of the red yeast, Phaffia rhodozyma (Xanthophyllomyces dendrorhous), in thepresence of tricarboxylic acid cycle intermediates. *Biotechnology Letters* Vol. 23, pp. 1005–1009, ISSN 0141-5492

Armstrong, G. A. & Hearst, J. E. (1996). Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis. *The FASEB Journal*, Vol.10, No.2, pp. 228-237, ISSN 0892-6638

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., Jouve, C.M. & Nicaud, J.M. (2009) Yarrowia lipolytica As a model for biooil production. *Progress in Lipid Research* Vol.48, pp. 375–387, ISSN 0163-7827

Bhosale, P. (2004). Environmental and cultural stimulants in the production of carotenoids from microorganisms. *Applied Microbiology and Biotechnology*, Vol.63, 351–361, ISSN 0175-7598

Bhosale, P. & Gadre, R. V. (2001a). Production of ß-carotene by a mutant of *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology,*Vol.55, No.4, pp. 423-427, ISSN 0175-7598

Bhosale, P. & Gadre, R. V. (2001b). β-Carotene production in sugarcane molasses by a *Rhodotorula glutinis* mutant. *Journal of Industrial Microbiology and Biotechnology*, Vol.26, No.6, pp. 327-332, ISSN 1367-5435

Breierova, E.; Gregor, T.; Marova, I.; Certik, M. & Kogan, G. (2008). Enhanced antioxidant formula based on a selenium-supplemented carotenoid-producing yeast biomass. *Chemistry and Biodiversity*, Vol.5, No.3, pp.440-446, ISSN 1612-1872

Britton, G.; Liaen-Jensen, S. & Pfander H. (1998): *Carotenoids.* Volume 3: Biosynthesis and Metabolism, pp. 14-191. Birkhäuser Verlag Basel-Boston-Berlin ISBN 3-7643-5829-7

Buzzini, P. (2001). Batch and fed-batch carotenoid production by *Rhodotorula glutinis–Debaryomyces castellii* co-cultures in corn syrup. *Journal of Applied Microbiology*, Vol.90, No.5, pp. 843-847, ISSN 1364-5072

Cerda-Olmedo, E. (2001). Phycomyces and the biology of light and color. *FEMS Microbiology Reviews* Vol.25, pp.503–512, ISSN 0168-6445

Certik, M.; Hanusova, V.; Breierova, E.; Marova, I. & Rapta P. (2009). Biotechnological production and propertiesof carotenoid pigments In: *Biocatalysis and Agricultural Biotechnology* (415 p.), Chapter 25, pp.358-373. Tailor and Francis Group LLt, ISBN 978-1-4200-7703-2

Dai, C.; Tao, J.; Xie, F.; Dai, Y. & Zhao, M. (2007). Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity. *African Journal on Biotechnology* Vol.6, No.18, pp.2130–2134, ISSN 1684-5315

Davoli, P.; Mierau, V. & Weber R. W. S. (2004). Carotenoids and Fatty Acids in Red Yeasts *Sporobolomyces roseus* and *Rhodotorula glutinis. Applied Biochemistry and Microbiology*, 2004. 40(4): p. 392-397, ISSN 0003-6838.

Dimitrova, S.; Pavlova, K.; Lukanov, L. & Zagorchev P. (2010). Synthesis of Coenzyme Q10 and β-carotene by yeastsisolated from Antarctic soil and lichen in response to UV and visible radiations. *Applied Biochemistry and Biotechnology* Vol.162, pp.795–804, ISSN 0273-2289

Ducrey Sanpietro, L.M. & Kula, M.R. (1998). Studies of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* (Phaffia rhodozyma). Effect of inhibitors and low temperature. *Yeast* Vol.14, pp.1007–1016, ISSN 0749-503X

Dufosse, L. (2006). Microbial Production of Food Grade Pigments. *Food Technology and Biotechnology*, Vol.44,pp. 313–321, ISSN 1330-9862.

