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ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

VALORIZATION OF FOOD WASTE MATERIALS BY USING SELECTED MICROORGANISMS

VALORIZACE POTRAVINÁŘSKÝCH ODPADŮ POMOCÍ VYBRANÝCH MIKROORGANISMŮ

DOCTORAL THESIS

DIZERTAČNÍ PRÁCE

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Doctoral Thesis Assignment

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Valorization of food waste materials by using selected microorganisms

Doctoral Thesis assignment:

The PhD thesis is focused on complex study of the production capabilities of carotenogenic yeast strains growing on various processed waste substrates.
Partial goals to achieve these objectives included:
1) Conducting a literature review on: waste substrates and their potential processing; on carotenogenic yeasts and their metabolism; cultivation methods; and metabolite analysis techniques.
2) Preparing various waste substrates, or their mixtures, and using them for the cultivation of selected yeast strains.
3) Testing selected cultivation conditions in laboratory-scale bioreactors.
4) Comparing the production characteristics of yeasts on different waste substrates.
5) Monitoring the adaptive changes during the initial stages of cultivation after transferring the culture of selected yeast strains from a complex medium to a mineral medium.
6) Analysing the live cultures using fluorescence techniques (flow cytometry and microscopy), as well as standard techniques (HPLC-PDA and GC-FID).

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Doctoral Thesis is necessary to deliver to a secretary of institute in the number of copies defined by the dean. This assignment is part of Doctoral Thesis.

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prof. Ing. Michal Veselý, CSc.
Dean

Abstract

The valorization of waste materials via microorganisms is currently a topic addressed in numerous scientific studies. This dissertation thesis focuses on the valorization of waste from the food industry and biofuel production available in a landlocked country (Czech Republic), as it does not deal with the broader issue of waste from marine animal and plant processing. It also provides a literature review on the utilization of various wastes and their potential applications in biotechnology within the framework of a circular economy model.

The carotenogenic yeasts used in this study are oleaginous yeasts with a specific enzymatic profile capable of synthesizing carotenoids, which give the yeasts their distinct coloration. In addition, they are able to accumulate ergosterol, ubiquinone, beta-glucans, and lipids in significant quantities. These characteristics make them potential industrial producers of bioactive compounds or enriched biomass suitable for use in the food or pharmaceutical industries. The yeast genera used in this study included *Cystofilobasidium*, *Rhodotorula*, *Sporidiobolus*, *Rhodosporidium*, and *Phaffia*.

The experimental and practical, selected strains of carotenoid-producing yeasts were screened for their ability to valorize individual waste substrates. The waste substrates tested, including hydrolysed coffee grounds, hydrolysed brewers' spent grain, autolyzed brewers' yeast, hydrolysed poultry feathers, waste frying oil, coffee oil from grounds, poultry fat, animal fat, and waste glycerol. Under specific substrate and culture conditions, yeast growth and production characteristics were also evaluated in laboratory bioreactors, yielding promising results, such as a biomass yield of 40.15 g/l for *S. pararoseus* cultured on media containing coffee oil and coffee hydrolysate.

All yeast strains were assessed post-cultivation for growth characteristics, and analyses included HPLC-PDA for carotenoid, ubiquinone, ergosterol, and tocopherol quantification, as well as GC-FID for fatty acid profile and concentration. Select samples were also analyzed for beta-glucan content using enzymatic method.

A further focus of the study was a detailed investigation of the growth and metabolic responses of yeasts in the initial stages of growth in production media, utilizing fluorescence microscopy and flow cytometry. A key finding was that the lag phase of the studied strains shifted to an accelerated growth phase between 4 and 12 hours post-inoculation.

Key words: Carotenogenic yeasts, Oleaginous yeasts, Valorisation, Waste substrates, Lag-phases

Abstrakt

Valorizace odpadních materiálů pomocí mikroorganismů je v nynější době problematikou řešenou v mnoha odborných pracích. Tato disertační práce se zaměřuje na valorizaci odpadů z potravinářství a produkce biopaliv dostupných vnitrozemského státu (ČR), neboť se nezabývá rozsáhlou problematikou odpadů ze zpracování mořských živočichů a rostlin. Zároveň poskytuje rešerši na téma utilizace různých odpadů a jejich možné použití v biotechnologiích v rámci modelu cirkulární ekonomiky.

Karotenogenní kvasinky využité v této práci jsou oleogenní kvasinky se specifickou enzymovou výbavou schopnou syntézy karotenoidů, což kvasinkám dodává jejich specifickou barvu. Dále jsou schopné ve větší míře kumulovat ergosterol, ubichinon, beta-glukany a lipidy. Tyto vlastnosti z nich dělají potenciální průmyslové producenty bioaktivních látek, popřípadě obohacené biomasy použitelné v potravinářství, popřípadě ve farmaceutickém průmyslu. Použitými rody kvasinek byly *Cystofilobasidium, Rhodotorula, Sporidiobolus, Rhodosporidium* a *Phaffia*.

V experimentální a praktické části byly testovány formou screeningových experimentů vybrané druhy karotenogenních kvasinek k valorizaci jednotlivých odpadů. Jako odpadní substráty byly použity: hydrolyzovaná odpadní kávová sedlina, hydrolyzované odpadní pivovarské mláto, autolyzované odpadní pivovarské kvasnice, hydrolyzované odpadní drůbeží peří, odpadní fritovací olej, kávový olej ze sedliny, drůbeží tuk živočišný tuk a odpadní glycerol. V některých podmínkách a kombinacích substrátů byly růstové a produkční vlastnosti kvasinek testovány i v laboratorních bioreaktorech, kde dosahovaly i velmi dobrých výsledků, příkladem je nejvyšší produkce biomasy při kultivaci kmene *S. pararoseus* na médiu obsahujícím kávový olej a kávový hydrolyzát s výtěžkem biomasy 40.15 g/l.

Všechny kvasinkové druhy byly po kultivaci podrobeny stanovení růstových charakteristik a analýzám zahrnujícím HPLC-PDA pro stanovení karotenoidů, ubichinonu, ergosterolu a tokoferolu, dále pak GC-FID pro stanovení množství a profilu mastných kyselin, dále bylo u vybraných vzorků stanoveno množství beta-glukanů enzymovou metodou.

Dalším zaměřením práce byl detailnější pohled na růst a metabolické odpovědi kvasinek v prvních fázích růstu v produkčním médiu s použitím fluorescenční mikroskopie a průtokové cytometrie. Důležitým zjištěním bylo, že lag-fáze u studovaných kmenů přešla do fáze zrychleného růstu v rozmezí od 4-12 hodiny po zaočkování.

Klíčová slova: Karotenogenní kvasinky, Oleogenní kvasinky, Valorizace, Odpadní substráty, Lag-fáze

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Declaration

I declare that the dissertation thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the dissertation thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student’s signature

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1 INTRODUCTION

Ensuring food security for the expanding global population necessitates a sustained increase in both the quantity and efficiency of agricultural output, as well as the processing of food raw materials. One approach for enhancing production efficiency involves the innovative processing of waste generated by the food and agricultural sectors. A substantial portion of biomass is currently underutilized, frequently incinerated or disposed of in landfills, which contributes to environmental pollution through harmful emissions and the leaching of toxic substances into soil and water systems.

From a circular economy perspective, it is advantageous to regard waste materials as by-products of production processes that can be repurposed in subsequent manufacturing processes. The circular economy concept in food and agriculture emphasizes more effective biomass management, promoting its reuse – for instance as a compost for soil enrichment or as substrates for animal or microbial valorisation into high-value products.

In this context, significant attention is directed towards the valorisation of waste materials (substrates) by using of carotenoid-producing and lipid-accumulating yeasts. These yeasts possess the capability to accumulate substantial lipid concentrations within their biomass while simultaneously generating valuable metabolites including carotenoids, ergosterol, and ubiquinone.

2 THEORETICAL PART

2.1 Valorisation of waste materials

Given the escalating environmental burden caused by mass production and population growth, it is essential to seek sustainable solutions that fulfil meet human demands through renewable resources. This necessity has spurred a concerted effort to implement the principles of the circular economy in practical applications. This approach involves repurposing by-products or waste from one production process as input materials for subsequent processes, ultimately yielding products with enhanced added value compared to the original materials [1; 2].

In biotechnology, the principles of valorisation and the circular economy are applied to waste materials, which, after appropriate treatment, can be further processed through a method known as biotransformation into valuable products. This approach primarily targets biodegradable waste generated from various sectors, including food production, agriculture, wood processing, leather manufacturing, and other industries handling materials of animal or plant origin.

2.2 Food industry and agricultural waste materials

These materials are residual by-products that generated during the production and industrial processing of food and agricultural products. Throughout the entire process of food production, from farm to final consumer, a significant amount of food waste is generated, which has the potential to be repurposed across various processing and manufacturing industries. This waste is produced at every stage of the food supply chain, including agricultural production, food processing, and the preparation and consumption of the final product [3]. Food waste can be categorized into several categories: harvest residues, mixed waste from processing industries, by-products from animal production, and inedible waste [4].

Annually, global food processing generates approximately 1.3 billion tons of waste materials [5]. Given the magnitude of this waste, it is imperative to develop more efficient processing methods rather than resorting to simple landfill disposal, which poses significant environmental risks. One potential approach involves repurposing this waste for energy production, utilizing the carbon stored in biological materials to generate energy or produce fuels. This encompasses methods such as biofuel production, pyrolytic processing, and anaerobic digestion in biogas facilities [6; 7]. Another effective strategy for utilizing food waste is to reintegrate organic material back into the human food chain. Potential methods include livestock feeding, composting, or biotechnological processing employing microorganisms [5; 8].

2.2.1 Lignocellulosic materials

The waste materials in question are primarily composed of cellulose, hemicellulose, and lignin polymers, which are predominantly derived from plant sources. These materials are significant as renewable resources, exhibiting both biodegradability and biocompatibility [9].

Lignocellulosic wastes, primarily found in the wood processing industry, agriculture, and the food sector, are rich in carbon and hold potential as substrates for microbial applications when subjected to appropriate treatment. However, due to their water insolubility and challenging processability, these wastes necessitate pre-treatment to enhance their suitability for further utilization. The valorisation of these materials through microbial cultivation typically requires extensive hydrolysis prior to microbial application. While certain microorganisms, such as filamentous fungi, possess the necessary enzymes to directly break down cellulose without substantial chemical modification, yeasts require more intensive pre-treatment. This process may involve mechanical grinding, extraction of lipid compounds using non-polar solvents, acid-catalysed hydrolysis, and enzymatic hydrolysis employing enzymes from filamentous fungi. Following these steps, separation of solid components is performed, allowing the dissolved monosaccharides to be utilized as substrates for microbial growth [10].

2.2.1.1 Coffee grounds

Coffee is one of the most traded commodities in the world. Data from the International Coffee Organization indicates a significant increase in coffee production over the past 30 years, with annual global coffee bean production reaching 9.9 million tons [11]. As coffee consumption rises, so does the production of coffee grounds, with approximately 650 kg of coffee grounds generated for every ton of green coffee beans processed [12; 13]. The highest contribution to coffee grounds production comes from the manufacturing of instant coffee beverages. Coffee grounds are the solid residue left after the water extraction of ground roasted coffee and consist of hemicellulose (30–40 wt. %), lignin (25–33 wt. %), oil (10–20 wt. %), cellulose (8.6–13.3 wt. %), proteins (6.7–13.6 wt. %), and polyphenols (2.5 wt. %) [14].

Due to the presence of antimicrobial and toxic substances, such as polyphenols and caffeine, coffee grounds are highly indigestible when directly incorporated into animal feed. However, composting significantly reduces the levels of these compounds, allowing coffee grounds to be utilized as fertilizer within composted biomass [15]. Given its wide range of nutrient-rich compounds, coffee grounds represent a lucrative resource for biotechnological and energy applications. They can be utilized to the production of bioethanol [16], polyhydroxyalkanoates (PHA) [14], carotenoids [17] and biodiesel [18].

2.2.1.2 Wood chips

The material in question is a type of biomass residue originally derived from wood. The wood chip, or wood shred, primarily consists of cellulose, hemicellulose, and lignin. The exact composition varies depending on the type of wood from which it originates. Residues from forestry operations differ in composition due to the presence of branches, foliage, and bark, compared to wood chips from the wood processing industry. Several methods for valorising wood chips have been identified. These include the production of wood-plastic composite materials, wood pellets, and thermochemical conversion into syngas, bio-oil, or biochar. Alternatively, biological pathways can be employed to produce bioethanol or biogas [19].

2.2.1.3 Brewing wastes (spent grains and waste yeasts)

Spent grain, or malt, is a lignocellulosic by-product of cereal grains that results from the separation of the endosperm during the brewing process. During mashing, the malt is filtered from the wort in lauter tuns. This raw malt contains 70–80 % water and constitutes up to 85 % of the total solid waste in brewing. The malt is composed of cellulose (17–25 %), hemicellulose (22–42 %), lignin (12–28 %), and proteins (15–25 %) [20]. Additionally, residual fermentable sugars from mashing are present in the malt, which, combined with its higher moisture content, increases its susceptibility to microbial contamination. Therefore, without prior treatment or preservation methods, it is not possible to store malt long-term (drying, cooling, or freezing). Malt is commonly used for direct animal feed or as an additive in animal feed. It can also be used for biogas production, bioethanol production, pyrolysis, or composting [21].

Brewer's yeasts, although not classified as a lignocellulosic material, are included here for a comprehensive overview of waste substrates in brewing. This substance consists primarily of spent yeasts after beer fermentation. It contains 74–86 % water and constitutes up to 15 % of the total solid waste in brewing. Its composition is approximately 45–60 % proteins, 36–42 % carbohydrates, around 7 % minerals and vitamins, and 4 % lipids; macro elements include 30–35 % oxygen, 5 % nitrogen, 5 % hydrogen, and 1 % phosphorus. With appropriate processing, it could serve as a complex supplement for cultivation purposes and can also be used for producing yeast extract or as feed biomass [20; 22].

2.2.1.4 Agricultural wastes

Agricultural residues from the harvesting and processing of grains contain a high proportion of cellulose and hemicellulose. These residues primarily include cereal straw, corn stalks, nut shells, rice straw, waste grass biomass, and bagasse from sugarcane. These lignocellulosic residues can be utilized for composting or bioethanol production [23; 24].

2.2.2 Oily materials

These are nonpolar materials generated in food processing and agricultural production, rich in fatty acids and glycerol. These wastes can originate from animal, plant, or microbial sources. They are a substantial carbon source and are suitable for use in the energy sector or within the framework of circular economy practices in the food and feed industries. Oily wastes are often highly amenable to biotechnological processing, as many microorganisms exhibit lipase activity, enabling them to utilize these wastes as carbon substrates.

2.2.2.1 Waste coffee oil

The coffee industry is expanding annually, resulting in increased production of waste material in the form of coffee grounds contain a significant proportion of oily components (10-20 % by weight). This fraction can be easily extracted using non-polar solvents and further utilized as a distinct source of carbon compounds. Waste coffee oil is rich in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, making it a suitable nutritional source for use in food products and cosmetics, as well as a potential substrate to the production of enriched biomass with lipid content or biopolymers [14; 17].

2.2.2.2 Waste frying oil

As a readily available and inexpensive lipid waste substrate is used frying oil. This waste frying oil is also rich in MUFA and PUFA; however, it contains compounds generated during the overheating process of frying [25; 26]. This type of waste is generated from the production of fried foods in restaurants and manufacturing facilities that produce pre-fried products. The annual global production of used cooking oil is between 0.9 and 1.5 million tons [17]. Used cooking oil is commonly utilized in biogas plants to the production of biogas or biodiesel; however, these products are often burned, thereby contributing to the overall carbon burden on the environment [27; 28]. An alternative approach involves repurposing used cooking oil as a nutrient source, where appropriately processed waste material serves as a substrate for the growth and propagation of microorganisms. These microorganisms, through biotransformation, convert a significant portion of the material into biomass or valuable metabolites usable in the human food chain, packaging industry, or cosmetics [17; 29; 30; 31].

2.2.2.3 Waste fat

In Europe, the annual production of fatty by-products from animal production is approximately 16 million tons, with the largest producers being Germany, France, the United Kingdom, and Spain. Of this, 10 million tons are suitable for human consumption or animal feed. Waste fats from slaughterhouse processing are not intended for direct human consumption but can be used in animal feed, provided they are free from transmissible spongiform encephalopathy (TSE) contaminants. In such cases, the material is handled according to the TSE regulation (Regulation 999/2001) [32; 33]. Waste fats are high in saturated fatty acids, making their use in animal feed less desirable. To enhance the quality of category 3 fats with valuable unsaturated fatty acids, biotechnological processes can be employed. These processes use microorganisms or enzymes to transform/bio-transform lipid-based substrates into more nutritionally valuable materials.

With the growing emphasis on a healthy lifestyle, which prioritizes high-quality diets low in saturated fatty acids and cholesterol, there is a need for standardized production of triglycerides with defined parameters. These triglycerides could serve as food fortifiers for both human and animal diets. Standardized conditions can be achieved using lipases, which, unlike chemical methods, specifically target the cleavage and transesterification of fatty acids from triglycerides. A significant advantage of lipase-catalysed reactions is the reduction of waste materials compared to alcoholate-based transesterification. However, a limitation of these processes is the high cost of the required enzymes [34].

2.2.2.4 Glycerol

Waste glycerol is a by-product of biofuel production. During the transesterification process, fatty acids bound in triglycerides are transesterified with a short-chain alcohol, producing esters, most commonly methyl esters or ethyl esters of fatty acids, and glycerol [35]. This raw glycerol can be further processed using various chemical and microbiological methods. Utilizing raw glycerol, which is produced in large quantities, presents a viable option as a carbon substrate for biotechnological valorisation [36]. Biotechnological valorisation of raw glycerol focuses on obtaining valuable products such as lipids, fatty acids, and lipidic substances [37]. Also noteworthy is the biotechnological production of polymer precursors, such as 1,3-propanediol, used in the textile and chemical industries for the production of the polymer PTT (poly-tri-methylene terephthalate) [38; 39].

2.2.3 Animal materials

Based on European Union regulations concerning animal by-products (Regulations 1069/2009 and 142/2011), animal by-products are classified into three categories. The first category includes the lowest quality materials, which can only be used for energy purposes. The second category consists of slaughter by-products, fats, and parts of deceased animals, which are suitable for both energy and technical applications. The third category comprises materials intended for human consumption, specifically edible parts of selected animals obtained during slaughter processing [33].

2.2.3.1 Feathers

Feathers are an animal material based on keratin, primarily produced in the poultry industry. Annual feather production is estimated at around 770 kilotons [40]. Feathers represent a highly valuable, complex nitrogen source with potential applications in biotechnology. Chicken feathers consist of more than 90 % protein, with keratin as the main component—a fibrous and insoluble protein, richly cross-linked by disulfide and other bonds. Feathers account for up to 10 % of a chicken's total weight. Feather keratin has a high cysteine content, and elevated levels of amino acids such as glycine, glutamic acid, proline, alanine, serine, and valine, but lower amounts of lysine, methionine, and tryptophan [41]. Due to the rigid structure of keratin, where protein chains are tightly packed and stabilized by hydrophobic interactions and disulfide bonds, degradation by common proteolytic enzymes such as pepsin, papain, or trypsin is unachievable [42]. Keratin belongs to the group of scleroproteins, and due to its water-insoluble nature, it is difficult to utilize in its native form by microorganisms without a specific enzymatic apparatus. However, in nature, there are microorganisms capable of utilizing this material due to their specific enzymatic capabilities. These are primarily keratinophilic microorganisms belonging to the Fungi kingdom, capable of producing specific keratin-degrading enzymes, such as those produced by human skin pathogens [43]. For this reason, technological processes often include pre-treatment of feathers using alkaline or acid-catalysed hydrolysis [41; 42].

2.2.3.2 Whey

Whey is a by-product of cheese or curd production and is categorized as either acid whey or sweet whey. Sweet whey is derived from cheeses coagulated with rennet, with a pH around 5.6. Acid whey comes from cheese produced by acid coagulation, with a pH around 5.1 [44]. Whey is a liquid residue after cheese curdling with a high water content (93 %). Whey represents 85-95 % of the original milk volume and retains up to 55 % of all nutrients present in the milk. Up to 20 % of the proteins originally present in milk remain in the whey [45]. Due to the high nutritional value of this material, whey can be utilized in numerous food and biotechnological applications. Whey protein serves as a suitable dietary supplement in both animal feed and human nutrition. Additionally, whey is rich in lactose, which can act as a carbon substrate for microbial growth. For microorganisms incapable of hydrolysing lactose, enzymatic pre-treatment with lactases is necessary [46].

2.3 Types of waste material valorisation and processing

2.3.1 Chemical treatment and valorisation

As previously mentioned regarding various waste materials, there are numerous ways to derive valuable products from used biomass. One potential approach is the processing of waste materials followed by valorisation through chemical methods such as pyrolysis, biofuel production, synthesis gas, biogas, or energy recovery. A potential issue with these methods is the consumption of materials that could otherwise be utilized in the food industry. Chemically valorised materials re-enter the food chain only through the emitted carbon dioxide during the disintegration of these materials.

2.3.2 Biological valorisation of waste

The primary motivation for this type of valorisation is to reintegrate carbon and other macro and micronutrients contained in waste materials back into the food chain without the step of mineralization. This involves the processing of biological materials using living systems, such as composting, feeding, or utilizing microbial cultures or their components to valorise waste and by-products from the food industry.

2.3.3 Examples of biotechnological valorisation of waste

There is a wide range of examples of biotechnological processing of various biologically degradable wastes. This discussion aims to highlight that this is a rapidly developing field. Given the increasing demands on the food industry, where crop yields are dependent on weather and climatic conditions, biotechnological valorisation offers a solution to inefficiency problems in production, as it can be conducted independently of climatic conditions.

2.3.3.1 Protein production

Waste materials from the food industry can also be processed into valuable microbial protein. An example of this is protein produced by the yeast *Saccharomyces cerevisiae* on a mixed waste substrate of both plant and animal origin. In a specific study, residues from fish processing and fruit peels were used. The yeast biomass in this experiment was able to produce protein suitable for animal feed [47].

2.3.3.2 Bioethanol production

Bioethanol can be produced from suitably pretreated lignocellulosic wastes. After saccharification of the waste using a combination of acid and enzymatic hydrolysis, conventional ethanol-producing strains, such as the yeast *Saccharomyces cerevisiae* or the bacterium *Zymomonas mobilis*, can be cultured on the substrate. The produced bioethanol can be utilized in various industries, such as energy production or transportation as a biofuel [48; 49].