Easterling, E.R.; French, W.T.; Hernandez, R. & Licha, M. (2009) The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. *Bioresource Technology* Vol. 100, pp.356–361, ISSN 0960-8524

Fang, TJ., Wang, J.M. (2002). Extractibility of astaxanthin in a mixed culture of a carotenoid over-producing mutant of *Xanthophyllomyces dendrorhous* and *Bacillus circulans* in two-stage batch fermentation, *Process Biochemistry* Vol. 37, pp.1235–1245, ISSN 1359-5113

Flores-Cotera, L.B. & Sanchez, S. (2001). Copper but not iron limitation increases astaxanthin production by *Phaffia rhodozyma* in a chemically defined medium. *Biotechnology Letters* Vol.23, pp.793–797, ISSN 0141-5492

Frengova, G. I. & Beshkova, D. M. (2009). Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance. *Journal of Industrial Microbiology & Biotechnology*, Vol. 36, No.2, pp. 163-180, ISSN 1367-5435

Frengova, G.; Simova, E. & Beshkova, D. (2004). Use of whey ultrafiltrate as a substrate for production of carotenoids by the yeast *Rhodotorula rubra*. *Applied Biochemistry and Biotechnology* Vol.112, pp.133–141, ISSN 1367-5435

Gasch, A. P. & Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Functional & Integrative Genomics*, September, Vol. 2, No.4-5, pp. 181-192, ISSN 1438-793X

Gasch, A. P.; Spellmann P. T.; Kao C. M.; Carmel-Harel, O.; Eisen M. B., Storz, G.; Botstein, D. & Brown, P. O. (2000). Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. *Molecular Biology of the Cell*, December, Vol. 11, No.12, pp. 4241-4257, ISSN 1059-1524

Hassan, M.; Blanc, P.J.; Granger, L.M.; Pareilleux, A. & Goma, G. (1996). Influence of nitrogen and iron limitationson lipid production by *Crytococcus curvatus* grown in batch and fed-batch culture. *Process Biochemistry* Vol.31, No.4, pp.355–361, ISSN 1359-5113

Hibbett, D.S. (2007). A higher level phylogenetic classification of the *Fungi. Mycological Research* Vol.111, No.5, pp.509–547, ISSN 0953-7562

Hohmann, S. & Mager, W.H. (2003). *Yeast Stress Responses*. Berlin: Springer-Verlag. 389 p. ISBN 3-540-43926-9

Hoog, G. S.; De Guarro, J.; Gene, J. & Figueras, M. J.(2001). *Atlas of Clinical Fungi.* Second edition. December 1, Washington: ASM Pressp. ISBN 9070351439

Hu, Z.C., Zheng, Y.G., Wang, Z. & Shen, Y.C. (2005). Effect of sugarfeeding strategies on astaxanthin production by *Xanthophyllomyces dendrorhous*. *World Journal of Microbiology and Biotechnology* Vol.21, pp.771–775, ISSN 0959-3993

Hu, Z.C., Zheng, Y.G., Wang, Z. & Shen, Y.C. (2007) Production of astaxanthin by *Xanthophyllomyces dendrorhous*ZJUT46 with fedbatch fermentation in 2.0 m3 fermentor. *Food Technology and Biotechnology* Vol. 45, pp.209–212, ISSN 1330-9862

Kim, J.H.;, Kang, S.W.; Kim, S.W. & Chang, H.I. (2005). High-level production of astaxanthin by *Xanthophyllomyces dendrorhous* mutant JH1 using statistical designs. *Bioscience Biotechnology and Biochemistry* 69:1743–1748.

Kim, J.H.; Choi, S.K.; Choi, S.Y.; Kim, H.K. & Chang, H.I. (2005). Suppresive effect of astaxanthin isolated from the *Xanthophyllomyces dendrorhous* mutant on ethanol-induced gastric mucosal injury in rats. *Bioscience Biotechnology and Biochemistry* Vol.69, pp.1300–1305, ISSN 0916-8451

Kuranda K, Grabinska K, Berges T, Karst F, Leberre V, Sokol S, François J, Palamarczyk G. (2009). The YTA7 gene is involved in the regulation of the isoprenoid pathway in the yeast Saccharomyces cerevisiae. *FEMS Yeast Research*, Vol.9, No.3, pp. 381-390, ISSN 1567-1364

Latha, B.V.; Jeevaratnam, K.; Murali, H. S & Manja, K. S. (2005): Influence of growth factors on carotenoid pigmentation of *Rhodotorula glutinis* DFR-PDY from natural source. *Indian Journal of Biotechnology*, Vol.4, pp. 353-357, ISSN 0972-5849

Leathers, T.D., Jones, J.D., Hohn, T.M. (2004): System for the sequential, directional cloning of multiple DNA sequences. *US patent 6,696,282*

Li, M.; Liu, G.L.; Chi, Z. & Chi, Z.M. (2010). Single cell oil production from hydrolysate of cassava starch by marine- derived yeast *R. mucilaginosa* TJY15a. *Biomass Bioenergy* Vol.34, pp.101–107, ISSN 0961-9534

Libkind, D.,, Brizzio, S. & van Broock, M. (2004). *Rhodotorula mucilaginosa*, a carotenoid producing yeast strain from a Patagonian high-altitude lake. *Folia Microbiologica* Vol.49, No.1, pp.19–25, ISSN 0015-5632.

Libkind, D., Sommaruga, R.; Zagarese, H. & van Broock M. (2005). Mycosporines in carotenogenic yeasts. *Systematic and Applied Microbiology*, Vol.28, No.8, pp. 749-754, ISSN 0723-2020

Libkind, D. & van Broock, M. (2006). Biomass and carotenoid pigment production by patagonian native yeasts. *World Journal of Microbiology & Biotechnology*, 2006, 22, 687–692, ISSN 0959-3993

Libkind, D.; Gadanho, M.; van Broock M., & Sampaio J. P. (2008). Studies on the heterogeneity of the carotenogenic yeast *Rhodotorula mucilaginosa* from Patagonia, Argentina. *Journal of Basic Microbiology*, Vol. 48, No.2), pp. 93-98, ISSN 0233-111X

Lukacs, G., Linka, B., Nyilasi, I. (2006). *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*: Astaxanthin-producing yeasts of biotechnological importance. *Acta Alimentaria*, Vol.5, pp.99–107, ISSN 0139-3006

Maldonade, I.R.; Rodriguez-Amaya, D.B. & Scamparini, A.R.P.(2008). Carotenoids of yeasts isolated from the Brazilian ecosystem. *Food Chemistry* Vol.107, pp.145–150, ISSN 0308-8146

Mann, J. (1990). S*econdary Metabolism*. 2nd edition. September 27, USA: Oxford University Press. 390 p. ISBN 0198555296

Mantzouridou, F.; Roukas, T. & Kotzekidou, P. (2002). Effect of the aeration rate and agitation speed on b-Carotene production and morphology of *Blakeslea trispora* in a stirred tank reactor: mathematical modeling. *Biochemical Engineering Journal,* Vol.10, pp.123–135, ISSN 1639-703X

Marova, I.; Breierova, E.; Koci, R.; Friedl, Z.; Slovak, B & Pokorna J (2004) Influence of exogenous stress factors on production of carotenoids by some strains of

carotenogenic yeasts. *Annals of Microbiology*, Vol. 54, No.1, pp. 73-85, ISSN 1590-4261

Marova, I.; Carnecka, M.; Halienova; A.; Koci, R. & Breierova, E.(2010). Production of carotenoid/ergosterol-supplemented biomass by red yeast *Rhodotorula glutinis* grown under external stress. *Food Technology and Biotechnology, Vol.*48, No.1, *p* p.56-61. ISSN 1330-9862

Marova, I.; Carnecka, M.; Halienova; Dvorakova, T. & Haronikova A.(2011). Use of several waste substrates for carotenoid-rich yeast biomass production. *Journal of Environmental Managemen*t. Accepted 8.6.2011. DOI: 10.1016/j.jenvman.2011.06.018 ISSN 0301-4797