2.3.3.3 Production of lipidic metabolites

Oleaginous microorganisms are able to accumulate a significant percentage of lipids in their biomass (more than 20 % of their biomass content). They are also capable of accumulating a wide range of lipidic metabolites, including carotenoids, ergosterol, and ubiquinone. Yeasts, for example, can be utilized for this phenomenon in the valorisation of food industry wastes. Lipids produced by microbial biomass can be used to produce third-generation biofuels; however, this form of processing competes with the human food chain. Therefore, microbial lipids are suggested for use as feed additives or in the food industry, for instance, as dietary supplements [50; 51].

2.4 Red yeasts and their metabolites

Yeasts are typically unicellular heterotrophic eukaryotic microorganisms classified within the Fungi kingdom. They are divided into two main groups: Ascomycota (sac fungi) and Basidiomycota (club fungi). These are unicellular microorganisms characterized by forms of vegetative asexual reproduction through budding and sexual reproduction without forming fruiting bodies [52].

Yeasts belonging to the Ascomycota group are the most numerous, including genera such as *Saccharomyces*, *Pichia*, and *Candida*. During sexual reproduction, these yeasts form an ascus, a sac that encases the meiotically produced ascospores [53].

The strains primarily focused on in this work belong to the Basidiomycota group, many of which are strictly aerobic. Under insufficient oxygen saturation in the culture, these strains may exhibit stunted growth or even perish. Representatives of this group include genera such as *Cystofilobasidium*, *Rhodotorula*, *Sporobolomyces*, *Rhodosporidium*, and *Xanthophyllomyces* (also known as *Phaffia*). All these genera are known for producing lipidic metabolites. They are recognized for their ability to accumulate high lipid content (over 70 % of biomass weight) and many of them are considered oleaginous [54]. The lipidic substances produced by these yeasts include various isoprenoid metabolites, which are the products of a well-developed mevalonate metabolic pathway. This pathway yields many carotenoid pigments, which are characterized by orange to red colours. For this reason, these strains are often referred to as red or carotenoid-producing yeasts. Other products of this pathway include ergosterol, which is incorporated into cell membranes, and coenzymes Q5-Q10, which are widely used in cosmetics and pharmaceuticals [53; 55].

2.5 Yeast cytology

2.5.1 Structure of yeast cells

Yeast cells are surrounded by three enveloping layers. The cell membrane consists of a lipid bilayer primarily composed of phospholipids and sterols, mainly ergosterol and zymosterol, with sterols providing rigidity and phospholipids contributing to membrane fluidity. The membrane also contains embedded transport proteins and biosynthetic proteins for the cell wall. The periplasm, located outside the cell membrane, is an interlayer containing secreted glycoproteins and enzymes, which primarily include hydrolases and invertases that pre-process various substrates. The cell wall, which makes up 80-90 % of the cell's polysaccharide content, mainly consists of β-glucans (with β-1,6 and β-1,3 linkages), with minor amounts of chitin and embedded mannoproteins. This rigid structure can constitute up to 25 % of the dry biomass content [56; 57; 58; 59]. The interior of the cell, known as the cytosol, is a colloidal environment containing various cellular structures and organelles such as the nucleus, endoplasmic reticulum, Golgi apparatus, vacuoles, mitochondria, and peroxisomes. The nucleus, which serves as the cell's control centre, contains genetic information in the form of chromosomes. The yeast genome size ranges from approximately 10-15 Mb. Within the nucleus is the nucleolus, which is involved in ribosome production [56; 57]. The yeast nucleus is surrounded by the endoplasmic reticulum, an organelle composed of a dense network of membranes. The endoplasmic reticulum is divided into two subunits: rough and smooth. The rough endoplasmic reticulum is studded with ribosomes, where proteins are produced. The smooth endoplasmic reticulum is involved in sterol production and lipid biosynthesis [56; 60]. The Golgi apparatus is a series of vesicles that serves as the central transport hub for the cell, where metabolites are sorted, and post-translational protein modifications occur [56; 57]. Vacuoles are vesicles that serve as storage sites for metabolites. They also facilitate the degradation of metabolites using hydrolases such as phosphatases, lipases, nucleases, and peptidases, and store amino acids, polyphosphates, and certain cations [56; 57]. Mitochondria are the energy centres of the cell, where aerobic respiration occurs via the electron transport chain, leading to ATP production. Additionally, mitochondria are involved in fatty acid synthesis and the Krebs cycle [56; 57; 61]. Peroxisomes are specialized organelles involved in the beta-oxidation of lipids and the detoxification of harmful substances, such as the breakdown of hydrogen peroxide into water and molecular oxygen via catalase, thus protecting the cell from oxidative stress [56; 58]. A schematic representation of a yeast cell is shown in Figure 1.

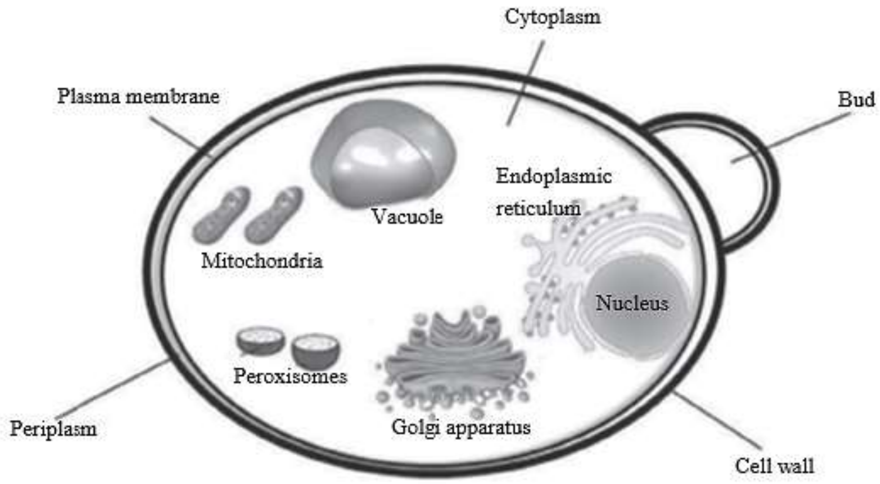


Figure 1: Diagram of a yeast cell [57].

2.6 Produced metabolites

2.6.1 Lipids

The term „lipids“ refers to a wide range of compounds directly or indirectly related to fatty acids. They are characterized by very poor solubility in water and high solubility in polar solvents. In organisms, lipids perform many essential functions:

- **Storage function:** Lipids serve as the storage of chemical energy in cells. Energy is stored in lipids in the form of fatty acids bound in triacylglycerols. During nutrient surplus, fatty acids are biosynthesized and stored, while in conditions of nutrient deficiency, the organism breaks down lipid reserves through β-oxidation.
- **Structural Function:** Lipids also serve as structural components of organisms. A specific example is the formation of bio-membranes, which are essential for cell survival. Bio-membranes are typically composed of phospholipids (phosphoacylglycerols and sphingomyelins), glycosphingolipids (cerebrosides and gangliosides), and sterols (cholesterol).
- **Metabolic Function:** The metabolic role of lipids includes the utilization of non-polar substances, for which lipids act as solvents, particularly for lipophilic vitamins (A, D, E, K) and hormones.
- **Protective Function:** In higher organisms, lipids, especially in the form of adipose tissue, fulfil thermo-insulating and mechanical protective functions.

Lipids are further classified based on their composition into:

- **Simple lipids:** These are fatty acids esterified with an alcohol. In the case of fats and oils, the alcohol is glycerol. For waxes, they are esters of fatty acids and higher monofunctional alcohols.
- **Complex lipids:** These are lipids that contain, in addition to esterified fatty acids, another group attached to the alcohol. The main groups of complex lipids are phospholipids and glycolipids, along with other complex lipids such as lipoproteins, sulfolipids, or aminolipids.

- **Derived lipids and precursors:** This is a diverse group of substances that share a lipophilic character. It includes steroids, alcohols, non-polar vitamins, hormones, fatty acids, glycerol, fatty aldehydes, and ketone bodies [62].

2.6.1.1 Fatty acids

Fatty acids are an integral part of organisms. They are organic acids with carbon chains ranging from 4 to 26 atoms, with varying degrees of unsaturation. An example of a saturated fatty acid is stearic acid (18:0), and its monounsaturated counterpart is oleic acid ($18{:}1^{\Delta 9}$), as shown in Figure 2. Yeasts exclusively produce fatty acids with an even number of carbon atoms. The production of fatty acids requires an adequate supply of acetyl-CoA, which is obtained from carbon-rich nutrients, such as sugars and fats. The biosynthesis of fatty acids occurs more intensively when the organism is well-nourished [63].

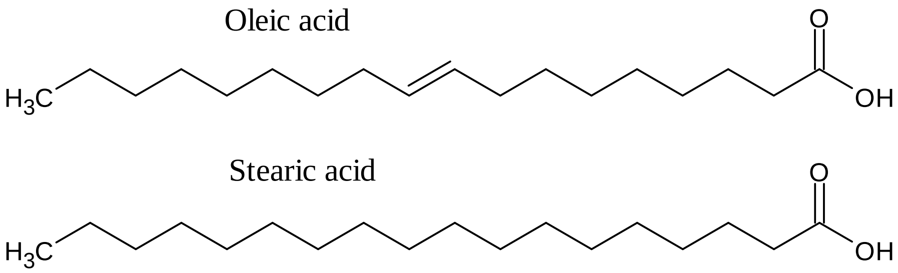


Figure 2: Structural formulas of Oleic and Stearic acids

Fatty acid biosynthesis takes place in the cytosol of cells, specifically in the endoplasmic reticulum, where enzyme complexes responsible for the individual steps of biosynthesis are located. These enzyme complexes ensure that the reactions are sequentially linked, allowing the process to occur with high efficiency. The multi-enzyme complex consists of two subunits connected by a disulfide bridge. The first trio of enzymes includes acetyl-trans-acylase, malonyl-trans-acylase, and β-oxo-acyl-synthase. Acetyl-trans-acylase catalyses the formation of acetyl-ACP, malonyl-trans-acylase facilitates the formation of malonyl-ACP, and β-oxo-acyl-synthase catalyses the synthesis of acetoacetyl-ACP from malonyl-ACP and acetyl-ACP, with simultaneous decarboxylation of malonyl-ACP. During the catalysis by the next trio of enzymes (β-oxo-acyl-reductase, β-hydroxy-acyl-dehydratase, enoyl-reductase), butyryl-ACP is formed. In this process, β-oxo-acyl-reductase reduces aceto-acetyl-ACP to D-3-hydroxybutyryl-ACP using the reducing coenzyme $NADPH+H^+$, which is then dehydrated by the enzyme β-hydroxy-acyl-dehydratase to form α,β-trans-butenoyl-ACP, which is further reduced by enoyl-reductase and the reducing coenzyme $NADPH+H^+$ to produce the aforementioned butyryl-ACP. The resulting acyl group is then transported within the reaction chain for further elongation by the enzyme β-oxo-acyl-synthase (reactions 2a and 3 in Figure 4). This elongation cycle repeats until the desired acyl length is achieved (typically C16). To convert the final acyl group into a fatty acid, it is subjected to the action of the enzyme acyl-esterase, which adds water and cleaves ACP. A complete scheme of palmitate biosynthesis can be found in Figure 4. Unsaturated fatty acids are biosynthesized from saturated fatty acids through the action of desaturases [62; 63].

Fatty acids are a very rich source of energy, so it is important to also mention the reverse metabolic pathway leading to their breakdown, known as β-oxidation, or Lynen’s spiral. Unlike biosynthesis, β-oxidation occurs in the mitochondrial matrix, where fatty acids are transported via the carnitine transport system across the mitochondrial membrane. It involves the activation of the fatty acid by attaching coenzyme A in place of the hydroxyl group on the carboxyl carbon. This activation is catalysed by the enzyme acyl-CoA synthetase and is only possible with the consumption of energy from ATP. Essentially, β-oxidation can be considered as the reverse of biosynthesis, as the acyl group is oxidized and progressively shortened by two-carbon acetyl units. Acyl-CoA (C_{n+2}) is first dehydrogenated by acyl-CoA dehydrogenase with the oxidizing coenzyme FAD, forming enoyl-CoA. Enoyl-CoA then undergoes hydration by enoyl-CoA hydratase, and the resulting L-3-hydroxyacyl-CoA is subsequently dehydrogenated by the enzyme β-hydroxy-acyl-CoA dehydrogenase to produce β-oxo-acyl-CoA, from which acetyl-CoA is cleaved, yielding acyl-CoA (C_n). A simplified comparison of β-oxidation and fatty acid biosynthesis is illustrated in Figure 3 [62; 64].

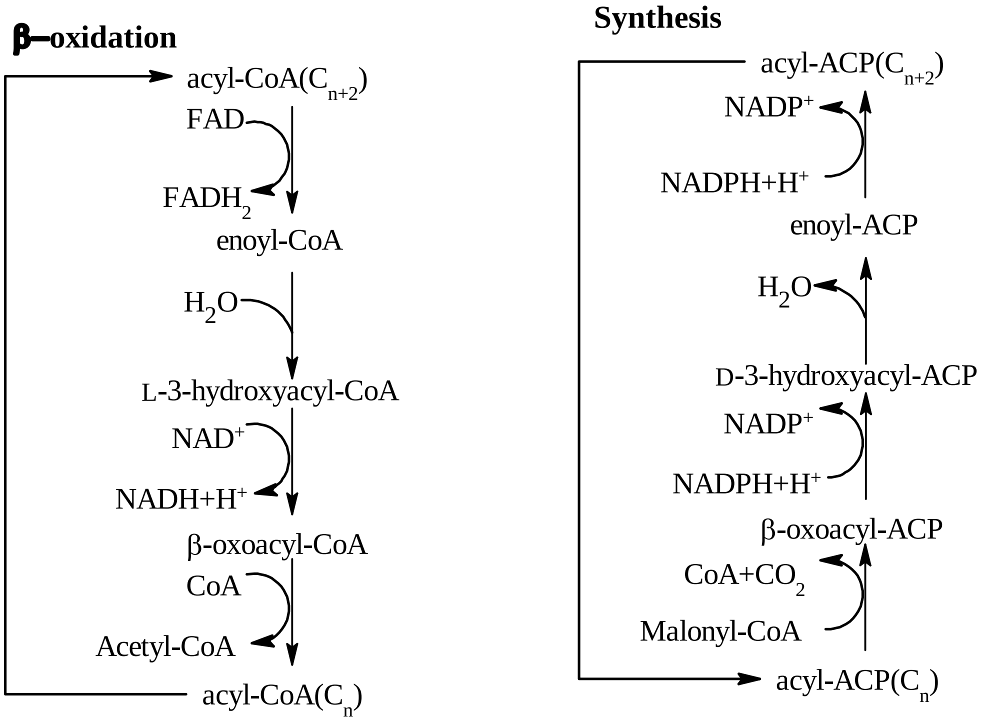


Figure 3: Diagrams of β-oxidation and fatty acid biosynthesis [64]

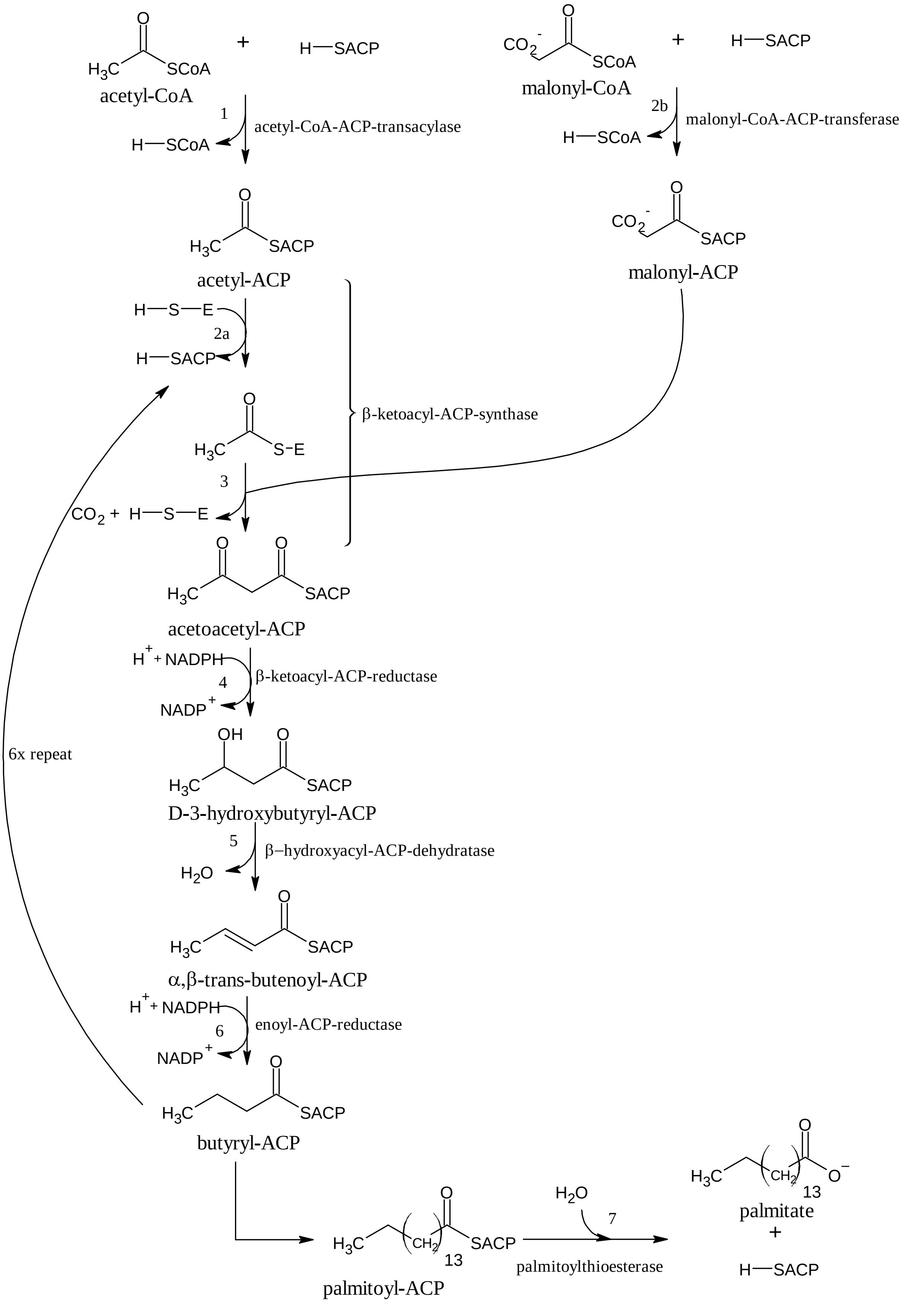


Figure 4: Diagram of fatty acid synthesis, specifically Palmitic acid [65]

2.6.2 Carotenoids

Carotenoids are natural lipophilic pigments found in plants and microorganisms, characterized by red, orange, and yellow coloration. Due to their lipophilic nature, they are localized within the non-polar regions of cells, primarily within membranes. Carotenoids play a role in photosynthesis as accessory photoactive pigments, capable of absorbing light at wavelengths between 450-550 nm. Their high antioxidant activity, attributed to the presence of numerous double bonds, significantly contributes to the reduction of oxidative stress and the scavenging of free radicals within the cellular environment. Free radicals can induce permanent alterations in essential compounds within cells, such as nucleic acids or proteins, potentially leading to undesirable mutations or cell death. In the animal kingdom, carotenoids not only serve as antioxidants but also fulfil a biosynthetic function, acting as precursors to vitamin A (retinol), which is, for example, crucial for proper vision function [66].

2.6.2.1 Structure of carotenoids

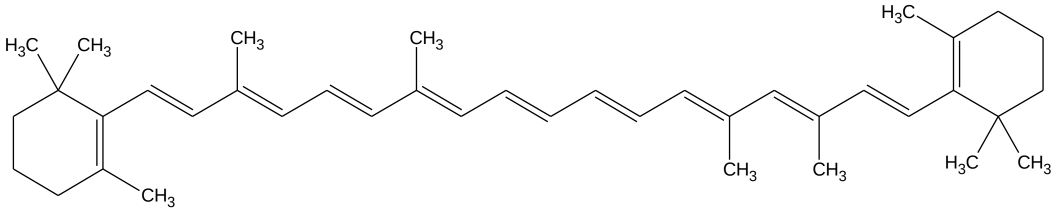
Carotenoids belong to the class of isoprenoids, specifically tetraterpenes, as they are composed of eight isoprene units. Their basic structure typically consists of 40 carbon atoms in a polyene chain. The characteristic absorption spectrum of carotenoids is due to a system of up to 15 conjugated double bonds in the polyene chain, which are predominantly in the all-trans configuration. Based on their elemental composition, carotenoids are divided into two main groups:

- **Carotenes**: Carotenes consist solely of unsubstituted hydrocarbon chains, exhibiting red and orange coloration. The primary representative is lycopene (see Figure 5), an aliphatic carotene that serves as a precursor for other carotenoids. During the biosynthesis of other carotenes, lycopene undergoes cyclization at one or both ends of the chain. Depending on the extent of cyclization, carotenoids can be further classified as acyclic, monocyclic, or dicyclic. Other significant carotenes include β-carotene and torulene (see Figure 5).
- **Xanthophylls**: Xanthophylls are derived from carotenes by hydroxylation or oxidation of the molecule. As a result, in addition to the hydrocarbon backbone, xanthophylls contain at least one oxygen atom attached to their chain. Notable examples of xanthophylls include torularhodin (see Figure 5) and lutein.

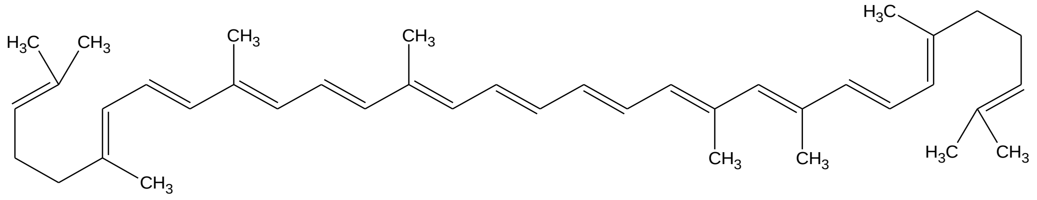
Carotenoids can also be classified based on the degree of cyclization of the polyene chain into:

- **Acyclic carotenoids**, which do not contain any cyclic structure within their chain. Examples include lycopene, δ-carotene, and neurosporene.
- **Monocyclic carotenoids**, which have a cyclic structure at one end of their chain, such as γ-carotene, torulene, or torularhodin.
- **Dicyclic carotenoids**, where both ends of the polyene chain are terminated with cyclic structures, with β-carotene being the most significant representative [63; 67; 68].

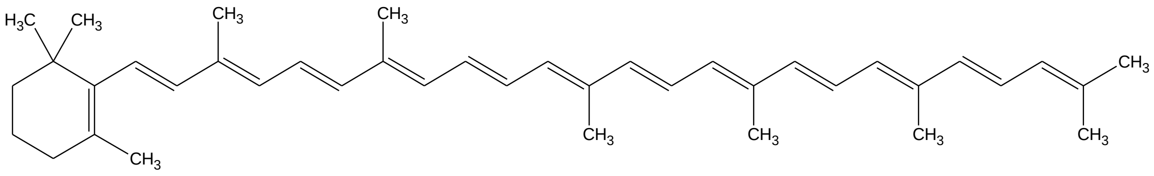
β-carotene:



Lycopene:



Torulene:



Torularhodin:

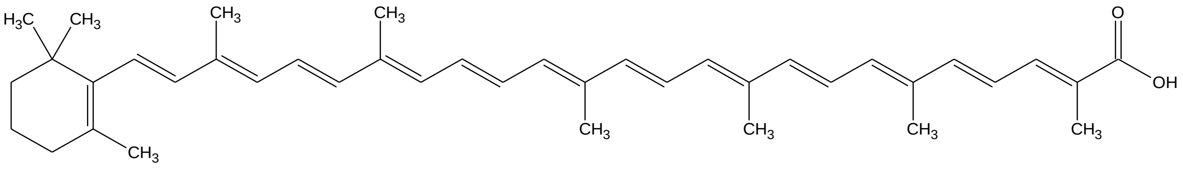


Figure 5: Examples of specific carotenoid structures [67]

2.6.2.2 Carotenoid biosynthesis

Carotenoids are synthesized in red yeast cells via the mevalonate pathway [69]. The process involves the sequential condensation of two-carbon acetyl units in the form of acetyl-CoA, which is derived from glycolysis or the β-oxidation of fatty acids. Initially, acetoacetate is formed, which then condenses with another acetyl-CoA to produce 3-hydroxy-3-methyl-glutaryl-CoA. This compound is subsequently reduced to mevalonate. Mevalonate is the precursor to isopentenyl pyrophosphate, also known as the „active isoprene unit," which is formed through the decarboxylation and double phosphorylation of mevalonate. The active isoprene units are joined in a „head-to-tail" fashion, forming geranyl pyrophosphate, which then condenses with two molecules of isopentenyl pyrophosphate to yield the intermediate farnesyl pyrophosphate and the final product, geranylgeranyl pyrophosphate. Two molecules of geranylgeranyl pyrophosphate undergo a reductive condensation in a „tail-to-tail" manner to form phytoene, the simplest C_{40} carotenoid. This synthesis is illustrated in Figure 6 [70; 71].

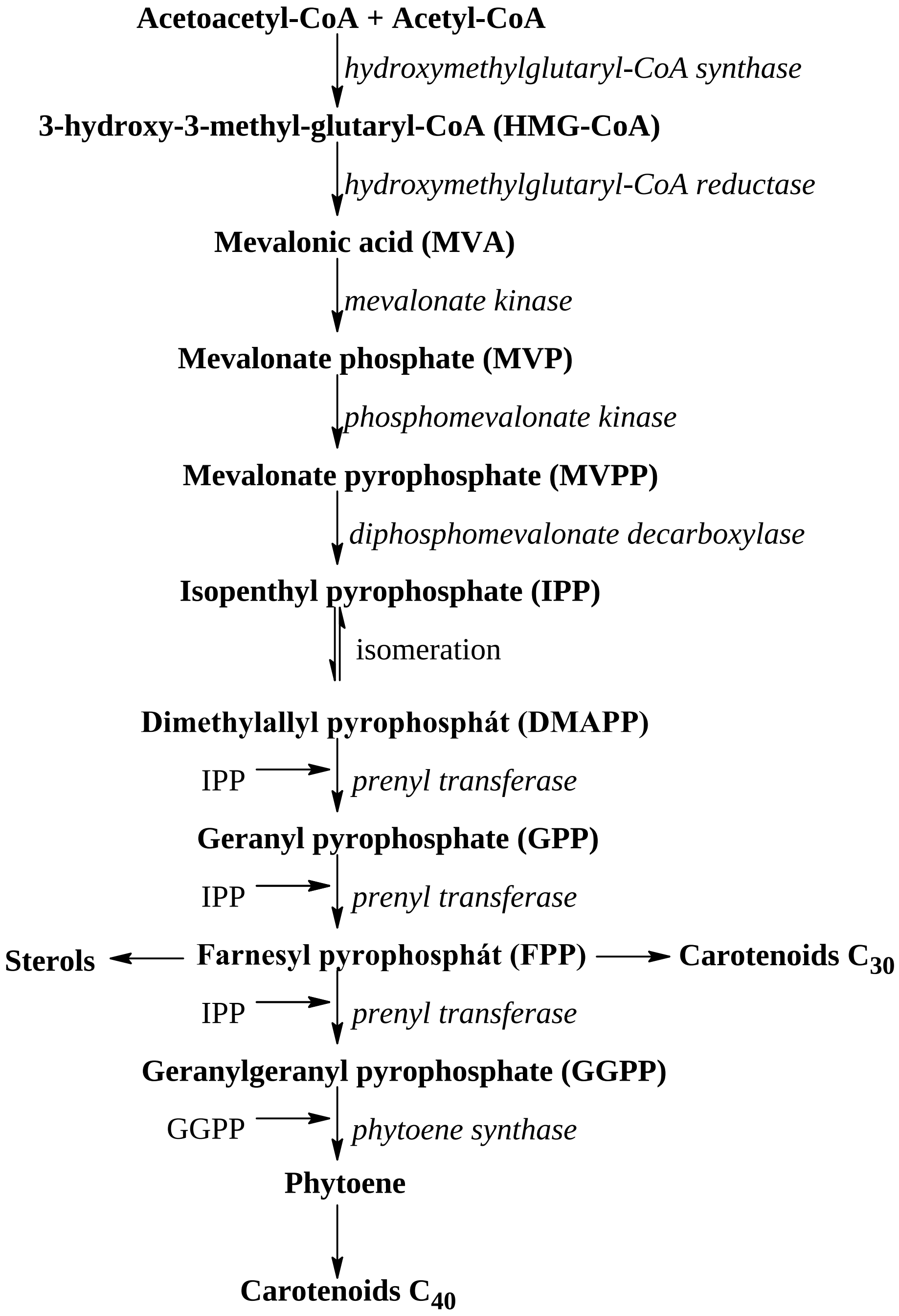


Figure 6: Schematic of carotenoid synthesis via the Mevalonate pathway [72]

In red yeasts, the predominant carotenoids are β-carotene, torulene, and torularhodin. Their synthesis proceeds via phytoene, which undergoes dehydrogenation to form neurosporene and then lycopene. Lycopene is converted to monocyclic γ-carotene by a cyclase enzyme. Alternatively, γ-carotene can also be formed through the dehydrogenation of β-zeacarotene, which is a product of neurosporene cyclization. β-carotene is synthesized from γ-carotene via the catalytic action of β-lycopene cyclase. Γ-carotene also serves as a precursor for torulene biosynthesis, where it retains a monocyclic structure but becomes more unsaturated through the action of desaturase enzymes. Further hydroxylation and oxidation of torulene lead to the formation of torularhodin, as depicted in Figure 7 [71; 72].

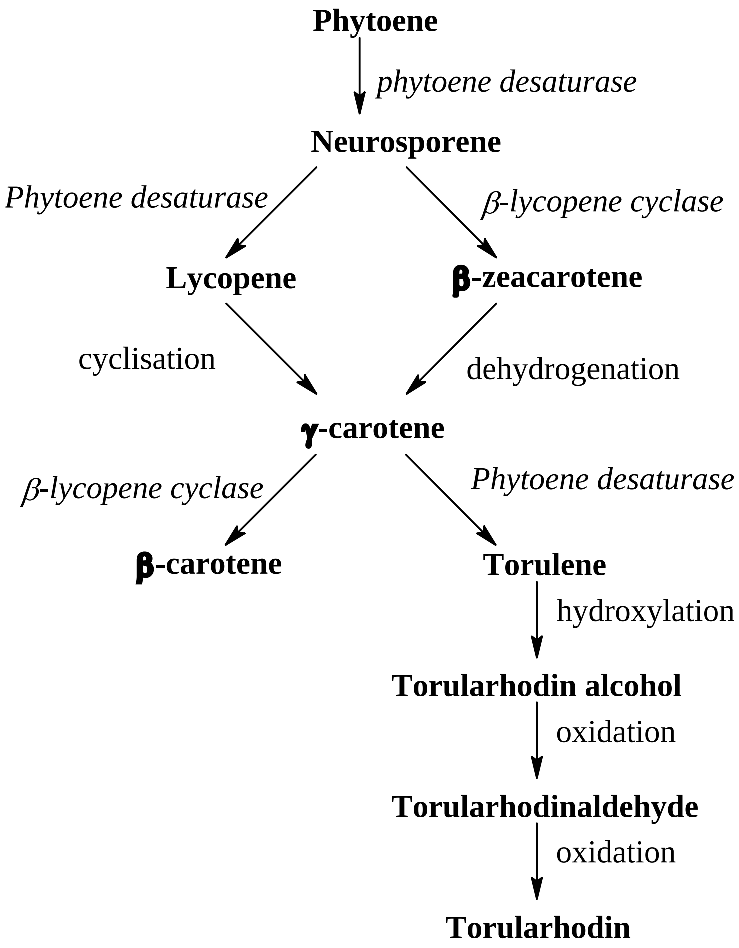


Figure 7: Biosynthesis pathway of β-Carotene, Torulene, and Torularhodin [72]

2.6.3 Ubiquinone

Also known as coenzyme Q, ubiquinone belongs to a distinct group of compounds characterized by an isoprenoid side chain attached to a benzoquinone ring, with the side chain length being specific to different organisms. For example, coenzyme Q_{10} has a side chain composed of ten isoprenoid units attached to the quinone ring (see Figure 8). Coenzymes Q function as hydrogen and electron carriers within the respiratory chain, linking NADH and flavoproteins with cytochrome b. Under aerobic conditions, coenzymes Q exist in the quinone state, while in anaerobic conditions, they are reduced to quinols. They are also found in association with vitamin E in the lipid compartments of cells, where they prevent lipid peroxidation [62; 73].

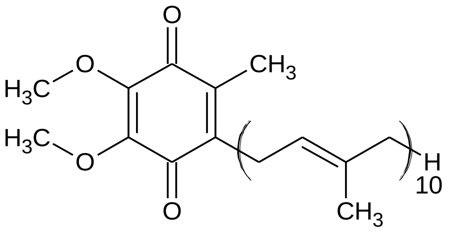


Figure 8: Structural Formula of Coenzyme Q_{10}

2.6.4 Ergosterol

Ergosterol is a nonpolar lipid found in fungi and yeast (see Figure 9). It is the yeast equivalent of cholesterol and serves as a precursor to vitamin D2, or ergocalciferol. The biological role of ergosterol includes maintaining membrane fluidity and permeability, as well as regulating the activity of membrane-bound enzymes. In yeast, ergosterol acts as a cytosolic regulator of protein kinase, which is necessary for initiating cell growth. The biosynthesis of ergosterol strictly requires aerobic conditions. Facultatively anaerobic organisms become auxotrophic for ergosterol production in the absence of oxygen. Due to its presence in fungi, particularly moulds, ergosterol is used as an indicator of food contamination. When exposed to UV light, the B-ring of ergosterol is cleaved between positions 9 and 10, resulting in the formation of ergocalciferol, which is used as a vitamin fortifier in milk or as a dietary supplement [65; 74].

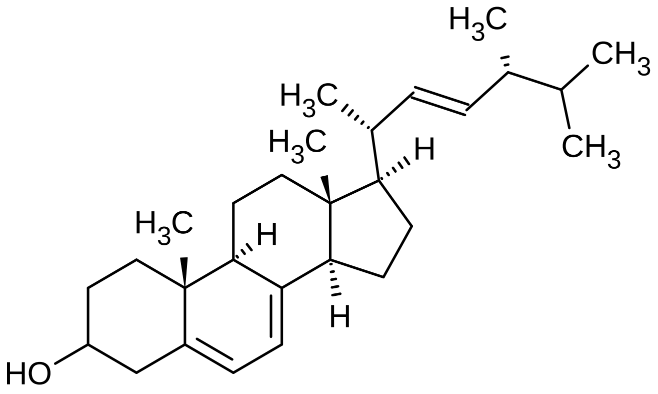


Figure 9: Structural Formula of Ergosterol

2.6.5 β-glucans

The yeast cell wall (see Figure 10) is composed of up to 85 % polysaccharides and up to 15 % proteins. From a monosaccharide perspective, it consists of 80-90 % of glucose, 1-2 % of N-acetylglucosamine, and 10-20 % of mannose [75]. One of the most significant components of the yeast cell wall are β-glucans. These are homopolymer structures made up of D-glucose units arranged in linear chains with β-1,3 linkages, and with glucose units in the branching points connected by β-1,6 linkages. Yeast β-glucans have immunomodulatory and immunostimulatory effects, particularly in their native helical conformation. Due to their stable structure, β-glucans can pass through the stomach almost intact and reach the intestines, where they elicit an immune response. In this process, the breakdown products of β-glucans, generated by macrophage activity, bind to immune cell receptors and enhance the organism's immune response [76; 77].

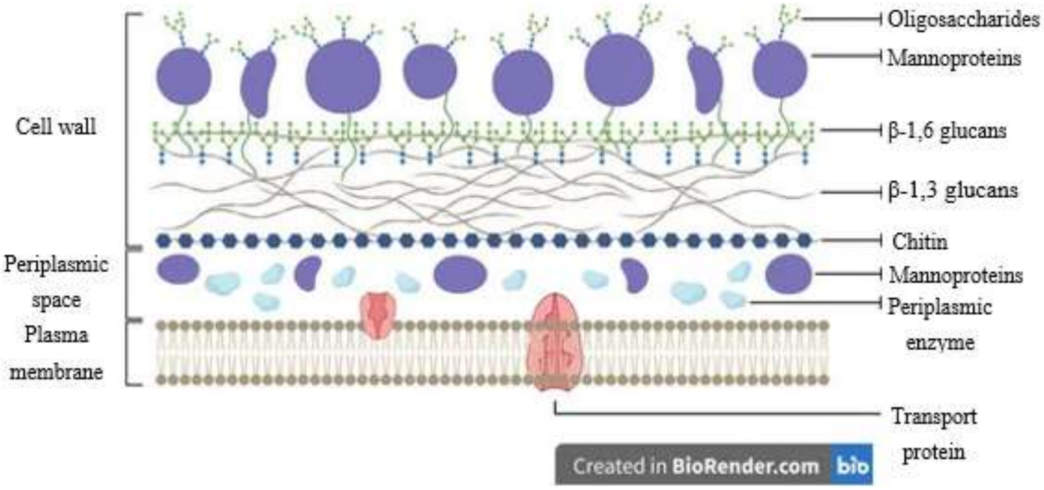


Figure 10: Schematic of yeast cell wall [78]

2.7 Cultivation strategies for waste utilization

2.7.1 Overview of selected fungal strain suitable for waste valorisation

2.7.1.1 Cystofilobasidium Genus

Yeasts of the genus *Cystofilobasidium* belong to the group Heterobasidiomycetes. They are capable of both sexual and asexual reproduction and can occasionally ferment sugars. In asexual reproduction, diploid or haploid oval-elongated cells undergo budding. Originally, the *Cystofilobasidium* genus was classified under the *Rhodosporidium* genus, but it exhibited distinct characteristics, such as the ability to assimilate inositol into starch-like compounds. The *Cystofilobasidium* genus is further defined by the formation of teliospores. Differentiation from other yeast strains is also marked by the production of carotenoid pigments and the formation of endospores in the mycelium. Colonies of this genus are glossy, with colours ranging from orange to salmon-pink, and sometimes even cinnamon-hued. Notable species in this genus include *C. macerans*, *C. infirmominiatum*, and *C. capitatum* [52; 79; 80].

2.7.1.2 Rhodotorula Genus

The *Rhodotorula* genus represents the anamorphic form of the *Rhodosporidium* genus. Strains of the *Rhodotorula* genus are classified under the division Basidiomycota, with some falling into the subphylum Pucciniomycotina and others into Ustilaginomycotina. This is a ubiquitous genus characterized by round, elliptical, or elongated cell shapes that do not form ballistoconidia. Cells reproduce through multilaterally or polar budding. They are capable of sexual reproduction via teliospores and the production of pseudomycelia. The most of strains of this genus cannot assimilate inositol. During the exponential growth phase, they can produce glycogen and accumulate significant amounts of lipids and carotenoid pigments within the intracellular biomass. These are strictly aerobic strains and, therefore, unable to ferment sugars. Notable strains include *Rhodotorula kratochvilovae*, *Rhodotorula mucilaginosa*, and *Rhodotorula minuta* [52; 55; 81; 82].

The *Rhodosporidium* genus represents the teleomorphic form of *Rhodotorula*. These carotenoid-producing yeasts are capable of synthesizing significant amounts of carotenoids and coenzymes Q_9 and Q_{10}

within their biomass. The colour of the colonies may vary depending on the types of pigments accumulated, ranging from shades of red to orange or yellow. During growth, strains of the *Rhodosporidium* genus require oxygen, classifying them as strictly aerobic, and thus, they are unable to ferment sugars into alcohol. They also cannot utilize lactose as a carbon substrate. The *Rhodosporidium* genus can exist in both unicellular (yeast) and mycelial forms. As yeasts, they reproduce by budding egg-shaped cells, which can form pseudomycelium. Reproduction can also occur sexually, either homothallically or heterothallically. In homothallic reproduction, teliospores are produced from a diploid cell. Through meiotic division, dikaryotic mycelium is formed, which precedes the formation of the teliospore, where karyogamy occurs. In heterothallic reproduction, the conjugation of sexual cells leads to the formation of dikaryotic mycelium, which then forms teliospores where karyogamy occurs. Meiotically germinating teliospores can also develop lateral and terminal basidia, from which diploid basidiospores arise. Representatives of this genus include *Rhodosporidium babjevae*, *Rhodosporidium toruloides*, and *Rhodosporidium fluviale* [52; 81; 83].

2.7.1.3 Sporidiobolus Genus

The *Sporidiobolus* genus represents the teleomorphic variant of the *Sporobolomyces* genus. Colonies on solid media exhibit a rough or smooth surface that can be either glossy or semi-matte, with pink to red coloration due to the presence of carotenoids. The texture is soft, sometimes slimy or even liquid-like. Cells are oval, spherical, or elongated, growing individually, in pairs, small clusters, or short chains. Reproduction in this genus can occur through asexual budding with potential pseudomycelium or mycelium formation or through ballistoconidia, as well as sexually via homothallic or heterothallic mechanisms. The *Sporidiobolus* genus cannot ferment sugars or produce starch. Notable species include *Sporidiobolus pararoseus*, *Sporidiobolus johnsonii*, and *Sporidiobolus salmonicolor* [52; 84].

2.7.1.4 Phaffia Genus

The *Phaffia* genus is defined by a single species, *Phaffia rhodozyma*. It represents the anamorphic variant of the *Xanthophyllomyces* genus. This strain may have arisen from incomplete pairing of basidiospores during sexual reproduction of the teleomorphic strain *Xanthophyllomyces dendrorhous*. The *Phaffia* genus is notable for producing substantial amounts of astaxanthin within its biomass, giving the biomass a red to salmon-red colour. Cells of the *Phaffia rhodozyma* strain are ellipsoidal and appear individually, in pairs, or short chains. These cells cannot form true mycelium, although rudimentary pseudomycelium may be present [52; 85].

2.7.2 Cultivation methods applicable to yeasts

2.7.2.1 Small-scale cultivations

In laboratory settings, a basic method for cultivation is the use of Erlenmeyer flasks. This method for cultivating axenic cultures has been widely used since the 20th century, where cultures were shaken on reciprocal or orbital shakers to ensure medium homogeneity and oxygen supply, particularly for aerobic microorganisms. The main drawbacks of these cultivations were the inability to consistently maintain optimal cultivation conditions (pH, pO_2, temperature) and the inability to monitor internal processes in the medium throughout the cultivation period. In the context of screening experiments for industrial biotechnological applications, flask cultivation may lead to significantly different or skewed results compared to large-scale regulated cultivation [86; 87].

With advancements in analytical chemistry instrumentation, it is possible to reduce cultivation volumes to one-tenth or one-hundredth of the original volumes used in Erlenmeyer flasks by utilizing micro-cultivation techniques. A system known as the “micro flask” system can be used, combining a microtiter plate with a “sandwich” cover. The entire cover consists of a stainless-steel lid, a sterile filter, microfiber inserts, and a flexible silicone sealing layer to ensure sufficient oxygen supply to individual wells, each serving as a separate bioreactor. This system allows for reproducible results, more efficient screening, and cost reduction for essential medium components [77; 88; 89].

2.7.2.2 Bioreactor cultivation

In the context of scale-up during the optimization of cultivation methods for operational conditions, bioreactor cultivation is worth mentioning. A bioreactor is a biotechnological device designed to the production of metabolites or biomass using microbial or cell cultures or their components. Many types of bioreactors exist, but for yeast cultivation, a classic batch system with possible nutrient feed into the submerged medium is suitable. A key condition in cultivation is the temperature optimum, which is determined based on temperature probe measurements and is regulated by a heating element or cooler. The amount of dissolved oxygen, which decreases with increasing medium temperature, also depends on the medium’s temperature. To ensure sufficient oxygen saturation of the culture, the bioreactor must be equipped with an aeration system. To enhance oxygen dissolution and distribution in the medium, mixing is applied using an agitator, which breaks the air introduced into the medium into small bubbles, thereby improving the oxygen transfer process into the aqueous phase. This also ensures medium homogeneity and prevents air bubble coalescence. A key component in regulating oxygen concentration is the oxygen probe, which provides feedback to the aeration and mixing systems based on the measured pO2 value. When working with a bioreactor, it is essential to maintain the process’s pH optimum, regulated based on feedback between the pH electrode and pumps that dose pH-adjusting substances (acids, bases) into the working space [17; 90].