Metzler D.E. (2003): Biochemistry. *The Chemical Reactions of Living Cells*. Vol.2, p.1226-1271. Academic Press, Elsevier Science, USA. ISBN 0-12-492543-X

Papp, T.; Velayos, A.; Bartók, T.; Eslava, A.P; Vágvölgyi, C. & Iturriaga, E.A. (2006). Heterologous expression of astaxanthin biosynthesis genes in *Mucor circinelloides*. *Applied Microbiology and Biotechnology* Vol.69, pp.526–531, ISSN 0175-7598

Parajo, J.C.; Santos, V. & Vazajez, M. (1998). Production of carotenoids by *Phaffia rhodozyma* growing on media made from hemicellulosic hydrolysates of *Eucalyptus globulus* wood. *Biotechnology and Bioengineering* Vol. 59, No. 4, August 20, ISSN 0006-3592

Park, K.M., Song, M.W. & Lee, J.H. (2008). Production of carotenoids by β-ionone-resistant mutant of *Xanthophyllomyces dendrorhous* using various carbon sources. *Biotechnology and Bioprocess Engineering* Vol.13, pp. 197-203, ISSN 1226-8372

Perrier, V.; Dubreucq, E. & Galzy, P.(1995). Fatty acid and carotenoid composition of *Rhodotorula* strains. *Archives of Microbiology*, Vol. 164, No3, pp.173-179, ISSN 0302-8933.

Rapta, P., Polovka, M., Zalibera, M., Breierova, E., Zitnanova, I., Marova, I. & Certik, M. (2005). Scavenging andantioxidant properties of compounds synthesized by carotenogenic yeasts stressed by heavy metals – EPR spin trapping study. *Biophysical Chemistry*, Vol.116, pp.1-9, ISSN 0301-4622

Ratledge, C. & Wynn, J.P.(2002). The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Advances in Applied Microbiology* Vol. 51, pp.1–51, ISSN

Rosa, C. & Peter, G. (2005). *Biodiversity and Ecophysiology of Yeasts (The Yeast Handbook)*. December 7, Berlin, Heidelberg, New York: Springer. 580 p. ISBN 3540261001

Sakaki, H.; Nakanishi, T.; Tada, A., Miki, W. & Komemushi, S. (2001) Activation of torularhodin production by *Rhodotorula glutinis* using weak white light irradiation. *Journal of Bioscience and Bioengineering* Vol. 92, pp.294–297, ISSN 1389-1723

Sandmann, G. (2001). Carotenoid Biosynthesis and Biotechnological Application. Archives *Biochemistry and Biophysics* Vol. 385, pp. 4–12, ISSN 0003-9861

Schmidt, I.; Schewe, H.; Gassel, S.; Jin, C.; Buckingham, J.; Hümbelin, M.; Sandmann, G. & Schrader, J. (2011).Biotechnological production of astaxanthin with *Phaffia*

rhodozyma/Xanthophyllomyces dendrorhous. *Applied Microbiology and Biotechnology* Vol.89, No.1, pp.555–571, ISSN 0175-7598

Schmidt-Dannert, C. (2000). Engineering novel carotenoids in microorganisms. *Current Opinion in Biotechnology* Vol.11,pp.255–261, ISSN 0958-1669

Simova, E.D., Frengova, G.I., Beshkova, D.M. (2004). Synthesis of carotenoids by *Rhodotorula rubra* GED8 co-cultured with yogurt starter cultures in whey ultrafiltrate. *Indian Journal of Microbiology and Biotechnology* Vol. 31, pp. 115–121, ISSN 0972-5849

Somashekar, D. & Joseph, R (2000). Inverse relationship between carotenoid and lipid formation in *Rhodotorula gracilis*according to the C/N ratio of the growth medium. *World Journal of Microbiology & Biotechnology*, Vol.16, pp.491–493, ISSN 0959-3993