Regarding system concepts, bioreactors can be continuous, fed-batch, or batch. A continuous system is designed so that fresh medium is continuously fed into the reaction space while culture is continuously removed from the reactor. This concept is suitable, for example, for immobilized enzymes or biomass. Another type is the batch system, which is a closed system where, apart from gas exchange and regulatory fluids, no medium is added or removed from the system. In this regard, the fed-batch system differs from the batch system, where the fed-batch bioreactor is either continuously or periodically supplied with substrate, but the medium is not removed from the system. This process prolongs the cultivation time and increases potential yields [90; 91].

2.7.3 Metabolic demands of microorganisms and strategies for utilizing waste substrates

Lipidic waste substrates can serve as a valuable carbon source for microbial cultures. In the degradation process, triacylglycerol-based substrates must be broken down into acyls and glycerol, which are then processed in the cells to form acetyl-CoA. Fatty acids are metabolized via β-oxidation in peroxisomes or the mitochondrial matrix, while glycerol is processed through glycolysis in the cytosol. The degradation of fatty acids is depicted in Figure 11 [62; 92].

Hydrolysed lignocellulosic wastes are broken down through hydrolytic pre-treatments into basic carbohydrate units, primarily pentoses and hexoses. These sugars are metabolized intracellularly through the pentose phosphate pathway and glycolysis to pyruvate (see Figure 12), and subsequently to acetyl-

CoA. Acetyl-CoA serves as the main fuel for the glyoxylate cycle, which functions as an anaplerotic pathway for the citric acid cycle [93].

Waste feathers and yeasts are hydrolysed into oligopeptides and individual amino acids, which can be directly utilized for metabolic processes by the culture. In the absence of other carbon sources, cells can convert amino acids into acetyl-CoA via transamination to keto acids or through deamination and subsequent processing of the carbon skeleton.

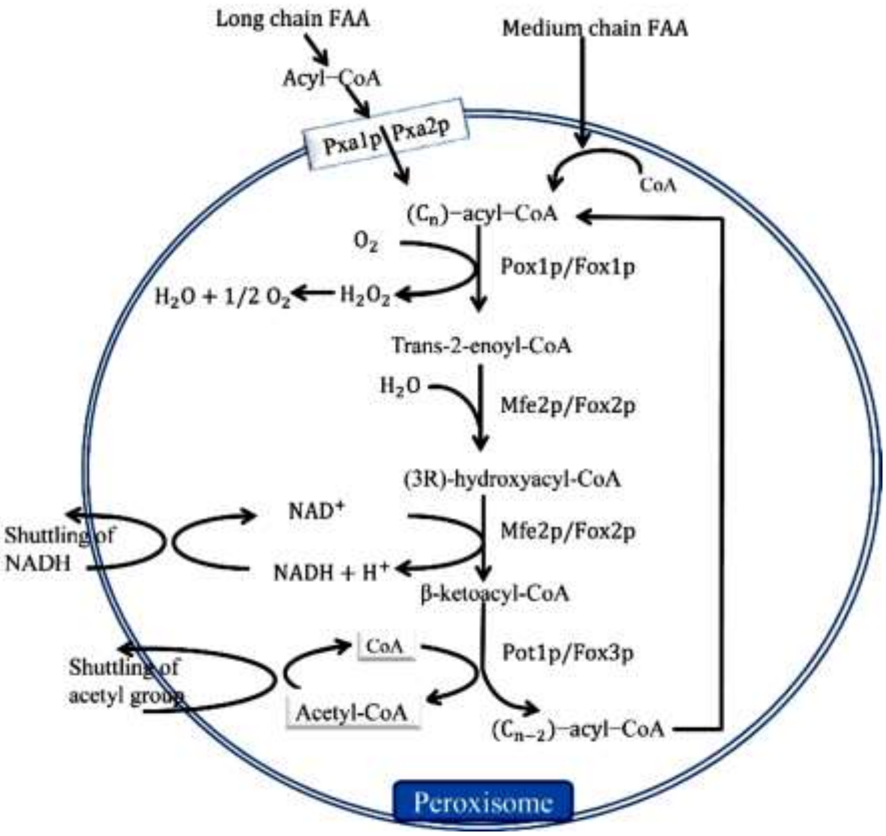


Figure 11: ***Diagram of Peroxisomal Beta-Oxidation.*** *Labels: Pxa1p; Pxa2p – Transmembrane transporters of long-chain fatty acids; Pox1p/Fox1p – Acyl-CoA oxidase; Mfe2p/Fox2p – Enoyl-CoA hydratase; Pot1p/Fox3p – β-oxoacyl thiolase [92]*

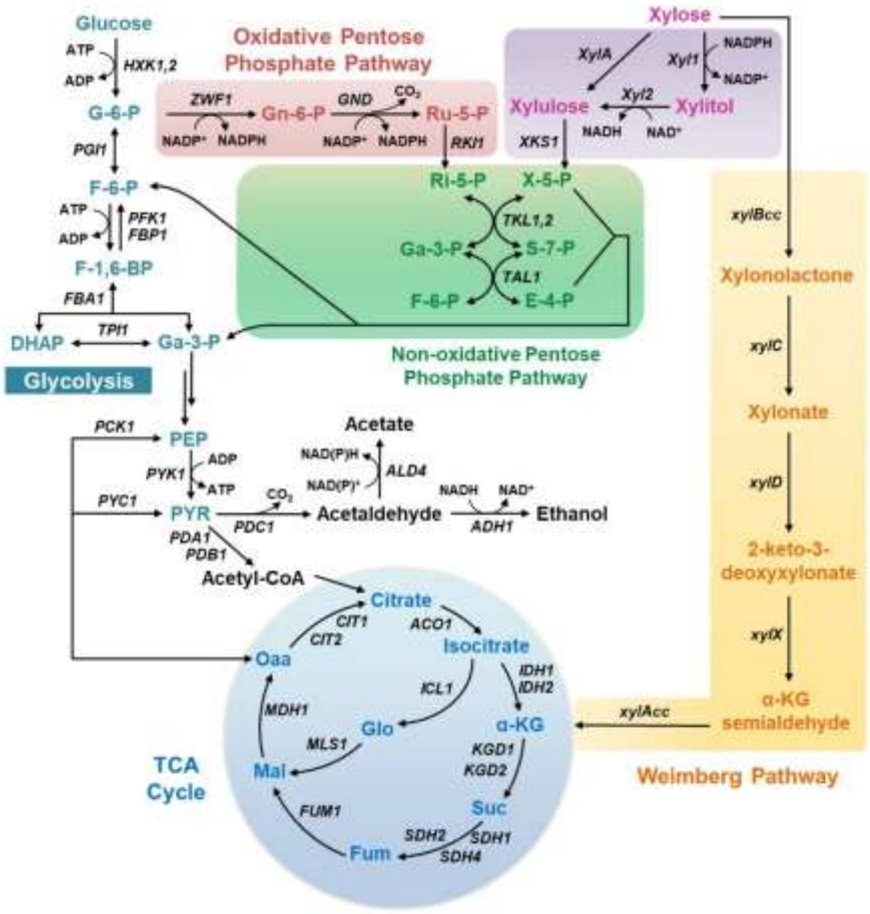


*Figure 12: **Diagram of the Metabolic Pathways of Glycolysis and the Pentose Phosphate Pathway in the Utilization of Various Saccharide Substrates by Different Yeast Species.** Metabolite abbreviations: G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; Ga-3-P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; α-KG, α-ketoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; Oaa, oxaloacetate; Gn-6-P, 6-phosphogluconate; Ru-5-P, ribulose-5-phosphate; Ri-5-P, ribose-5-phosphate; X-5-P, xylulose-5-phosphate; S-7-P, sedoheptulose-7-phosphate; E-4-P, erythrose-4-phosphate [94]*

2.7.4 Metabolic effectors influencing culture growth

Every organism is limited by external conditions that define its ability to survive and reproduce. This also applies to microbial cultures. For biotechnological applications, understanding the strain and the conditions under which it proliferates or produces desired metabolites is crucial. This is due to inducible enzymatic pathways that are activated in the presence of specific inducers (substrates) [83].

Microorganisms require suitable external conditions for growth and production. These conditions include temperature, oxygen availability, pH, osmotic pressure, nutrients (both macro- and microelements), light, etc. Yeasts, for example, have been observed to achieve a doubling time of approximately 2 hours under optimal conditions [83]. For more effective yeast cultivation, it is beneficial to supplement macro elements (C, H, O, P, N, S, K, Mg) with microelements, which can enhance the strain's production properties by enabling higher biomass yields in a shorter time. Additionally, under certain physicochemical conditions, yeasts can incorporate higher concentrations of minerals into their biomass than their usual physiological concentrations [95].

Another method to increase metabolite production is by using appropriate stress factors. In nature, yeasts and other microorganisms have to adapt to various changes in external conditions, often involving multiple growth parameters simultaneously. Consequently, yeasts have developed different strategies to manage stress conditions, such as light, oxidative, or osmotic stress. Nutritional stress, induced by using waste substrates as alternative nutrient sources, can also affect the production of enzymes and both extracellular and intracellular metabolites, depending on the type of substrate. In carotenoid-producing yeasts, stress responses typically result in the overproduction of ergosterol, carotenoids, and ubiquinone, which are valuable metabolites in various consumer industries, adding further value to waste utilization [53; 96].

2.7.5 Cultivation dynamics and initial cellular changes during the Lag phase

During cultivation, numerous biochemical changes occur within the cells of the culture, as the culture is typically inoculated into a new type of medium at the early stage, leading to the lag phase. The lag phase represents the metabolic adaptation of cells to utilize the substrates present in the production medium. During this phase, cells enlarge while maintaining their number. From an industrial application perspective, this phase is economically inefficient, and efforts are made to minimize its duration. In a conventional batch culture model, microorganisms then transition to the exponential growth phase, where their enzymatic machinery has adapted to the new conditions, and the culture begins to proliferate. The generation time decreases, leading to the next growth phase. During the exponential growth phase, the generation time shortens to its minimal value, and the biomass primarily produces primary metabolites. As nutrient concentrations in the medium decline, the culture enters the stationary phase, characterized by slowing growth. In this phase, secondary metabolites are produced, particularly in response to substrate limitation-induced stress. This phase culminates in the death phase, during which the culture depletes nutrients in the medium and gradually dies. These phases are described by the generalized growth curve model, as shown in Figure 13 [83; 90].

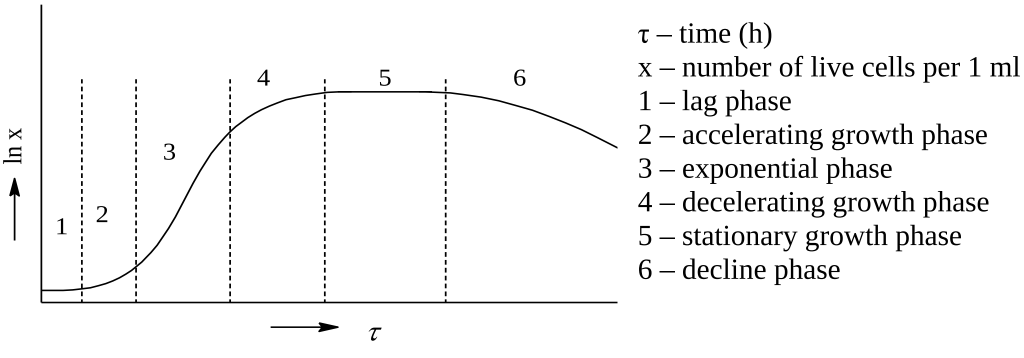


Figure 13: Model of growth curve [83]

The model differs from reality in several ways: for instance, when multiple substrates are used, the culture goes through several lag phases, initially utilizing simpler substrates and gradually assimilating more complex ones. When transitioning to more complex substrates or when conditions in the medium change during cultivation, the culture experiences adaptive lag phases as it metabolically "switches" between different metabolic pathways—illustrated in Figure 14 [97; 98].

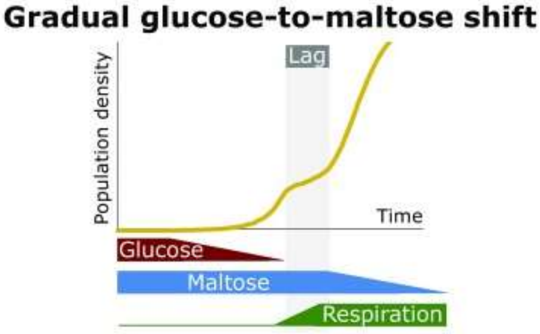


Figure 14: Model of Lag phase during transition from anaerobic to aerobic conditions with a substrate shift from glucose to maltose by the yeast Saccharomyces cerevisiae [97]

2.7.6 Application of carotenoid-accumulating yeasts in the processing of food waste

2.7.6.1 Utilization for animal fat

With the increase in animal production, there is also a rise in the production of animal waste in the form of waste fats. This substrate can be extensively processed into biofuels, but this is a competing sector with food production [99]. Integrating this material back into the food chain in a health-compatible form is highly desirable. Carotenoid-accumulating yeasts exhibit lipase activity and are capable of accumulating high concentrations of lipids in their biomass. Therefore, their use is particularly suitable for the degradation of lipidic waste materials from the food industry. The production of lipidic metabolites is further favoured due to the waste substrate acting as a stress factor, as it often presents a more complex material with more challenging nutrient availability compared to complex media. A disadvantage of animal fat is its higher melting temperature, making it often present as coagulated clusters at lower cultivation temperatures [100].

2.7.6.2 Utilization for coffee waste grounds

Coffee grounds can be considered a valuable substrate containing both lipid and saccharide components. Coffee grounds are initially ground and subjected to extraction with a non-polar solvent. Hexane has been found to be the most effective solvent for extracting the lipidic phase [101]. After the removal of lipids from the coffee grounds, the material undergoes acidic catalysis followed by enzymatic hydrolysis to obtain usable sugars [102]. When cultivating on a combined waste substrate of coffee oil and coffee hydrolysate, there is stimulation of enzymatic pathways supporting the pentose phosphate pathway alongside glycolysis for the assimilation of carbon from sugars and beta-oxidation, together with activated lipases that break down TAGs into usable components for the cells. Additionally, tocopherol from the coffee oil enters the cells, serving as protection against oxidative stress [103].

2.7.6.3 Utilization for frying oil

Frying oil is a waste oil from food processing establishments, rich in MUFAs and PUFAs, but it contains some microbiologically inhibitory substances formed during overheating. These are originally plant-based oils suitable for processing into biofuels or in biogas plants. However, these methods of processing increase the carbon footprint in the atmosphere due to CO_2 emissions. Using carotenoid-accumulating or oleaginous yeasts as biotechnological intermediaries for the valorisation of waste oil is appropriate. Yeasts, through lipases, break down the waste oil into fatty acids and glycerol, which can be processed further through metabolic pathways or incorporated into their biomass, for example, as membrane lipids. The advantage of waste frying oil is its lower solidification temperature, making it liquid under cultivation conditions and achieving better and more uniform distribution within the medium [17; 104; 105].

2.7.6.4 Utilization for feathers

Keratin is the third most abundantly produced biopolymer in the world, after lignocellulose and chitin. Therefore, its use as a waste material from poultry has lucrative significance, as it can serve as a valuable complex nitrogen source after appropriate pre-treatment. Prior to cultivation, preparatory steps are performed, where the material is stripped of lipids and then subjected to basic hydrolysis [106; 107]. Current research is investigating the impact of adding feather hydrolysate to the cultivation medium on the composition and condition of biomass after cultivation. The results of this study will determine whether the resulting hydrolysate is suitable for use in cultivating carotenoid-accumulating yeasts as a complex substrate or if it is also usable in combination with other waste substrates.

2.7.6.5 Utilization for whey

Whey is a complex by-product of dairy product manufacturing. Its composition varies depending on the chosen curdling method but contains a significant amount of dissolved lactose and proteins. From a biotechnological perspective, it is an interesting material that, after appropriate pre-treatment, can be used as a substrate for microbial cultivation. For carotenoid-accumulating yeasts, it is necessary to hydrolyse the lactose present in whey into glucose and galactose during pre-treatment, due to the absence of lactase in their enzymatic apparatus [108; 109; 110].

2.7.6.6 Utilization for brewer's yeast

Waste brewer's yeast is a biological material rich in proteins. To utilize this material, it must be converted into a usable substrate through hydrolysis. Since yeasts naturally contain a range of hydrolytic enzymes, they can be autolyzed at suitable temperatures, ionic strength, and pH to produce a valuable autolysate for cultivation purposes. This material is prone to contamination, so the resulting autolysate must be filtered and sterilized [111; 112; 113].

2.8 Analytical methods in yeast culture growth studies

Analytical methods include both quantitative and qualitative determinations of analytes in samples. When analysing various samples, it is important to consider the suitability of the methods used for determination. Additionally, attention must be paid to the precision of sample preparation and the sensitivity and selectivity of the methods employed. For the identification and quantification of metabolites from yeasts, chromatographic methods are used. For the determination of isoprenoid metabolites, HPLC-PDA methods can be employed, while GC-FID is suitable for fatty acid determination [114].

When studying metabolic and growth activities during different phases of growth, it is advisable to analyse yeast biomass in its native state, without subjecting it to destructive modifications. Techniques such as spectral and optical methods, including turbidimetry and microscopy, are suitable for this purpose.

2.8.1 Spectrophotometric methods

2.8.1.1 UV-VIS absorption spectrophotometry

This analytical method is based on the interaction of photons from a light source with the sample. When continuous or monochromatic radiation passes through the sample, photons are absorbed by electrons in the molecular orbitals of chromophores, exciting them to higher energy levels. The intensity of the remaining transmitted radiation is measured by a detector. Specific absorption spectra allow for the identification of analyte components, which can be quantified based on calibration standards. This method is a part of many analytical techniques, such as the determination of reducing sugars according to Dubois in waste substrates or the determination of proteins using the biuret method [115; 116; 117].

2.8.1.2 Fluorescence techniques

Fluorescence techniques measure the fluorescence value in the analyte, similarly to UV-VIS absorption spectrophotometry, but instead of measuring absorption, fluorescence caused by the transition of electrons to lower energy states is measured. This emitted fluorescence has lower energy and therefore a longer wavelength compared to the excitation radiation. The fluorescence phenomenon lasts only a few nanoseconds after excitation and is measured perpendicularly to the direction of the excitation radiation. The lower energy of the emitted fluorescence is called the Stokes shift. The increased wavelength of the emitted radiation is due to the dissipation of energy to the surroundings in the form of heat [115; 118]. Red yeasts contain natural fluorophores in the form of carotenoids or natural co-factors, which are responsible for cell autofluorescence. This property of carotenoids offers the possibility of tracking and locating them within cells, for example, using laser scanning confocal microscopy (LSCM) [119].

2.8.1.3 Turbidimetry and nephelometry

For rapid determination of biomass growth during online measurements in bioreactors or off-line measurements in the laboratory, turbidimetry or nephelometry can be used. These spectroscopic methods work on the principle of light scattering by particle forms of the analyte. In turbidimetry, the decrease in light transmission through the sample is measured and is related to the concentration of the analyte using the Beer’s law. In nephelometry, the intensity of scattered light is measured, which changes linearly with the concentration of the analyte [114; 120].

2.8.2 Optical methods

2.8.2.1 Flow cytometry

Flow cytometry is a non-destructive technique for monitoring intracellular metabolites within a culture. It involves measuring at the single-cell level, where a cell passing through a measurement chamber is illuminated by a laser of a specific excitation wavelength, exciting specific molecules within the cells. Subsequent fluorescence of the fluorophores is detected in several directions and amplified using photon multipliers to produce an observable signal. As the cell passes through the excitation source, the laser beam scatters in all directions. Light scattering at small angles (FC, FACS) is collected in the axis of the laser beam by a photodiode or photon multiplier; the size of the forward scatter correlates with the relative size of the cells [121].

Flow cytometry could be used for studying different growth phases and metabolic activities in yeasts. By staining specific metabolites with fluorophores, this method could be applied to study the metabolic adaptation of cultures to a new substrate during the lag phase. Based on the obtained data, cultivation techniques could be optimized to reduce the duration of the lag phase [122; 123].

2.8.2.2 Fluorescence microscopy

Laser scanning confocal microscopy is a method for visualizing and quantifying fluorophores within cells. This method involves irradiating the sample with a focused laser beam. The microscope's objective then focuses the beam into the sample. The emitted fluorescence is then directed through the objective, through a confocal pinhole to a detector, typically a photon multiplier. A planar image is obtained by scanning the field of view with the laser beam [124].

This method can be used to localize natural fluorophores (e.g., carotenoids) or to observe metabolites that are not naturally fluorescent by using appropriate staining agents. For instance, lipidic clusters within cells can be effectively localized. Similarly to flow cytometry, this method can help identify changes in yeast cells occurring during different growth phases [119; 125].

2.8.3 Chromatographic methods

Chromatographic methods are separation techniques based on the principle of affinity between the components of an analyte and two different phases. The analyte is carried by the mobile phase through the stationary phase. Components of the analyte interact with both phases simultaneously and are separated based on their differing affinities for each phase. Components with a higher affinity for the mobile phase pass through to the detector first, while those preferring the stationary phase are eluted later.

2.8.3.1 HPLC-PDA

High-Performance Liquid Chromatography (HPLC) is a type of column chromatography suitable for separating more complex samples. In HPLC, the liquid or solid sample is dissolved in an appropriate solvent. The mobile phase is continuously pumped into the system to prevent fluctuations in flow or pressure. The high resistance of the stationary phase results in a significant increase in system pressure, which the pump must overcome to push the mobile phase and analyte through to the detector. After injection, the sample is passed through a chromatographic column with the liquid mobile phase. Separation is determined by the interaction between the solvent and the stationary phase, including liquid-solid adsorption and liquid-liquid partitioning. Columns are typically made of stainless steel with an internal diameter of 2.1 to 4.6 mm and lengths ranging from 3 to 30 cm. They are packed with porous silica particles ranging from 3–10 µm in diameter, which may have irregular or spherical shapes and are usually surface-modified for specific separations. To extend the column's lifespan, guard columns are used to prevent clogging by unwanted particles; these are shorter and less expensive but contain a similar packing material to the main column [114].

For the analysis of isoprenoid compounds from biomass, a gradient elution procedure with PDA (photodiode array) detection is suitable. Extracts from cultured biomass contain a range of carotenoids and isoprenoid compounds that can be separated using this method. These include torularhodin, torulene, lycopene, beta-carotene, ergosterol, and ubiquinone. Carotenoids are detected at wavelengths between 450-550 nm, while ubiquinone and ergosterol are detected in the UV range at 280 nm. Suitable columns for analysis are modified with C18 to C30 [126; 127; 128].

2.8.3.2 HPLC-RI

This type of liquid chromatography is suitable for analysing substances that alter the plane of polarized light. RI here stands for Refractive Index detector, which detects changes in the refractive index of polarized light. Unlike HPLC-ELSD, HPLC-RI is not suitable for gradient elution because it measures the difference between a measurement cell containing the mobile phase with the analyte and a reference cell with pure mobile phase. The detector has an internal thermostat to maintain both cells at a constant temperature. This method is effective for measuring dissolved sugars, alcohols, organic acids, peptides, lipids, and polymers that alter the plane of polarized light [129; 130].

2.8.3.3 GC-FID

Gas Chromatography with Flame Ionization Detection (GC-FID) is a chromatographic method developed for volatile compounds with low boiling points. Similarly to HPLC, it is a type of column chromatography; however, the sample is in the gaseous phase during the analysis, achieved by vaporizing the sample upon injection into the column. A carrier gas continuously flows through the column, transporting the analyte toward the detector. During the analysis, the analyte interacts with the stationary phase of the capillary column, leading to separation. Capillary columns are made of quartz with an inner diameter of a few hundred micro-meters and are coated internally with a thin layer of stationary phase. These columns typically have lengths ranging from tens to up to 100 meters, providing a large separation area and thus high separation efficiency. The column is maintained at predetermined temperature conditions by a thermostat [114].

For the analysis of yeast fatty acids, the analytes are trans-esterified into methyl esters (FAMEs), which have much lower boiling points than TAGs. Methyl esters are transferred into a suitable non-polar solvent (hexane) and analysed. During this process, individual FAMEs are separated based on a temperature gradient. Flame Ionization Detection (FID) is commonly used for the detection of FAMEs [114; 131; 132; 133].

3 THE AIMS OF THE THESIS

One of the primary objectives of this work was to assess the production capabilities of carotenogenic yeast strains on waste substrates and to ensure the suitability of these waste materials through appropriate pretreatments, making them utilizable by the yeasts. Partial goals to achieve these objectives included:

- Conducting a literature review on: waste substrates and their potential processing; carotenogenic yeasts and their products; cultivation methods; and metabolite analysis techniques.
- Preparing various waste substrates, or their mixtures, and using them for the cultivation of selected yeast strains.
- Testing selected cultivation conditions in laboratory-scale bioreactors.
- Comparing the production characteristics of yeasts on different waste substrates.

Another primary objective was to monitor the adaptive changes during the initial stages of cultivation after transferring the culture from a complex medium to a mineral medium. Partial goals to achieve this included:

- Conducting a literature review on the growth phases of yeast cultures.
- Selecting appropriate conditions for the experiment.
- Cultivating selected yeast strains and subsequently analysing the live cultures using fluorescence techniques (flow cytometry and microscopy), as well as standard techniques (HPLC-PDA and GC-FID).

4 EXPERIMENTAL PART

4.1 Materials

4.1.1 Yeast strains used

The yeast strains were obtained from the Culture Collection of Yeasts (CCY) at the Slovak Academy of Sciences in Bratislava, Slovakia.

Strain	**CCY code**	**shortcut**
Rhodotorula (Rhodosporidium) toruloides	CCY 62-2-4	RT
Rhodotorula kratochvilovae	CCY 20-2-26	RK
Rhodotorula mucilaginosa (20-9-7)	CCY 20-9-7	RM(20-9-7)
Rhodotorula mucilaginosa (19-4-6)	CCY 19-4-6	RM(19-4-6)
Cystofilobasidium macerans	CCY 10-1-2	CM
Cystofilobasidium infirmominiatum	CCY 17-18-4	CI
Sporobolomyces (Sporidiobolus) pararoseus	CCY 19-9-6	SP
Sporidiobolus metaroseus	CCY 19-6-20	SM
Sporidiobolus salmonicolor	CCY 19-6-4	SS
Phaffia rhodozyma	CCY 77-1-1	PR

4.1.2 Waste materials used in work

- Coffee Grounds (lignocellulose and lipid waste material used as source of carbon for media preparation, local sources, Czech Republic)
- Waste Poultry Fat (lipid waste acquired through the ByPROValue project – used as carbon source for media preparation, Norway)
- Wate feathers (waste feathers obtained from local bedding company – used as nitrogen source for media preparation, Czech republic)
- Waste Brewery Yeast (spent biomass from local brewery – used as nitrogen source, Czech Republic)
- Whey (waste whey from local diary – used as complex source for media preparation, Czech Republic)
- Waste glycerol (carbon source from local biofuel producers – used for media preparation, Czech Republic)

4.2 List of chemicals

4.2.1 Chemicals for yeast culture media preparation

- Agar (Duchefa Biochemie, Netherlands)
- Antifoam (Sigma-Aldrich, Czech Republic)
- Bacteriological Peptone (Roth, Germany)
- D-Glucose Monohydrate p.a. (VWR, Czech Republic)
- Potassium Dihydrogen Phosphate p.a. (Penta, Czech Republic)
- Phenol (Lach-ner, Czech Republic)
- Anhydrous Glycerol p.a. (Penta, Czech Republic)
- Hexane 95 % (Penta, Czech Republic)

- Potassium Hydroxide p.a. (Lach-ner, Czech Republic)
- Sodium Hydroxide p.a. (Lach-ner, Czech Republic)
- Immersion Oil (Roth, Germany)
- Isopropanol p.a. (Penta, Czech Republic)
- Yeast Autolysate (Roth, Germany)
- Sulfuric Acid 96 % p.a. (Penta, Czech Republic)
- Urea p.a. (Penta, Czech Republic)
- Yeast Autolysate (Roth, Germany)
- Ammonium Sulphate p.a. (Lach-ner, Czech Republic)
- Magnesium Sulphate Heptahydrate (Lach-ner, Czech Republic)

4.2.2 Chemicals for extraction and analysis of metabolites by HPLC and GC

- Acetonitrile for HPLC (Chem-Lab, Belgium)
- Ethyl acetate for HPLC (Chromservis, Czech Republic)
- Hexane for HPLC (Chromservis, Czech Republic)
- Heptadecanoic acid (Sigma-Aldrich)
- Chloroform stabilized with 1 % ethanol p.a. (Penta, Czech Republic)
- Hydrochloric acid 36 % (VWR, Czech Republic)
- Sulfuric acid 96 % p.a. (Penta, Czech Republic)
- Methanol p.a. (Penta, Czech Republic)
- Methanol for HPLC (Chem-Lab, Belgium)
- Nile red (Sigma-Aldrich, Czech Republic)
- Tris-HCl (Lach-Ner, Czech Republic)

4.3 Equipment and instruments

- FreeZone 4.5 Freeze Dryer (Labconco, USA)
- BeadBug 6 Homogenizer (Benchmark, USA)
- RV 06 Vacuum Evaporator (IKA, Germany)
- HPLC Filters, PRE-CUT (Alltech, USA)
- Laboratory Bioreactor RALF3,7 (Bioengineering, Switzerland)
- Laboratory Bioreactor RALF6,7 (Bioengineering, Switzerland)
- HPLC/PDA System (Thermo Fisher Scientific, USA):
  - Dionex Ultimate 3000 Series (Thermo Fisher Scientific, USA)
    - Dionex Ultimate 3000 Series Pump
    - Dionex Ultimate 3000 Series Autosampler
    - Thermo Fisher Vanquish Detector
    - Chromeleon Data Analysis System
  - Kinetex EVO C18 Column, 150 x 4.6 x 2.6 mm (Phenomenex, USA)

- Dry Block Heater (Ohaus, USA)
- Analytical Balances, Pioneer (Ohaus, USA)
- Centrifuge, MIKRO 120 (Hettich, Germany)
- Vapodest 500, C. (Gerhardt GmbH & Co. KG, Germany)
- Kjeldatherm Block-Digestion Unit (Gerhardt GmbH & Co. KG, Germany)
- Shaker, SHR-2D (Witeg, Germany)
- Aurora Flow Cytometer (Cytek, USA)
- Optical Microscope, Lx500 (Labomed, USA)
    - Camera, EOS 1100D (Canon, Japan)
    - Imaging Software, Labphoto 3.0
- Fluorescence Microscope, Eclipse E400 (Violab, Italy)
    - Imaging Software (NIS-Elements D, Japan)
    - G-2A Green Excitation Filter (Nikon, Japan)
    - B-2A Blue Excitation Filter (Nikon, Japan)
    - DAPI Ultraviolet Excitation Filter (Nikon, Japan)
- TRACE GC/FID (Thermo Fisher Scientific, USA)
    - Lion LN FAME GC Capillary Column, 30 m x 0.32 mm; 0.25 µm (Chromservis, Czech Republic)
- HPLC/RI System:
    - Dionex Ultimate 3000 Series (Thermo Fisher Scientific, USA)
        - Ultimate DAD Detector
        - ERC RefractoMax 520
        - Ultimate Pump, SmartFlow
        - Column Oven LCO 101 (ECOM, Czech Republic)
    - Luna Omega Sugar Column, 250 mm x 4.6 mm, 2.6 µm (Phenomenex, USA)

4.4 Substrate preparation

4.4.1 Coffee oil

Coffee grounds were sourced locally, consisting of a blend of Arabica and Robusta varieties. The waste grounds were dried in an oven at 70 °C for 24 hours and subsequently ground into fine particles. Coffee oil was then extracted from the dried, ground coffee grounds, with 60 g of the grounds subjected to extraction using a Soxhlet apparatus with a 50:50 mixture of IPA and hexane for 90 minutes. The extraction solvent was then evaporated using a rotary evaporator under vacuum. The extracted coffee oil was stored at low temperature in a refrigerator.

4.4.2 Coffee hydrolysate

The pre-extracted coffee grounds were first dried in an oven at 80 °C for 24 hours. Then, 200 g of the dried grounds were mixed with 1 liter of 1 % sulfuric acid in a 2-liter sterilization flask. This mixture was subjected to acid hydrolysis in an autoclave at 130 °C for 90 minutes. After cooling, the pH of the mixture was adjusted to the optimal level for cellulase and hemi-cellulase activity, which were then added. The mixture was stirred and maintained at the enzymatic hydrolysis optimal temperature for 24 hours. Subsequently, the mixture was filtered through filter paper under reduced pressure. The carbohydrate concentration in the hydrolysate was determined using the Dubois method [116]. The coffee hydrolysate was stored at a low temperature to prevent contamination for further use.

4.4.3 Waste frying oil

The used frying oil was filtered under reduced pressure through filter paper to remove coarse impurities before being utilized as a substrate. The oil was then transferred to a separatory funnel, and sodium chloride was added, resulting in the formation of two distinct phases. The lower aqueous phase was separated and discarded. The dewatered oil was subsequently stored at a low temperature for further use.

4.4.4 Poultry fat

The waste poultry fat was obtained through the ByPROValue project in collaboration with Norway food processing industry. This material was used without further processing for the preparation of production media and was stored at a low temperature.

4.4.5 Waste glycerol

Waste glycerol obtained from local sources was measured by HPLC-RI method (chapter 4.6.5) to acquire the information of glycerol concentration in the waste material. Based on the concentration, the filtered glycerol was then used directly in media.

4.4.6 Spent grain hydrolysate

Spent grain obtained from a local brewery was dried in an oven at 70 °C and subsequently ground. For hydrolysis, 400 g of the ground spent grain were mixed with 1500 ml of 1 % sulfuric acid in distilled water. The hydrolysis was carried out in an autoclave at 130 °C for 90 minutes. After cooling, the pH of the hydrolysate was adjusted to 5, and hemi-cellulase and cellulase enzymes were added. The mixture was stirred at 40 to 45 °C for 48 hours. The resulting enzymatic hydrolysate was filtered under reduced pressure, and the filtrate was stored in a freezer. The total carbohydrate content in the hydrolysed spent grain was determined using the Dubois method (chapter 4.6.2).

4.4.7 Whey

Waste whey, sourced locally, was promptly transferred into five-liter sterilization flasks and sterilized in an autoclave at 115 °C for 15 minutes to prevent microbial growth. Due to the precipitation of whey proteins at high temperatures, the whey was filtered under reduced pressure using a Büchner funnel after cooling. The filtered whey was then stored in a freezer. The removal of precipitated whey proteins was necessary, as in previous cultivations, these proteins mixed with yeast biomass, preventing accurate analysis of the produced yeast biomass. The sugar content in the whey was subsequently determined using the Dubois (chapter 4.6.2) method and HPLC-RI, while the nitrogen content was assessed by the Kjeldahl method (chapter 4.6.1).

4.4.8 Poultry feathers

Purified, defatted poultry feathers were sourced locally. For cultivation purposes, a basic feather hydrolysate was prepared by mixing 800 ml of 1 M NaOH with 80 g of purified feathers in a 1-liter sterilization flask. The mixture was then sterilized in a pressure cooker at 140 °C for 1 hour. The resulting product was stored at a low temperature. The nitrogen content in the hydrolysate was determined using the Kjeldahl method (chapter 4.6.1).

4.4.9 Spent brewer's yeast

Yeast obtained from a local brewery was centrifuged at 7500 rcf for 5 minutes. The cells were then washed twice with cold water and once with a phosphate buffer. The phosphate buffer was prepared as a 150 mM solution with a pH of 5. The yeast was mixed with the phosphate buffer to form a suspension with a 10 % w/v concentration. In the first part of the experiment, this suspension was stirred using magnetic stirrers at 300 rpm and a temperature of 50 °C for 24 hours.

Another suspension was prepared using commercially available granulated yeast in a 150 mM phosphate buffer with a pH of 6, with the granules constituting 20 % w/v. The composition of the granulated brewer's yeast, as provided by the manufacturer, included dried yeast (*Saccharomyces cerevisiae*), wheat meal, corn meal, and rapeseed oil, with a declared crude protein content of 25.50 %.

After incubation, the final mixtures were centrifuged in 250 ml centrifuge tubes, and the nitrogen content in the resulting liquid autolysates was determined by the Kjeldahl method (chapter 4.6.1). The autolysate was then stored at a low temperature for further use.

4.5 Cultivation techniques

4.5.1 Inoculation

First, the YPD agar medium (Table 1) was sterilized in sterilization bottle in pressure cooker and poured into sterile Petri dishes. After cooling, 100 µl of stock cultures from cryovials were inoculated onto the agar plates. Following two to three days of optimal cell growth, the cultures on the agar plates were ready to be transferred into liquid YPD inoculation media, which had been prepared and sterilized in Erlenmeyer flasks. The transfer was carried out at a ratio of one loopful of culture per 10 ml of medium. The composition of the YPD medium is shown in the referenced table.

For screening cultivations, a dual inoculation method was used. The first inoculation medium (I1) consisted of 50 ml in a 250 ml Erlenmeyer flask, while the second inoculation medium (I2) consisted of 125 ml in a 500 ml Erlenmeyer flask, with 25 ml from I1 being inoculated into I2. An inoculation ratio of 1:5 was always maintained. Production media, with a volume of 50 ml, were inoculated with 10 ml of the inoculation medium. For large-scale cultivations, the dual inoculation method was also applied, but the final volume of I2 was 250 ml.

Table 1: Composition of YPG agar medium and liquid YPG media (without agar) on 1000 ml of distiled water

Component	Amount [g/l]
Agar	20
Peptone	20
Yeast autolysate	10
Glycerol	20

4.5.2 Standard flasks cultivations

From the I2 media, production media (50 ml in 250 ml Erlenmeyer flasks) were inoculated under sterile conditions in a laminar flow hood at a 1:5 ratio. These flasks were then placed on a reciprocal shaker (115 rpm) for 96 hours. The composition of the media is detailed in the corresponding experiments within the results section. During some experiments, 10 ml samples were taken to monitor the culture's condition over time. After 96 hours, cultivation was terminated, and the biomass was centrifuged at 7,500 rcf and stored in a freezer for further processing and analysis.

4.5.3 Bioreactor cultivations

Cultivations in laboratory bioreactors were conducted with working volumes of 2.2 and 5 liters. During cultivation, the pO_2 was maintained at a stable level of 25 % by adjusting the stirrer speed. The pH was controlled based on feedback from a pH probe, using two peristaltic pumps to dose either acid (10 % H_2SO_4) or base (10 % KOH), keeping the pH at 6.5. Temperature was regulated at 25 °C using an internal cooling finger and a heating jacket. Before each cultivation, the oxygen probe was calibrated to 0 % after autoclaving and then to 100 % after cooling, with intensive aeration and stirring at 600 rpm. Inoculation was always carried out at a 1:10 ratio, with the inoculum pumped into the bioreactor under pressure. Antifoam was added as needed using a sterile syringe.

4.5.4 Cultivation for lag-phase testing

To test the lag phase, a standard inoculation was performed in I2 medium, which was incubated on a reciprocal shaker at 115 rpm for 24 hours. Subsequently, under sterile conditions in a laminar flow hood, 10 ml of culture was transferred into pre-sterilized 15 ml tubes containing 4.62 g of glycerol. The tubes were carefully sealed with lids and secured with parafilm. The contents were thoroughly mixed, and the tubes were stored in a deep-freeze at -80 °C. For each strain tested, 12 tubes were prepared. Glycerol in these tubes served as both a cryoprotectant and a carbon source for the production medium.

For experimental use, the tubes were thawed in lukewarm water, and after thoroughly washing the exterior of tubes with 70 % ethanol, their contents were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of nutrient salt solution. Inoculations into the flasks were carried out at the following time intervals: 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 12 h, and 24 h. The last tube served as a control for the original inoculum. The yeast cultures were subsequently analysed using flow cytometry, fluorescence microscopy, HPLC with a PDA detector, and GC-FID (see 4.6).

4.6 Analytical techniques for substrate and biomass metabolite analysis

4.6.1 Determination of nitrogen by the Kjeldahl method

Feather and yeast samples collected during hydrolysis were analysed using the Kjeldahl method to determine the total nitrogen content. The precise amount of each sample was weighed or measured into digestion tubes, to which Weininger's catalyst and sulfuric acid were added. The samples were then digested using a Kjeldahl digestion unit (Kjeldatherm, Gerhardt GmbH & Co. KG). After digestion, phenolphthalein indicator was added to the digest, and the tube was placed into a VAPODEST 500 unit, where ammonia was distilled using steam. The distilled ammonia was collected in a titration flask containing a precisely measured amount of sulfuric acid. After the distillation process was completed, the remaining sulfuric acid was titrated with a standardized sodium hydroxide solution using Tashiro's indicator. The total nitrogen content was calculated based on the consumption of the titration solution [134].

4.6.2 Determination of residual sugars by the Dubois method

The filtered carbohydrate substrate samples were diluted 1000-fold. Then, 1 ml of the diluted sample was transferred into a glass test tube, and 1 ml of 5 % phenol solution and 5 ml of 96 % sulfuric acid were added. The mixture was immediately shaken and allowed to stand for 30 minutes at room temperature. Absorbance was then measured at 490 nm in a glass cuvette, using distilled water as the blank. Based on a previously established calibration curve using mannose (μg/ml) as the standard (see Table 2), the total sugar concentration was determined [116].

Table 2: Calibration parameters

Calibration equation	**R^2 value**
$y = 0.0099x$	0.9981

4.6.3 Gravimetric analysis

Gravimetric determination of dry matter was conducted by sampling 1 ml of culture into a 2 ml microcentrifuge tube, where the culture was centrifuged several times and washed with distilled water. The resulting sediment was then frozen, lyophilized, and subsequently analysed gravimetrically.

4.6.4 Flow cytometry and fluorescence microscopy

For cell visualization post-cultivation, a fluorescence cytometer (Aurora, Cytek) equipped with blue and violet excitation lasers was used. From each cultured sample, 1 ml was taken and transferred into two 2 ml plastic microcentrifuge tubes. The samples were centrifuged three times and washed with 100 mM phosphate buffer (pH 6) to remove the supernatant. Subsequently, they were diluted 100-fold into new 2 ml microcentrifuge tubes. To one set of samples, 1 μl of Nile red solution (1 mg/ml) was added, and these samples were incubated for 15 minutes. The samples were then analysed using the flow cytometer to collect 10 000 events.

Undiluted samples prepared for cytometry were further utilized for visualization under a fluorescence microscope at 100x objective magnification and 10x ocular magnification. The samples were observed under ultraviolet, green, and blue excitation light, using a mercury vapor lamp as the light source. The samples were imaged both in their native state and after a 15-minute incubation with Nile red solution (2 mg/ml) at 2 μl/ml, which was added prior to measurement.

4.6.5 HPLC-RI

For the analysis of dissolved sugars and alcohols, the samples were first centrifuged and subsequently filtered through a nylon filter with a pore size of 0.45 μm. From the filtered samples, 20 μl was injected into the HPLC-RI system. The analysis was performed using a Dionex Ultimate 3000 HPLC system with an RI detector, supplied by Thermo Fisher Scientific. The analytical conditions included a temperature of 35 °C and separation on a Luna Omega Sugar column (250 mm x 4.6 mm, 2.6 μm particle size) with a mobile phase flow rate of 1.0 ml/min, using mixture of Acetonitrile:Milli-Q water in ratio 75:25 as the mobile phase [135].

4.6.6 HPLC-PDA

4.6.6.1 Extraction of lipidic antioxidants

Carotenoids, ergosterol, tocopherol and ubiquinone were extracted together from a single sample using the Folch extraction method [136]. Biomass, weighing between 5 and 20 mg, was first measured on an analytical balance into 2 ml screw-cap plastic vials. The biomass was hydrated with 1 ml of distilled water for 30 minutes. After hydration, the biomass was centrifuged, and the supernatant was discarded. Approximately 0.5 ml of glass beads and 1 ml of methanol were added to the hydrated biomass. The vials containing the mixture were vigorously shaken on a multi-position vortex mixer for 20 minutes.

Subsequently, the entire vial contents were transferred to a 15 ml screw-cap tube, to which 2 ml of chloroform was added. The mixture was vortexed again for 20 minutes. Then, 1 ml of distilled water was added, and the sample was centrifuged (300 rcf, 1 minute). After phase separation, the lower chloroform phase was collected using a pipette and transferred to another 15 ml tube. The chloroform was evaporated on a heating block at 35 °C using compressed nitrogen. The resulting residue was dissolved in 2 ml of an HPLC solvent mixture consisting of acetonitrile and ethyl acetate in a 1:2 ratio. This mixture was thoroughly mixed on a vortex and filtered through a 0.45 μm PTFE filter into a glass vial for HPLC/GC analysis. The prepared sample was either stored in the freezer or immediately analysed.