Squina, F.M. & Mercadante, A.Z. (2005). Influence of nicotine and diphenylamine on the carotenoid composition of *Rhodotorula* strains. *Journal of Food Biochemistry* Vol.29 pp. 638–652, ISSN 0145-8884

Subramaniam, R.; Dufreche, S.; Zappi, M. & Bajpai R. (2010). Microbial lipids from renewable resources: production andcharacterization. *Indian Journal of Microbiology and Biotechnology, Vol*. 37, pp.1271–1287, ISSN 0972-5849

Talaro, K. P. & Talaro, A. (2001). *Foundations in Microbiology*. Pasadena: McGraw-Hill. 889 p. ISBN 0-07-248864-6

Tan, T.; Zhang, M. & Gao, H. (2003). Ergosterol production by fed-batch fermentation of *Saccharomyces cerevisiae. Enzyme and Microbial Technology* Vol.33 pp. 366–370. ISSN 0141-0229

Tinoi, J.; Rakariyatham, N. & Deming, R.L. (2005). Simplex optimization of carotenoid production by *Rhodotorula glutinis* using hydrolyzed mung bean waste flour as substrate. *Process Biochemistry*, 40(7): p. 2551-2557, ISSN 0032-9592

Verdoes, J.C.; Sandmann, G.; Visser, H.; Diaz, M.; Van Mossel, M. & Van Ooyen, A.J.J. (2003) Metabolic engineering ofthe carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous*. *Applied and Environmental Microbiology* Vol.69, pp.3728–3738, ISSN 0099- 2240

Verwaal, R.; Wang, J.; Meijnen, J.P.; Visser, H.; Sandmann, G.; Van den Berg, J. & Van Ooyen, A.J.J. (2007). High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. *Applied and Environmental Microbiology* 73:4342–4350, ISSN 0099- 2240

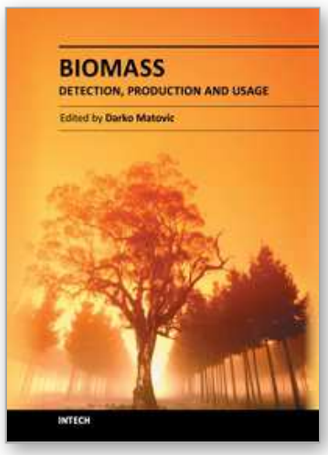
Walker, G. M. (1998). *Yeast physiology and biotechnology*. West Sussex, England: John Willey & Sons Ltd. 362 p. ISBN 0-471-964468

Wang, C.; Oh, M.K. & Liao, J.C. (2000). Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production. *Biotechnology Progress* Vol.16, pp.922–926 ISSN 1520-6033

Xiao, W. (2005). *Yeast Protocols (Methods in Molecular Biology)*. Second edition. September 1, Totowa, New Jersey: Humana Press. 392 p. ISBN 1588294374

Xue, F.; Miao, J.; Zhang, X. & Tan T.(2010). A New Strategy for Lipid Production by Mix Cultivation of *Spirulina platensis* and *Rhodotorula glutinis*. *Applied Biochemistry and Biotechnology* Vol. 160, pp.498–503, ISSN 0273-2289

Yurkov, A.M.; Vustin, M.M.; Tyaglov, B.V.; Maksimova, I.A. & Sinekoiy S.P. (2008). Pigmented *Basidiomycetous* Yeasts are a promising source of carotenoids and ubiquinone Q10. *Microbiology,* Vol. 77, No. 1, pp. 1–6.ISSN 0026-2617



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Biomass has been an intimate companion of humans from the dawn of civilization to the present. Its use as food, energy source, body cover and as construction material established the key areas of biomass usage that extend to this day. Given the complexities of biomass as a source of multiple end products, this volume sheds new light to the whole spectrum of biomass related topics by highlighting the new and reviewing the existing methods of its detection, production and usage. We hope that the readers will find valuable information and exciting new material in its chapters.

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