4.6.6.2 HPLC analysis

Samples containing carotenoids, ergosterol, and ubiquinone were analyzed using an HPLC system from Thermo Fisher Scientific, equipped with a Kinetex EVO C18 column (150 mm x 4.6 mm, 2.6 μm particle size). Elution was performed in gradient mode with a mobile phase flow rate of 1.2 ml/min, and the total analysis time was 25 minutes. The composition of the mobile phases is shown in Table 3, and the gradient changes of the mobile phase on the column are described in Table 4. Detection was carried out using a PDA detector, with carotenoids detected at a wavelength of 450 nm and ergosterol and ubiquinone at 280 nm. Identification and quantitative determination were based on previously measured standards and their calibration curves. Data analysis was performed using Chromeleon software [137].

Table 3: Composition of mobile phases

Mobil Phase (MP)	**Component**	**% (vol)**
A	Methanol	2
	Acetonitril	84
	100 mM TrisHCl buffer (pH 8)	14
B	Methanol	60
	Ethyl acetate	40

Table 4: Gradient changes during analysis

Retention time [min]	**MP A [%]**	**MP B [%]**
0	100	0
13	0	100
19	0	100
20	100	0
25	100	0

4.6.7 GC-FID

4.6.7.1 Transesterification and sample preparation

For the analysis of lipids and the fatty acid profile (SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids), 10–15 mg of microbial biomass from each sample was weighed into 2 ml glass crimp vials. Then, 1.8 ml of a transesterification mixture, containing 15 % sulfuric acid in methanol and 0.5 mg/ml of the internal standard heptadecanoic acid, was added. The vials were sealed with aluminium caps and incubated on a heating block for 120 minutes at 85 °C. After cooling, the contents of the vials were transferred to larger 5 ml glass vials, where 0.5 ml of 0.05 M NaOH solution and 1 ml of hexane were added. After vigorous shaking and phase separation, 100 µl of the upper hexane phase was pipetted into a 2 ml screw-cap vial and diluted to 1 ml with the addition of 900 µl hexane. The prepared sample was analysed using GC-FID instrumentation [137].

4.6.7.2 GC analysis

The analysis was performed using a TRACE 1300 TM chromatograph, with an injection volume of 1 µl and a split ratio of 10:1. Separation was conducted on a Lion LN FAME GC column with dimensions of 30 m x 0.25 mm x 0.2 µm. Hydrogen was used as the carrier gas at a flow rate of 0.5 ml/min. A temperature gradient, as described in Table 5, was applied during the analysis. Data evaluation was carried out using Chromeleon 7.2 software, where individual fatty acids were identified based on previously measured standards and quantified using processing method [137].

Table 5:Temperature program of gas-chromatographic analysis

retention time [min]	temperature gradient [°C/min]	target temperature [°C]	temperature hold [min]
0	-	-	-
1	0	80	1
5	15	140	0
21.7	3	190	0
24.5	25	260	1
25.5	Stop	-	-

4.6.8 Beta-glucan determination

The determination of beta-glucans was performed using a dual analysis method, measuring both total glucans and alpha-glucans. The beta-glucan content in the samples was calculated by subtracting the amount of alpha-glucans from the total glucans. The analysis was conducted using an enzymatic kit from MEGAZYME, specifically the K-YBGL kit [138]. To reduce chemical consumption, the procedure was slightly modified as outlined below.

4.6.8.1 Determination of total glucans

Samples of yeast biomass, weighing 13–15 mg, were measured on an analytical balance and placed into glass reaction tubes. To each tube, 300 µl of 12 M sulfuric acid was added, and the tubes were placed in an ice bath for 2 hours, during which they were vortexed every 30 minutes. After this period, 1.5 ml of water was added to each tube, and the samples were vortexed again. The tubes were then incubated in a heating block set to 100 °C for 2 hours. After cooling to room temperature, neutralization was performed by adding 450 µl of 10 M KOH and 5.25 ml of 200 mM acetate buffer at pH 5.

Subsequently, 1 ml of the sample was transferred into a centrifuge tube and centrifuged at 16 000 rcf for 5 minutes. From the supernatant, 30 µl was transferred into a 96-well microplate, where 30 µl of a mixture of exo-1,3-β-glucanase and β-glucosidase in 200 mM acetate buffer was added. The mixture was vortexed and incubated at 40 °C for 60 minutes. After this incubation, 10 µl of the mixture was transferred to a second 96-well microplate, where 150 µl of GOPOD reagent was added. A second incubation followed at 40 °C for 20 minutes. During this time, a blank sample containing acetate buffer and GOPOD reagent was prepared, along with a standard D-glucose sample. Finally, absorbance was measured using an ELISA reader at a wavelength of 510 nm.

4.6.8.2 Determination of alpha-glucans

Samples of yeast biomass, weighing 13–15 mg, were measured on an analytical balance and placed into glass reaction tubes. Subsequently, 300 µl of 1.7 M NaOH was added to the samples, and the tubes were placed in an ice bath for 20 minutes. After this period, 1.2 ml of 1.2 M acetate buffer (pH 3.8) and 30 µl of invertase were added to the samples. The tubes were then transferred to a heating block and incubated at 40 °C for 20 minutes. Following incubation, 1 ml of the sample was transferred into a centrifuge tube and centrifuged at 16 000 rcf for 10 minutes.

From the resulting supernatant, 50 µl was transferred to a 96-well microplate, where 50 µl of 200 mM acetate buffer (pH 4,5) was added. From this mixture, 10 µl was transferred to a second 96-well microplate, where 150 µl of GOPOD reagent was added. The prepared plate was then incubated at 40 °C for 20 minutes. Finally, the absorbance was measured using an ELISA reader at a wavelength of 510 nm.

5 RESULTS

5.1 Experiments on waste substrates

The following experiments were conducted on various waste substrates or their combinations, primarily at C/N ratios of 13, 25, and 50 (see experiment descriptions for details). All cultivations were performed at least in duplicates using the same inoculum. In the case of multiple series, repeated control cultivations were conducted to demonstrate the variability of results with different inocula. For bioreactor cultivations, duplicate samples were taken at each time point. The deviations from the mean for the individual duplicates did not exceed 15 %; therefore, the values for deviations are not presented in the graphs or tables. The results of this work have been published in several scientific articles and conference papers. **The results of Chapter 5.1.1 were partially published in**: Szotkowski M., Holub J. et al., 2021. *Poster presentation; The EuroBiotech Journal.* DOI: 10.2478/ebtj-2021-0031. **The results of Chapter 5.1.2 were partially published in**: Szotkowski M., Plhalová Ž., Sniegoňová P., Holub J. et al., 2023. *Microorganisms*. *11*(4), 1013; DOI: 10.3390/microorganisms11041013. **The results of Chapter 5.1.4. were partially published in** Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321; Holub J. et al., 2022. *Proceedings of the 9th International Conference on Chemical Technology.* ISBN 978-80-88307-11-2; Holub et al., 2021. *Poster presentation; The EuroBiotech Journal.* DOI: 10.2478/ebtj-2021-0031. **The results of Chapter 5.1.5. were partially published in** Holub et al., Cultivation of carotenogenic yeast on various combination of waste nitrogenous and carbonaceous substrates. *Poster presentation; Eurobiotech Congress 2024*, 3.10.-5.10.2024, Istanbul - Turkey and Holub J. et al., 2024. *Book of abstracts of the 11th International Conference on Chemical Technology*. ISBN 978-80-88214-48-9.

5.1.1 Cultivation on a combination of oils and coffee hydrolysate

The initial experiments describe the cultivation of red yeast strains: *Rhodotorula mucilaginosa* (19-4-6) (CCY 19-4-6), *Rhodotorula (Rhodosporidium) toruloides* (CCY 62-2-1), *Cystofilobasidium macerans* (CCY 10-1-2), *Sporidiobolus pararoseus* (CCY 19-9-6), *Sporidiobolus metaroseus* (CCY 19-9-20), *Rhodotorula mucilaginosa* (20-9-7) (CCY 20-9-7), and *Rhodotorula kratochvilovae* (CCY 20-2-26). These yeast strains were cultivated on a combination of oils with coffee hydrolysate, specifically using coffee oil and frying oil. The composition of the production media and their descriptions are provided in Table 6 and Table 7. The media and their loadings were designed with a C/N ratio of 50.

Table 6: The composition of the mineral base media used in the experiment, excluding glycerol, which was utilized as the carbon substrate in the control media

Substance	**Amount**
KH_2PO_4	4 g
$MgSO_4 \cdot 7H_2O$	0.696 g
Urea	1.81 g
Distilled water	1000 ml
Glycerol (control 1 and 2)	92.51 g

Table 7: Labels indicating the ratios of hydrolysate to oil for each case, corresponding to the oil dosages per 50 ml of medium

Label	**Ratio**	**Oil [g]**
10 % hydr.	1:9	2.12
20 % hydr.	2:8	1.88
30 % hydr.	3:7	1.65
40 % hydr.	4:6	1.41
50 % hydr.	5:5	1.18

5.1.1.1 Sporidiobolus pararoseus (CCY 19-9-6)

Table 8 presents a comprehensive summary of the growth and metabolite production of the yeast *Sporidiobolus pararoseus* under various concentrations of coffee hydrolysate (hydr.) combined with coffee oil (co) and waste frying oil (fo). In the control medium, the yeast exhibited relatively low levels across all parameters, including carotenoids (0.138 mg/g) and ubiquinone (1.455 mg/g). The highest carotenoid production (0.334 mg/g) and a significant increase in lipid content (65.00 %) were achieved with coffee oil and a 30 % concentration of hydrolysate. The peak tocopherol level (45.791 mg/g) was observed at a 20 % hydrolysate concentration, indicating that moderate hydrolysate concentrations may enhance antioxidant synthesis. Optimal production of ubiquinone (5.738 mg/g) and ergosterol (2.370 mg/g) was recorded at a 40 % hydrolysate concentration, which also represented the maximum value for this group. The highest biomass increase was observed at a 10 % hydrolysate concentration with 90 % coffee oil (31.12 g/l). Notably, the highest lipid concentration (65.00 %) was measured with a 30 % hydrolysate and 70 % coffee oil composition. The greatest proportion of polyunsaturated fatty acids (45.29 %) was detected in the medium with 40 % hydrolysate and coffee oil. The highest accumulation of monounsaturated fatty acids at 78.32 % was recorded in the control medium of the second series, while the content of saturated fatty acids reached the 63.85 % in media consisting of coffee oil with 10 % of hydrolysate.

When using waste frying oil, the yeast achieved optimal results at different hydrolysate concentrations. The highest carotenoid (1.900 mg/g) and ubiquinone (8.280 mg/g) contents were observed at a 40 % hydrolysate concentration, demonstrating optimized conditions for the synthesis of these metabolites. The maximum ergosterol content (4.252 mg/g) was found at a 10 % hydrolysate concentration, while the highest biomass (28.85 g/l) was reached at a 30 % hydrolysate concentration. The highest PUFA content (22.56 %) was recorded at a 40 % hydrolysate concentration (Szotkowski M., Holub J. et al., 2021. *Poster presentation; The EuroBiotech Journal.* DOI: 10.2478/ebtj-2021-0031 – see attachment 5, pg. 90).

Table 8: Sporidiobolus pararoseus production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media

	Sporidiobolus pararoseus								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control 1	0.138	1.455	1.568	9.11	n.a.	21.44	75.37	3.19	20.94
10 % hydr.+co	0.234	0.154	0.378	31.12	28.653	63.85	7.25	28.9	64.50
20 % hydr.+co	0.031	0.467	0.348	5.41	45.791	50.77	9.29	39.94	61.60
30 % hydr.+co	0.334	0.204	0.205	22.09	21.872	51.0	9.42	39.58	65.0
40 % hydr.+co	0.21	5.738	2.37	25.09	3.987	45.26	9.45	45.29	55.0
50 % hydr.+co	0.041	3.722	1.631	9.85	28.760	55.64	8.52	35.84	47.0
control 2	0.761	1.536	3.086	18.58	n.a.	17.14	78.32	4.54	11.30
10 % hydr.+fo	1.213	4.175	4.252	24.41	1.264	13.16	69.12	17.72	18.03
20 % hydr.+fo	1.900	7.295	3.199	23.97	0.115	15.16	63.86	20.98	15.29
30 % hydr.+fo	0.687	4.361	1.725	28.85	0.194	16.22	66.16	17.63	23.35
40 % hydr.+fo	1.313	8.28	3.484	20.53	0.121	14.22	63.22	22.56	15.09
50 % hydr.+fo	1.112	5.172	2.614	18.12	0.131	19.24	64.36	16.41	14.78

5.1.1.2 Rhodotorula mucilaginosa (CCY 19-4-6)

The Table 9 presents production of various metabolites, biomass accumulation, lipid content, and fatty acid composition of *Rhodotorula mucilaginosa* (19-4-6). Among the metabolites, the highest carotenoid content (except for control 1 medium) was recorded in the “20 % hydr.+co” (1.341 mg/g) in the same medium the highest ubiquinone production was 4.759 mg/g. The highest ergosterol content was observed in the “40 % hydr.+fo” condition (5.999 mg/g). For tocopherol, the “20 % hydr.+co” condition exhibited the highest concentration (62.273 mg/g).

Table 9: Rhodotorula mucilaginosa (19-4-6) production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media

	Rhodotorula mucilaginosa (19-4-6)								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control 1	1.789	2.193	2.019	17.35	1.606	30.47	66.56	2.97	13.50
10 % hydr.+co	0.217	0.675	0.355	13.26	9.415	41.16	11.46	47.39	31.27
20 % hydr.+co	1.341	4.759	1.040	24.43	62.273	39.49	10.49	50.02	42.19
30 % hydr.+co	1.220	3.706	1.608	10.75	40.088	43.16	10.99	45.86	28.50
40 % hydr.+co	0.812	2.172	1.032	11.64	17.548	45.85	10.10	44.06	30.00
50 % hydr.+co	2.251	3.215	2.351	10.02	27.350	44.47	10.35	45.18	35.78
control 2	1.223	2.051	4.437	22.84	2.464	13.47	39.24	47.30	45.00
10 % hydr.+fo	0.243	3.410	5.347	11.71	0.215	13.45	40.85	45.70	30.00
20 % hydr.+fo	0.173	4.162	2.505	14.43	1.443	13.29	40.79	45.92	19.85
30 % hydr.+fo	0.220	3.127	2.812	13.92	1.382	13.79	40.46	45.75	27.00
40 % hydr.+fo	0.212	2.984	5.999	15.00	0.188	9.87	40.16	49.98	30.00
50 % hydr.+fo	0.498	3.843	3.661	19.95	0.186	25.41	11.85	62.74	18.00

Regarding lipid content and fatty acid profiles, the control condition using waste frying oil produced the highest total lipid content (45.00 %). For SFA, the “40 % hydr.+co” condition recorded the highest percentage (45.85 %). MUFA were most abundant in the control group using coffee oil (66.56 %), whereas PUFA were maximized in the “50 % hydr.+fo” condition (62.74 %).

5.1.1.3 Rhodosporidium toruloides (CCY 62-2-4)

The Table 10 provides data on the metabolite production, biomass accumulation, lipid content, and fatty acid composition of *Rhodotorula toruloides* under various conditions. Among the metabolites, the highest carotenoid content was observed in the “control 1” with 4.875 mg/g, and the maximum ubiquinone concentration was also noted in the “control 1” condition (5.657 mg/g). The highest ergosterol concentration was recorded in the control group with waste frying oil, reaching 3.176 mg/g. For tocopherol, the “30 % hydr.+co” condition showed the highest value (26.528 mg/g). In terms of biomass production, the “40 % hydr.+fo” condition achieved the highest yield at 24.16 g/l.

In the context of lipid content and fatty acid profiles, the “10 % hydr.+co” condition produced the highest total lipid content (36.14 %). The highest percentage of SFA was found in the “10 % hydr.+co” group (43.36 %). For MUFA, the control condition demonstrated the highest percentage (74.82 %), while the “40 % hydr.+fo” condition exhibited the greatest proportion of PUFA (47.85 %).

Table 10: Rhodosporidium toruloides production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media

	Rhodosporidium toruloides								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control 1	4.875	5.657	1.933	12.22	n.a.	20.35	74.82	4.83	11.44
10 % hydr.+co	0.459	3.285	1.209	3.25	25.597	43.36	12.55	44.10	36.14
20 % hydr.+co	0.243	3.100	0.483	5.26	19.083	42.25	11.22	46.53	31.00
30 % hydr.+co	0.399	3.806	0.566	8.13	26.528	44.31	11.50	44.19	29.27
40 % hydr.+co	0.226	0.801	0.399	6.78	8.675	41.17	13.25	45.58	29.94
50 % hydr.+co	0.471	1.157	0.678	10.79	10.336	42.50	16.49	41.02	13.86
control 2	3.346	2.617	3.176	19.41	n.a.	19.22	69.04	11.75	11.55
10 % hydr.+fo	0.316	3.255	0.488	14.84	1.223	16.16	37.31	46.53	30.00
20 % hydr.+fo	1.614	3.941	1.682	21.57	3.693	32.54	26.84	40.62	10.44
30 % hydr.+fo	1.730	3.196	2.188	23.60	3.555	24.32	30.03	45.65	16.11
40 % hydr.+fo	2.167	3.798	2.279	24.16	4.447	20.96	31.19	47.85	24.18
50 % hydr.+fo	0.602	2.342	1.175	13.54	1.391	26.22	31.49	42.30	8.20

5.1.1.4 Cystofilobasidium macerans (CCY 10-1-2)

The Table 11 presents the results of the cultivation of the yeast *Cystofilobasidium macerans*. Among the metabolites, the highest carotenoid content was observed in the “30 % hydr.+co” condition with 3.214 mg/g, while the maximum ubiquinone concentration was found in the “10 % hydr.+co” group at 6.408 mg/g. The highest ergosterol concentration was recorded in the “10 % hydr.+fo” condition, reaching 5.585 mg/g. For tocopherol, the “20 % hydr.+co” condition demonstrated the highest value of

40.178 mg/g. In terms of biomass production, the “20 % hydr.+fo” condition achieved the highest yield, with 27.65 g/l, indicating this combination is optimal for promoting growth in this yeast species.

Table 11: Cystofilobasidium macerans production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media

	Cystofilobasidium macerans								
Media	Carote-noids [mg/g]	Ubiqui-none [mg/g]	Ergoste-rol [mg/g]	Biomass [g/l]	Tocoph-erol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control 1	1.426	6.303	1.847	19.83	n.a.	44.87	50.28	4.85	16.23
10 % hydr.+co	1.254	6.408	2.514	12.06	31.711	43.55	9.90	46.55	45.00
20 % hydr.+co	0.778	2.884	1.720	11.42	40.178	43.55	10.46	46.00	41.53
30 % hydr.+co	3.214	5.279	4.426	14.21	14.504	43.50	10.79	45.71	41.23
40 % hydr.+co	1.532	3.185	2.820	11.08	24.324	39.69	12.85	47.47	23.70
50 % hydr.+co	2.678	4.399	4.074	12.00	12.086	40.38	10.49	49.14	30.00
control 2	1.818	3.218	2.710	26.57	n.a.	25.95	54.13	19.93	11.95
10 % hydr.+fo	0.803	1.421	5.585	17.13	1.094	12.23	66.79	20.98	24.64
20 % hydr.+fo	2.210	3.390	3.947	27.65	0.121	12.49	63.85	23.66	18.55
30 % hydr.+fo	0.598	1.745	2.919	28.04	0.229	15.55	57.99	26.47	13.35
40 % hydr.+fo	0.465	2.764	1.745	20.49	0.694	13.79	63.85	22.36	14.74
50 % hydr.+fo	0.452	3.461	4.499	23.96	0.536	18.46	51.79	29.76	8.50

In the context of lipid content and fatty acid composition, the “10 % hydr.+co” condition produced the highest total lipid content (45.00 %). The highest percentage of SFA was found in the control group (44.87 %), while the greatest proportion of MUFA was observed in the “10 % hydr.+fo” condition at 66.79 %. For PUFA, the “50 % hydr.+co” condition exhibited the highest percentage (49.14 %).

5.1.1.5 Sporidiobolus metaroseus (CCY 19-6-20)

Table 12 displays the results of the cultivation of the yeast *Sporidiobolus metaroseus*. Under control conditions, without the addition of hydrolysate, the yeast achieved relatively low levels of carotenoids (1.233 mg/g) and ergosterol (1.817 mg/g) but showed decent biomass production (20.83 g/l) and lipid content (14.40 %). The best results with coffee oil were observed at a 30 % hydrolysate concentration, where the highest carotenoid content (2.351 mg/g) was achieved, and in the “40 % hydr.+fo” condition, where the highest lipid content (36.49 %) was observed. A significantly increased content of ubiquinone (9.270 mg/g) and ergosterol (3.500 mg/g) was noted at a 10 % hydrolysate concentration also on this medium the highest content of PUFA was observed (48.83 %), indicating optimized synthesis of these metabolites at lower hydrolysate concentration, although, the highest MUFA (64.49 %) was observed on “20 % hydr.+co” medium.

When waste frying oil was used, the best results were obtained at higher hydrolysate concentrations. The highest carotenoid content (2.587 mg/g) was recorded at a 40 % hydrolysate concentration, while the maximum biomass production (20.830 g/l) was achieved at a 30 % hydrolysate concentration. The highest PUFA content (28.49 %) was observed at a 10 % hydrolysate concentration, while ergosterol and ubiquinone content peaked (3.836 mg/g and 11.249 mg/g respectively) under control conditions without hydrolysate.

Table 12: Sporidiobolus metaroseus production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media

	Sporidiobolus metaroseus								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control 1	1.233	2.560	1.817	20.83	n.a.	43.89	51.87	4.24	14.40
10 % hydr.+co	1.633	9.270	3.500	11.10	9.796	38.03	13.15	48.83	20.13
20 % hydr.+co	0.782	2.290	2.310	13.26	22.998	13.91	64.49	21.60	20.61
30 % hydr.+co	2.351	3.829	3.670	7.06	5.584	43.50	11.85	44.65	36.43
40 % hydr.+co	1.431	4.585	2.725	9.90	23.934	43.70	10.56	45.74	36.49
50 % hydr.+co	0.327	6.897	1.904	8.70	15.447	40.46	13.26	46.28	22.83
control 2	0.499	11.249	3.836	11.09	n.a.	17.47	58.99	23.55	7.01
10 % hydr.+fo	0.587	0.823	1.011	17.37	0.075	13.15	58.36	28.49	16.30
20 % hydr.+fo	1.601	3.430	2.821	19.14	0.699	19.98	62.35	17.67	22.14
30 % hydr.+fo	1.675	2.074	0.025	20.83	1.042	14.45	57.36	28.19	15.59
40 % hydr.+fo	2.587	1.812	0.036	20.25	0.414	14.03	58.35	27.63	16.42
50 % hydr.+fo	1.494	2.494	2.301	18.54	0.357	15.95	57.77	26.29	11.00

5.1.1.6 *Rhodotorula mucilaginosa (20-9-7)*

Table 13 provides the results of growth and metabolite production for the yeast *Rhodotorula mucilaginosa* (20-9-7). Under control conditions, the highest lipid content (79.00 %) was achieved, along with a high ubiquinone content (8.016 mg/g) and the greatest biomass increase (24.74 g/l), indicating a high baseline production of these metabolites and biomass.

Table 13: Rhodotorula mucilaginosa (20-9-7) production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media

	Rhodotorula mucilaginosa (20-9-7)								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control 1	1.350	8.016	3.461	24.74	n.a.	51.55	12.15	36.30	79.00
10 % hydr.+co	2.063	1.288	3.149	23.30	2.544	51.42	12.90	35.68	40.00
20 % hydr.+co	1.177	1.923	2.486	12.40	3.477	22.54	45.64	31.82	16.25
30 % hydr.+co	1.138	2.145	2.901	14.35	5.463	34.15	25.47	40.38	18.25
40 % hydr.+co	1.366	1.772	2.724	14.13	3.799	29.17	29.34	41.49	10.72
50 % hydr.+co	0.344	2.381	0.834	20.89	8.109	41.94	18.15	39.91	15.00
control 2	2.872	5.091	4.108	20.25	n.a.	19.16	61.22	19.63	10.30
10 % hydr.+fo	1.012	2.901	3.268	12.48	0.285	20.46	36.85	42.69	10.34
20 % hydr.+fo	1.484	2.304	2.636	22.52	0.022	16.15	36.32	47.53	16.08
30 % hydr.+fo	1.572	4.196	3.027	15.54	0.096	15.77	38.80	45.44	8.35
40 % hydr.+fo	1.141	2.166	3.020	12.14	0.045	18.46	36.65	44.88	12.00
50 % hydr.+fo	1.209	2.629	2.590	17.25	n.a.	19.16	35.46	45.39	6.18

With the use of coffee oil, the highest concentration of carotenoids (2.063 mg/g) was observed at a 10 % hydrolysate concentration, while the maximum tocopherol production (8.109 mg/g) was recorded at a 50 % hydrolysate concentration. The highest ergosterol content (3.149 mg/g) and relatively high biomass (23.30 g/l) were also observed at a 10 % hydrolysate concentration. When waste frying oil was used, the best results were achieved at different hydrolysate concentrations depending on the target metabolites. The highest carotenoid (2.872 mg/g) and ergosterol (4.108 mg/g) contents were recorded under control conditions without hydrolysate, while the maximum ubiquinone production (4.196 mg/g) was reached at a 30 % hydrolysate concentration. The highest biomass (22.52 g/l) and PUFA content (47.53 %) were observed at a 20 % hydrolysate concentration, suggesting optimal conditions for growth and the synthesis of these lipids and bioactive compounds.

5.1.1.7 Comparision of yeasts strains

Figure 15 shows the biomass growth results of various yeast strains. The strain *SP* with “10 % hydr.+co” shows an outstanding result of 31.12 g/l. For *RM* (19-4-6), the highest biomass is observed with “20 % hydr.+co”, yielding 24.43 g/l, while the *RT* strain achieves its peak (24.16 g/l) under the “40 % hydr.+fo” condition. The *CM* strain records the highest biomass at 28.04 g/l with “30 % hydr.+fo”, and similarly, *SM* reaches its maximum biomass growth of 20.83 g/l under the same condition. Finally, *RM* (20-9-7) shows the highest biomass of 24.74 g/l in the control condition.

A clear trend can be seen where certain strains, particularly *SP* and *CM*, perform better under lower hydrolysate concentrations (10-30 %) combined with either coffee oil or frying oil, while other strains like *RM* (19-4-6) and *RT* show enhanced biomass growth at higher hydrolysate concentrations, particularly with frying oil. This indicates that the optimal media composition for biomass production is strain-dependent, and careful selection of both hydrolysate concentration and oil type is essential to maximizing yield.

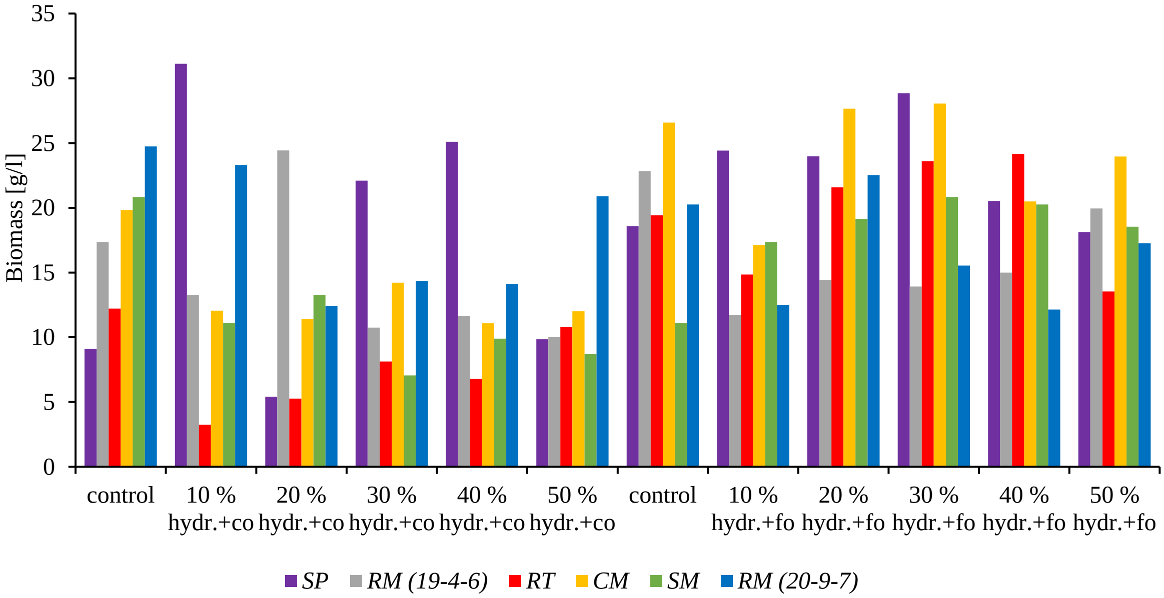


Figure 15: Comparison of biomass growth of yeast strains described in the previous chapters

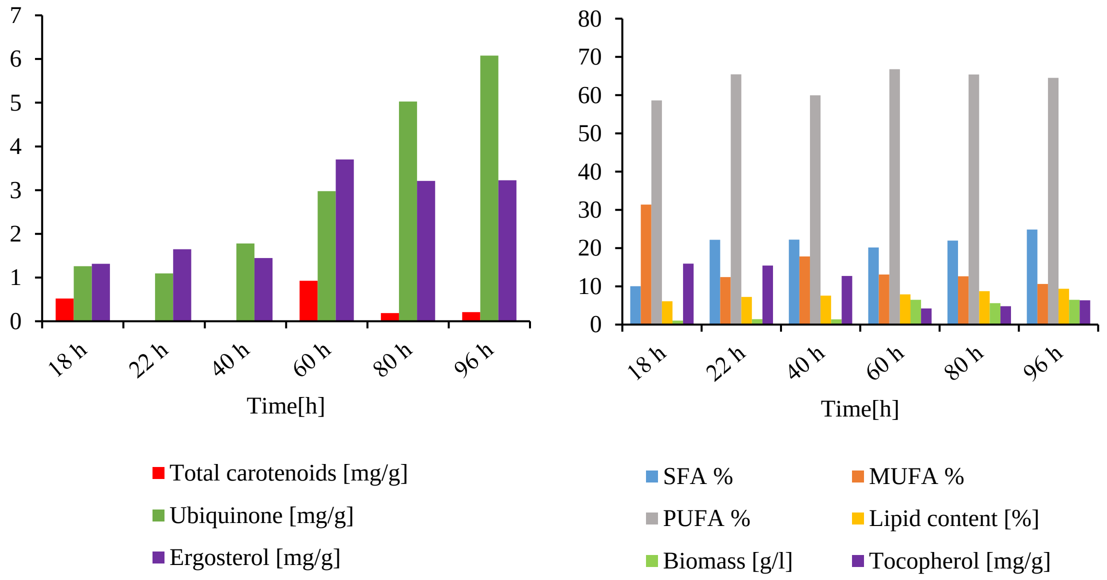
5.1.1.8 Sporidiobolus pararoseus (CCY 19-9-6) bioreactor cultivation

The cultivation was performed in a 2.2 l bioreactor. The composition of the bioreactor medium is shown in Table 14. The strain *Sporidiobolus pararoseus* with coffee oil and a 10 % addition of coffee hydrolysate was selected, as this cultivation showed the highest biomass increase among the screening cultivations.

Table 14: Bioreactor media composition.

Substance	**Amount/L of media**
KH_2PO_4	8 g
$MgSO_4 \cdot 7H_2O$	1.4 g
Urea	3.62 g
Coffee oil	42.4 g
Cofee hydrolysate	10 %

Figures 16 presents the growth dynamics and metabolite production of the yeast *Sporidiobolus pararoseus* in a bioreactor at various time intervals. Throughout the cultivation period, changes in cellular metabolite composition and biomass accumulation were observed. After 60 hours of cultivation, the yeast reached its highest carotenoid concentration (0.925 mg/g), suggesting that this time point represents optimal conditions for carotenoid synthesis. The ubiquinone content steadily increased, reaching its peak (6.079 mg/g) after 96 hours of cultivation, indicating enhanced production of this antioxidant metabolite over prolonged cultivation periods. Ergosterol content also showed a gradual increase, peaking after 60 hours (3.704 mg/g).



Figures 16: Sporidiobolus pararoseus production of various lipidic antioxidants, accumulation of lipids, and biomass growth in bioreactor condition

Biomass reached its maximum after 96 hours (6.50 g/l), demonstrating a gradual growth trajectory of the yeast in the bioreactor under extended cultivation conditions. The tocopherol content initially decreased, from the initial highest accumulation (15.924 mg/g), to 60th hour but then progressively increased, attaining a high level (6.338 mg/g) by 96 hours, suggesting a rise in antioxidant compounds with extended cultivation time. The proportion of polyunsaturated fatty acids was highest after 60 hours (66.76 %). Lipid content also showed a steady increase, peaking at 9.36 % after 96 hours, which implies that as biomass increases, there is a concurrent accumulation of lipid metabolites.

5.1.1.9 Rhodosporidium toruloides (CCY 62-2-4) bioreactor cultivation

On the same medium as in Table 14 was performed another cultivation in bioreactor with the yeast *Rhodosporidium toruloides*. At the early stages of cultivation (8 to 24 hours), *Rhodosporidium toruloides* exhibits high carotenoid production, with a peak at 8 hours (10.714 mg/g). Ubiquinone levels also reach a significant early peak (5.135 mg/g at 8 hours), and the biomass rapidly increases from 2.59 g/l to 6.63 g/l by 24 hours, indicating a strong initial growth phase. During this period, the lipid profile is characterized by a relatively high PUFA content, peaking at 53.94 % at 24 hours, suggesting robust fatty acid desaturation activity. The highest lipid accumulation peaked in 56th hour with value 25.38 %. The tocopherol accumulation peaked in 40th hour with the value 19.706 mg/g.

As the cultivation progresses into the mid-phase (40 to 72 hours), there is a notable fluctuation in metabolite production. Ergosterol and carotenoid concentrations generally increase, reaching secondary peaks at 144 hours (9.783 mg/g for ergosterol) and (10.757 mg/g for carotenoids). Biomass continues to rise steadily, achieving a maximum of 15.19 g/l at 120 hours.

The lipid content shows variability throughout the cultivation, with a general trend toward increased SFA and a decrease in PUFA percentages after 56 hours, reflecting shifts in lipid metabolism and cellular adaptation to prolonged cultivation conditions. By the end of the cultivation period (168 hours), there is a stabilization of biomass at 14,82 g/l and lipid profiles, indicating that *Rhodosporidium toruloides* has reached a balanced metabolic state under the provided bioreactor conditions.

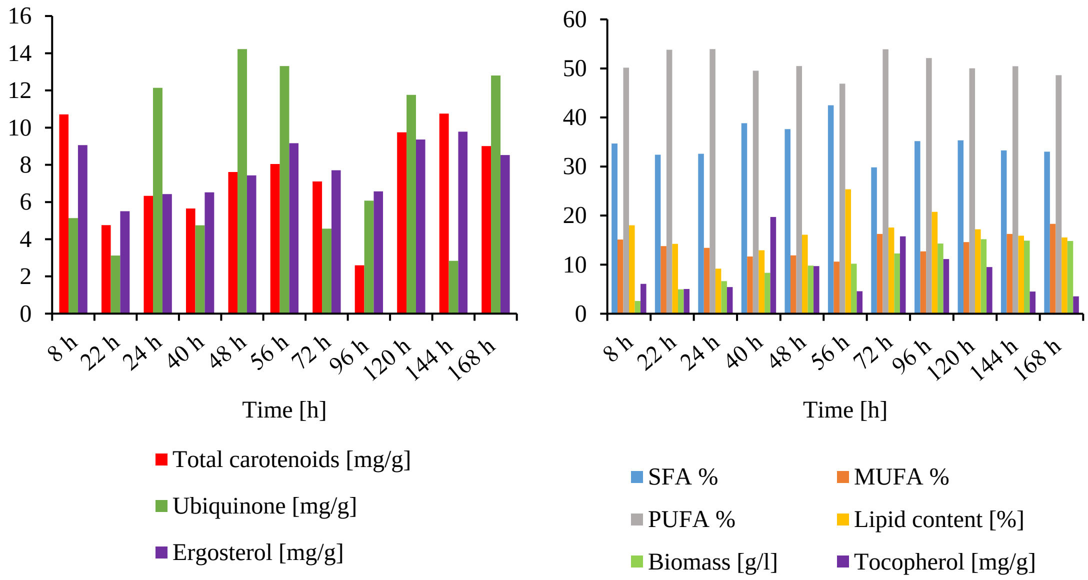


Figure 17: Rhodosporidium toruloides production of various lipidic antioxidants, accumulation of lipids, and biomass growth in bioreactor condition

5.1.2 Cultivation on waste lipid substrates in combination with glycerol

Cultivation of yeast on lipidic substrates in combination with glycerol, where a glycerol medium served as the control, was conducted on the following strains: *Rhodotorula mucilaginosa* (19-4-6) (CCY 19-4-6); *Rhodotorula (Rhodosporidium) toruloides* (CCY 62-2-1); *Cystofilobasidium macerans* (CCY 10-1-2); *Sporidiobolus pararoseus* (CCY 19-9-6); *Sporidiobolus metaroseus* (CCY 19-9-20); and *Rhodotorula kratochvilovae* (CCY 20-2-26). The composition of the lipidic components was variable, as the effect of adding mixtures of different lipidic substrates to the medium was tested. These mixtures were prepared prior to their addition to the medium by heating and continuous stirring for 10 minutes to ensure thorough mixing in a 50:50 ratio. The detailed description of each medium is provided in Table 15. The composition of the media and the amounts of substrates used are detailed in Table 16 and Table 17Table 17. The results of this chapter were partially published in: Szotkowski M., Plhalová Ž., Sniegoňová P., Holub J. et al., 2023. *Microorganisms*. *11*(4), 1013; DOI: 10.3390/microorganisms11041013.

Table 15: The content of carbonaceous substrates in the media and their naming (in the case of media containing combined greasy substrates, their 50:50 mixtures were used)

Medium	**Shortcut**
Control glycerol	control 1
Frying oil + Glycerol 10 %	FoG10
Frying oil + Glycerol 25 %	FoG25
Coffee oil + Glycerol 10 %	CoG10
Coffee oil + Glycerol 25 %	CoG25
Animal fat + Glycerol 10 %	Fat+G10
Animal fat + Glycerol 25 %	Fat+G25
Frying oil + Animal fat + Glycerol 10 %	Fo+F+G10
Frying oil + Animal fat + Glycerol 25 %	Fo+F+G25
Coffee oil + Animal fat + Glycerol 10 %	Co+F+G10
Coffee oil + Animal fat + Glycerol 25 %	Co+F+G25
Control glycerol	control 2
Frying oil	Fo
Coffee oil	Co
Animal fat	Fat
Frying oil + Animal fat	Fo+Fat
Coffee oil + Animal fat	Co+Fat
Control glycerol with induction of inoculum	control 3
Frying oil with induction of inoculum	Fo (I)
Coffee oil with induction of inoculum	Co (I)
Animal fat with induction of inoculum	Fat (I)
Frying oil + Animal fat with induction of inoculum	Fo+F (I)
Coffee oil + Animal fat with induction of inoculum	Co+Fat (I)

Table 16: The composition of the mineral base media used in the experiment

Substance	**Amount [g/l]**
KH_2PO_4	4
$MgSO_4 \cdot 7H_2O$	0.696
$(NH_4)_2SO_4$	3.98

Table 17: Amount and types of used carbonaceous substrates dedicated for C/N ratio 25

Medium	weight [g/50 ml media]	
	Glycerol	**Lipidic substrate**
control	2.31	0
Glycerol 10 %	0.23	1.06
Glycerol 25 %	0.58	0.88
Pure lipidic media	0	1.18

5.1.2.1 *Sporidiobolus pararoseus (CCY 19-9-6)*

The Table 18 provides an overview of the metabolic responses and growth parameters of *Sporidiobolus pararoseus* when cultivated in media supplemented with different oil and fat sources frying oil (Fo), coffee oil (Co), and waste animal fat (Fat or F) along with varying concentrations of glycerol (G, 10 % or 25 %). In control medium, the yeast achieved moderate levels of carotenoids (3.664 mg/g) and high ergosterol content (10.946 mg/g), with minimal lipid accumulation (5.34 %). When on medium FoG10, there was a significant increase in biomass (11.8 g/l) and a notable shift towards higher PUFA content (54.54 %) compared to control conditions.

Table 18: Sporidiobolus pararoseus production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with glycerol portion

	Sporidiobolus pararoseus frying oil/coffee oil/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	3.664	0.721	10.946	2.00	n.a.	10.08	78.46	11.46	5.34
FoG10	1.509	2.335	3.703	11.80	n.a.	11.22	34.24	54.54	21.38
FoG25	0.256	0.506	2.942	2.10	0.058	13.29	38.41	48.30	45.94
CoG10	1.772	2.032	4.263	5.60	13.971	71.67	5.80	22.53	80.22
CoG25	0.370	10.041	3.498	5.00	6.257	62.73	8.34	28.93	42.72
Fat+G10	1.053	12.601	4.700	9.20	n.a.	44.90	43.38	11.72	52.47
Fat+G25	0.661	1.493	2.016	6.10	0.015	67.72	26.47	5.82	57.88
Fo+F+G10	0.344	1.240	2.239	4.00	0.011	37.82	36.91	25.26	48.38
Fo+F+G25	0.254	0.706	1.169	3.50	n.a.	64.71	22.25	13.03	50.75
Co+F+G10	0.694	7.461	3.000	6.30	0.210	63.88	20.94	15.18	54.53
Co+F+G25	0.684	5.817	5.022	5.10	0.510	64.93	19.87	15.20	51.67

Coffee oil medium with 10 % glycerol addition (CoG10) resulted in the highest lipid, tocopherol and SFA accumulation (80.22 %, 13.971 mg/g and 71.67 %, respectively), while “CoG25” led to the high ubiquinone concentration (10.041 mg/g). The use of waste animal fat with 10 % glycerol (Fat+G10) produced the highest levels of ubiquinone (12.601 mg/g), indicating a significant enhancement in antioxidant capacity under these conditions. The combination of waste animal fat and 25 % glycerol (Fat+G25) resulted in substantial lipid accumulation (57.88 %), predominantly in saturated and monounsaturated forms. Overall, the data indicate that the type of oil or fat source and the concentration of glycerol significantly influence the metabolic output and growth of *Sporidiobolus pararoseus*. Higher glycerol concentrations generally favour lipid production, while specific combinations optimize the synthesis of secondary metabolites such ergosterol, reflecting the yeast's adaptive metabolic flexibility in response to different carbon sources and nutrient availability.

The Table 19 provides an overview of the growth characteristics and metabolite production of *Sporidiobolus pararoseus* under various conditions involving different oils: frying oil (Fo), coffee oil (Co), and waste animal fat (Fat or F) and combinations thereof, with some conditions including the induction of lipase by the addition of frying oil to the inoculum media indicated by (I). In the control conditions without any oil supplementation, the yeast showed a moderate biomass yield (2.00 g/l) with high ergosterol content (10.946 mg/g). When frying oil (Fo) was used, there was an increase in biomass (4.30 g/l) and lipid content (48.34 %), with a significant proportion of polyunsaturated fatty acids (46.51 %). The combination of coffee oil and waste animal fat (Co+Fat) led to higher biomass production (9.50 g/l) and a balanced lipid profile with a considerable PUFA content (27.06 %). The use of frying oil with induction "Fo (I)" resulted in the highest biomass yield (14.4 g/l) and a notable PUFA content (57.17 %), suggesting that lipase induction enhances both growth and specific metabolite production. The highest tocopherol and lipid cumulation were obtained from Co medium with values 8.893 mg/g and 62.63 % respectively.

Table 19: Sporidiobolus pararoseus production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with induction by Fo in I2 media

	Sporidiobolus pararoseus frying oil/coffee oil/animal fat+ inoculum induction								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	3.664	0.721	10.946	2.00	n.a.	10.08	78.46	11.46	5.34
Fo	0.678	3.280	5.241	4.30	0.179	15.39	38.09	46.51	48.34
Co	0.730	7.627	2.592	6.90	8.893	70.40	6.47	23.13	62.63
Fat	0.114	11.241	2.102	8.60	n.a.	49.82	41.73	8.45	39.74
Fo+Fat	1.574	2.581	3.724	3.90	0.396	73.68	16.26	10.06	62.38
Co+Fat	0.200	5.053	1.233	9.50	0.021	45.09	27.85	27.06	46.71
control	1.923	2.181	8.543	2.50	n.a.	14.08	36.87	49.05	19.95
Fo (I)	1.917	1.493	3.955	14.40	n.a.	10.81	32.03	57.17	29.13
Co (I)	0.584	21.141	4.885	7.50	6.814	61.76	8.53	29.72	56.80
Fat (I)	0.796	6.569	3.537	5.30	n.a.	42.07	44.78	13.14	40.09
Fo+F (I)	1.114	14.979	3.123	12.70	n.a.	27.05	41.39	31.56	41.14
Co+Fat (I)	0.300	4.751	1.443	6.10	0.242	73.76	14.58	11.66	51.50

When examining the effect of coffee oil with induction (Co (I)), a significant increase in ubiquinone content (21.141 mg/g) was observed, along with a high lipid accumulation (56.80 %) that was rich in polyunsaturated fatty acids (29.72 %). Additionally, the combination of frying oil and fat with induction

Fo+F (I) showed a substantial ubiquinone production (14.979 mg/g) and balanced lipid profile, highlighting the yeast's adaptive metabolic responses to various carbon sources and lipase induction strategies. Overall, the data indicate that the type of oil, fat, and the induction process significantly influence the metabolic pathway activation and growth parameters of *Sporidiobolus pararoseus*, with specific conditions promoting optimal production of desired metabolites such as ubiquinone, ergosterol, and lipids.

5.1.2.2 Rhodotorula mucilaginosa (CCY 19-4-6)

In Table 20 is presented the control condition, *Rhodotorula mucilaginosa* (19-4-6) exhibited moderate production of carotenoids (1.157 mg/g) and ergosterol (3.452 mg/g) with a high MUFA content (72.90 %) and low PUFA content (2.80 %), indicating a lipid profile rich in monounsaturated fats and a total lipid content of 7.03 %. When grown in media containing frying oil with 10 % glycerol (FoG10), the yeast showed a significant increase in PUFA content (50.51 %), reflecting a shift towards a more unsaturated fatty acid profile, but with lower carotenoid production (0.536 mg/g). The addition of coffee oil with 10 % glycerol (CoG10) resulted in the highest carotenoid production (5.263 mg/g) and a balanced lipid profile with substantial PUFA content (46.80 %), suggesting that this condition promotes both pigment synthesis and unsaturation of fatty acids. The "Co+F+G10" media resulted in the highest carotenoids production (7.114 mg/g). Media supplemented with waste animal fat and glycerol exhibited varied metabolic responses. The combination of waste animal fat and 25 % glycerol (Fat+G25) led to the highest biomass yield (8.60 g/l) and ergosterol content (7.587 mg/g), alongside a lipid profile dominated by MUFA (70.29 %). In contrast, the combination of coffee oil, waste animal fat, and 25 % glycerol (Co+F+G25) resulted in high carotenoid production (5.058 mg/g) and a substantial PUFA content (26.77 %), indicating a favourable condition for both carotenoid synthesis and fatty acid desaturation. These findings highlight how the type and combination of oils, fats, and glycerol concentrations significantly influence the metabolic pathways and lipid profiles of *Rhodotorula mucilaginosa* (19-4-6).

Table 20: Rhodotorula mucilaginosa (19-4-6) production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with glycerol portion

	Rhodotorula mucilaginosa (19-4-6) frying oil/coffee oil/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	1.157	3.526	3.452	8.50	n.a.	24.30	72.90	2.80	7.03
FoG10	0.536	9.180	2.802	3.80	n.a.	11.05	38.44	50.51	11.71
FoG25	0.253	4.701	3.688	7.40	n.a.	11.71	63.41	24.88	4.38
CoG10	5.263	6.661	5.340	4.40	1.951	31.21	21.99	46.80	7.65
CoG25	1.354	0.927	4.110	6.50	n.a.	16.85	49.39	33.76	4.91
Fat+G10	3.191	5.621	5.049	4.40	n.a.	11.77	68.43	19.80	3.99
Fat+G25	6.436	5.435	7.587	8.60	n.a.	11.54	70.29	18.18	3.85
Fo+F+G10	0.944	0.167	4.769	4.30	0.419	22.90	42.92	34.18	13.39
Fo+F+G25	n.a.	6.478	4.231	7.30	n.a.	9.08	58.31	32.62	5.91
Co+F+G10	7.114	0.117	5.085	5.60	0.054	23.46	42.12	34.42	5.55
Co+F+G25	5.058	0.128	6.014	7.00	0.046	17.39	55.84	26.77	4.61

Under control conditions without lipase induction, *Rhodotorula mucilaginosa* (19-4-6) exhibited high carotenoid production (4.826 mg/g) and moderate levels of ergosterol (4.058 mg/g) with a biomass yield of 10.10 g/l. The lipid profile was predominantly composed of MUFA (70.45 %) with a low PUFA content (3.13 %), and the total lipid content was relatively low at 6.51 %. When frying oil was added (Fo), there was a significant increase in PUFA content (48.85 %) and total lipid content (20.17 %), indicating enhanced fatty acid desaturation. The presence of coffee oil (Co) led to a substantial increase in ubiquinone (6.081 mg/g), ergosterol (5.919 mg/g) and tocopherol (4.422 mg/g) levels, suggesting a boost in antioxidant capacity, although the overall biomass yield was lower (2.40 g/l), as is presented in Table 21.

In control medium the highest biomass (11.10 g/l) and MUFA (72.13 %) was achieved by the *Rhodotorula mucilaginosa* (19-4-6). The conditions involving lipase induction (indicated by "I") resulted in different metabolic profiles. For instance, the combination of coffee oil and lipase induction "Co (I)" resulted in the highest ubiquinone concentration (9.384 mg/g) and significant carotenoid production (2.645 mg/g), reflecting an increase in both antioxidant and pigment synthesis. The addition of waste animal fat with lipase induction "Fat (I)" showed a balanced lipid profile with a moderate PUFA content (29.08 %) and high MUFA content (51.31 %), but with a lower biomass yield (3.90 g/l). The combination of coffee oil and waste animal fat with lipase induction "Co+F (I)" yielded the highest, except control, carotenoid production (3.561 mg/g) and a substantial ergosterol content (4.631 mg/g), indicating favourable conditions for the synthesis of valuable metabolites. Overall, the data demonstrate that the type of oil or fat, glycerol concentration, and lipase induction significantly influence the growth and metabolic output of *Rhodotorula mucilaginosa* (19-4-6), optimizing the production of specific metabolites and modulating lipid profiles.

Table 21: Rhodotorula mucilaginosa (19-4-6) production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with induction by Fo in I2 media

	Rhodotorula mucilaginosa (19-4-6) frying oil/coffee oil/animal fat+ inoculum induction								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	4.826	0.779	4.058	10.10	n.a.	26.42	70.45	3.13	6.51
Fo	0.615	2.175	3.324	3.50	n.a.	10.90	40.25	48.85	20.17
Co	1.911	6.081	5.919	2.40	4.422	37.76	20.57	41.67	9.93
Fat	1.112	6.437	5.851	2.60	n.a.	25.47	57.34	17.20	6.34
Fo+Fat	0.571	7.358	5.227	3.20	n.a.	23.19	44.04	32.77	13.93
Co+Fat	2.733	0.255	5.306	2.30	0.065	37.07	34.04	28.89	10.21
control	4.091	2.330	3.829	11.10	n.a.	20.71	72.13	7.17	5.99
Fo (I)	1.143	0.886	4.189	4.20	0.192	9.60	42.65	47.75	9.10
Co (I)	2.645	9.384	5.296	2.60	5.709	33.31	21.35	45.34	8.83
Fat (I)	2.345	7.279	5.211	3.90	n.a.	19.61	51.31	29.08	5.14
Fo+F (I)	0.299	5.781	5.359	2.70	0.069	20.45	45.65	33.90	7.28
Co+Fat (I)	3.561	5.987	4.631	5.10	0.031	44.66	26.37	28.96	12.97

5.1.2.3 *Rhodosporidium toruloides (CCY 62-2-4)*

The Table 22 provides an in-depth analysis of metabolite content, biomass production, lipid percentages, and fatty acid composition in *Rhodotorula toruloides* grown on media containing frying oil, coffee oil, animal fat, and glycerol. The highest concentration of carotenoids (11.454 mg/g) is observed in the "Fo+F+G25" medium, while the "Co+F+G25" medium shows the most elevated ubiquinone levels (9.532 mg/g). Ergosterol production reaches its peak (7.758 mg/g) in the "Fat+G10" medium. Biomass yield is highest in the control medium (12.19 g/l), surpassing all glycerol-containing media. However, significant tocopherol production (16.899 mg/g) is noted only in CoG10, highlighting a strong association between coffee oil and tocopherol synthesis.

In terms of lipid accumulation, the "Co+F+G10" medium achieves the highest lipid content (18.01 %), suggesting that a combination of coffee oil, frying oil, and animal fat supports enhanced lipid production. Regarding the fatty acid profile, the highest percentage of saturated fatty acids is found in CoG10" medium (40.32 %), while the "FoG25" medium has the most elevated monounsaturated fatty acids content (66.04 %). Polyunsaturated fatty acids are most abundant (42.97 %) in the "CoG10" medium, indicating a significant lipid variation depending on the media composition (Szotkowski M., Plhalová Ž., Sniegoňová P., Holub J. et al., 2023. *Microorganisms*. *11*(4), 1013; DOI: 10.3390/microorganisms11041013 – see attachment 1, pg. 11-14).

Table 22: Rhodosporidium toruloides production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with glycerol portion

	Rhodosporidium toruloides frying oil/coffee oil/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	5.288	1.585	2.461	12.19	n.a.	38.78	58.14	3.08	14.36
FoG10	2.987	1.363	6.059	6.41	n.a.	8.90	64.59	26.51	9.70
FoG25	6.179	1.214	4.667	8.76	n.a.	9.57	66.04	24.39	13.17
CoG10	4.544	3.741	4.464	5.59	16.899	40.32	16.72	42.97	15.90
CoG25	5.957	2.560	5.528	6.74	6.803	36.33	22.45	41.21	14.56
Fat+G10	8.110	1.289	7.758	5.40	0.389	11.60	55.88	32.52	6.13
Fat+G25	6.334	1.340	6.195	8.20	n.a.	18.53	59.73	21.74	8.63
Fo+F+G10	8.799	1.772	7.134	5.40	n.a.	10.15	52.56	37.29	6.89
Fo+F+G25	11.454	0.608	6.854	8.20	n.a.	14.69	55.33	29.99	8.49
Co+F+G10	7.576	1.889	7.114	5.70	0.022	37.55	32.31	30.14	18.01
Co+F+G25	10.912	9.532	7.739	8.00	n.a.	34.22	36.45	29.32	16.24

The Table 23 summarizes the effects of inoculum induction and different media compositions on metabolite production, biomass, lipid content, and fatty acid profiles in *Rhodosporidium toruloides*. The highest carotenoid concentration (9.323 mg/g) is observed in the "Fat (I)" medium, which also exhibits the largest ubiquinone production (4.917 mg/g) and the highest ergosterol concentration (8.644 mg/g). Biomass production peaks (12.20 g/l) in the control medium, followed closely by the control medium with inoculum induction (12.00 g/l). Tocopherol is only detectable in the "Co+Fat" (0.424 mg/g), "Co (I)" (5.636 mg/g), and Fo (I) (0.089 mg/g) media, with the most significant value in "Co (I)".

Table 23: Rhodosporidium toruloides production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with induction by Fo in I2 media

	Rhodosporidium toruloides frying oil/coffee oil/animal fat+ inoculum induction								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	6.684	2.086	2.318	12.20	n.a.	43.81	54.05	2.14	14.90
Fo	1.250	1.189	4.703	2.90	0.130	9.03	42.99	47.98	9.68
Co	6.827	0.613	7.080	1.50	0.365	23.77	21.37	54.86	7.17
Fat	4.474	1.541	7.097	3.10	n.a.	22.64	53.93	23.43	6.81
Fo+Fat	4.151	2.181	7.488	3.00	n.a.	12.35	49.23	38.42	4.79
Co+Fat	7.706	1.032	7.720	3.00	0.424	29.79	36.00	34.22	9.44
control (I)	4.505	2.310	2.987	12.00	n.a.	33.79	62.64	3.57	7.81
Fo (I)	1.534	2.797	5.066	3.30	0.089	6.40	42.72	50.88	9.71
Co (I)	6.966	4.606	6.558	3.70	5.636	31.74	19.79	48.47	10.04
Fat (I)	9.323	4.917	8.644	4.00	n.a.	9.55	52.57	37.88	6.50
Fo+F (I)	7.158	1.432	8.023	4.20	n.a.	8.31	54.48	37.21	5.40
Co+Fat (I)	8.206	1.131	8.393	3.40	n.a.	12.17	46.33	41.49	4.83

In terms of lipid content, the highest percentage is observed in the control medium (14.90 %), followed by “Fat (I)” (9.68 %) and “Fo (I)” (9.71 %). Regarding fatty acid composition, the control medium exhibits the highest saturated fatty acids percentage (43.81 %), while “Co (I)” has the second highest SFA content (31.75 %). Monounsaturated fatty acids peak (62.64 %) in the control medium with inoculum induction, and the “Co” medium displays the most abundant polyunsaturated fatty acids at 54.86 % (Szotkowski M., Plhalová Ž., Sniegoňová P., Holub J. et al., 2023. *Microorganisms*. *11*(4), 1013; DOI: 10.3390/microorganisms11041013 – see attachment 1, pg. 11-14).

5.1.2.4 Cystofilobasidium macerans (CCY 10-1-2)

The Table 24 provides a detailed overview of the metabolite production, biomass yield, lipid content, and fatty acid composition of *Cystofilobasidium macerans* grown in various media combinations, including frying oil, coffee oil, animal fat, and glycerol. Among the metabolites, the highest carotenoid concentration (3.340 mg/g) is observed in the “Fat+G10” medium, whereas ubiquinone production peaks (4.936 mg/g) in the “CoG25” medium. Ergosterol synthesis is most pronounced in the Fat+G10 medium (4.203 mg/g). Biomass production reaches its maximum in the “FoG25” medium (10.37 g/l), and tocopherol is significantly present only in the “CoG25” (4.438 mg/g) and “CoG10” (1.398 mg/g) media.

In terms of lipid content, the “CoG25” medium exhibits the highest lipid percentage (36.97 %), suggesting that the combination of coffee oil and glycerol promotes enhanced lipid accumulation. Regarding the fatty acid profile, the “CoG25” medium has the highest saturated fatty acid content (31.79 %), while the control medium displays the highest MUFA (59.30 %). Polyunsaturated fatty acids are most abundant in the “CoG10” medium (54.26 %), indicating a significant influence of coffee oil and glycerol on the production of unsaturated fatty acids.

Table 24: Cystofilobasidium macerans production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with glycerol portion

	Cystofilobasidium macerans frying oil/coffee oil/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	1.762	0.641	2.635	6.10	n.a.	24.07	59.30	16.64	12.19
FoG10	0.490	0.865	2.416	7.53	0.112	13.08	57.38	29.54	25.32
FoG25	1.045	1.266	1.566	10.37	0.372	15.68	57.29	27.03	23.19
CoG10	0.979	1.234	3.384	6.16	1.398	31.45	14.29	54.26	32.29
CoG25	0.570	4.936	2.215	8.28	4.438	31.79	16.65	51.56	36.97
Fat+G10	3.340	0.948	4.203	3.40	n.a.	16.66	46.84	36.50	11.65
Fat+G25	0.941	0.451	3.163	5.90	n.a.	21.34	46.26	32.40	13.94
Fo+F+G10	2.748	1.568	3.529	8.70	n.a.	14.08	42.33	43.59	29.01
Fo+F+G25	2.011	0.631	2.638	8.60	0.255	17.38	41.78	40.84	31.30
Co+F+G10	1.156	0.963	3.028	5.90	0.004	19.56	35.63	44.81	22.64
Co+F+G25	1.253	1.198	3.243	8.70	0.054	22.53	35.35	42.13	17.66

The Table 25 presents an evaluation of *Cystofilobasidium macerans* production abilities under various media compositions with inoculum induction. The highest carotenoid concentration (7.548 mg/g) is observed in the “Fat (I)” medium, which also exhibits substantial ubiquinone (4.100 mg/g) and ergosterol (4.768 mg/g) production. The tocopherol content peaks (2.131 mg/g) in the “Co” medium, highlighting the influence of coffee oil on tocopherol synthesis. The highest biomass yield (7.00 g/l) is recorded in the control medium, though many inoculum-induced conditions also show relatively high biomass yields. In terms of lipid content, the highest percentage is observed in the “Fo (I)” (44.58 %).

Table 25: Cystofilobasidium macerans production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with induction by Fo in I2 media

	Cystofilobasidium macerans frying oil/coffee oil/animal fat+ inoculum induction								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	1.702	1.084	2.266	7.00	n.a.	23.12	63.67	13.21	13.79
Fo	1.286	1.697	1.875	6.80	n.a.	8.01	34.78	57.20	20.47
Co	2.172	1.240	2.692	4.50	2.131	24.96	11.27	63.77	28.09
Fat	3.330	0.394	3.846	2.80	n.a.	30.08	40.09	29.83	9.53
Fo+Fat	3.020	0.595	3.159	6.40	0.005	14.02	42.01	43.97	16.37
Co+Fat	2.232	1.636	2.854	4.30	0.325	23.85	32.25	43.91	24.79
control	2.239	10.715	3.643	6.80	n.a.	21.58	37.31	41.11	14.82
Fo (I)	0.355	1.592	0.982	6.30	0.057	7.59	35.92	56.49	44.58
Co (I)	1.845	1.999	3.015	5.60	1.757	25.45	13.32	61.23	22.24
Fat (I)	7.548	4.100	4.768	4.00	n.a.	19.99	43.85	36.15	18.56
Fo+F (I)	2.450	1.932	3.171	4.60	0.151	23.62	29.69	46.70	21.95
Co+Fat (I)	1.353	17.626	3.844	5.80	0.296	15.49	39.35	45.16	25.83

Regarding the fatty acid composition, the highest percentage of saturated fatty acids is found in the “Fat” medium (30.08 %), while the “Co” medium exhibits the highest polyunsaturated fatty acids content (63.77 %). Monounsaturated fatty acids are most prevalent in the control medium (63.67 %), suggesting a diverse range of fatty acid biosynthesis pathways depending on the media composition.

5.1.2.5 Sporidiobolus metaroseus (CCY 19-6-20)

The Table 26 presents data on *Sporidiobolus metaroseus* cultivated in various media incorporating frying oil, coffee oil, animal fat, and glycerol. The highest carotenoid concentration (3.607 mg/g) is found in the “Co+F+G10” medium, while the “Co+F+G25” medium exhibits the highest ubiquinone content (8.457 mg/g). Ergosterol production reaches its peak (3.876 mg/g) in the “Co+F+G10” medium, and biomass is most substantial (11.80 g/l) in the “Fo+F+G25” medium. Tocopherol is detected in several media, with the highest concentration (1.655 mg/g) in CoG25. In terms of lipid accumulation, the highest percentage of lipids (26.27 %) is observed in the “Co+F+G25” medium, followed by “Fo+F+G25” (24.46 %). As for the fatty acid composition, saturated fatty acids peak (32.07 %) in “Co+F+G25”, whereas monounsaturated fatty acids reach their highest percentage (53.53 %) in “Fat+G10.“ Polyunsaturated fatty acids are most abundant (58.56 %) in the “CoG10” medium, suggesting a significant impact of coffee oil on the accumulation of unsaturated fatty acids.

Table 26: Sporidiobolus metaroseus production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with glycerol portion

	Sporidiobolus metaroseus frying oil/coffee oil/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	2.313	1.593	2.507	6.70	n.a.	22.82	49.45	27.72	14.13
FoG10	2.217	1.590	2.455	8.10	0.059	9.83	35.47	54.71	22.57
FoG25	2.351	1.273	2.488	10.50	0.049	16.97	34.36	48.67	22.47
CoG10	2.465	1.004	2.493	6.80	0.961	28.54	12.90	58.56	21.23
CoG25	0.715	6.540	2.342	8.40	1.655	26.99	15.44	57.56	21.56
Fat+G10	3.415	1.073	2.947	8.50	0.038	23.01	53.53	23.45	18.84
Fat+G25	3.105	0.933	3.043	6.30	n.a.	23.13	41.94	34.93	14.24
Fo+F+G10	2.758	0.722	2.761	9.00	0.051	16.42	41.90	41.68	22.71
Fo+F+G25	2.200	0.850	2.346	11.80	n.a.	20.43	39.02	40.55	24.46
Co+F+G10	3.607	0.916	3.876	7.60	0.052	26.89	31.45	41.65	18.23
Co+F+G25	3.306	8.457	2.592	10.00	0.053	32.07	31.44	36.50	26.27

The Table 27 presents an analysis of *Sporidiobolus metaroseus* metabolite production, biomass yield, lipid content under various media conditions with inoculum induction. The highest carotenoid concentration (11.554 mg/g) is observed in the “Co+F (I)” followed by “Fo+F (I)” medium (11.354 mg/g). Ubiquinone production peaks significantly (14.991 mg/g) in the Fo (I) medium, while the “Co+F (I)” medium also exhibits the highest ergosterol concentration (9.105 mg/g). The “control (I)” medium with inoculum induction produces the highest biomass (10.80 g/l). Tocopherol content is most prominent in the “Co (I)” medium (4.174 mg/g).

Table 27: Sporidiobolus metaroseus production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with induction by Fo in I2 media

	Sporidiobolus metaroseus frying oil/coffee oil/animal fat+ inoculum induction								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	2.313	1.593	2.507	6.70	n.a.	22.82	49.45	27.72	14.13
Fo	2.086	9.880	3.833	2.70	1.558	10.76	32.41	56.83	9.16
Co	1.882	0.808	1.895	4.80	0.725	31.20	11.82	56.98	25.95
Fat	2.624	11.477	4.548	3.00	2.119	18.24	40.01	41.75	8.10
Fo+Fat	3.202	1.336	3.393	5.80	0.007	13.39	39.81	46.79	13.96
Co+Fat	2.760	1.029	2.867	6.40	0.017	25.69	29.47	44.84	16.91
control (I)	5.477	1.328	3.763	10.80	n.a.	27.41	60.42	12.17	11.14
Fo (I)	0.266	14.991	4.001	4.20	n.a.	8.89	36.97	54.15	19.36
Co (I)	10.385	0.244	7.191	3.10	4.174	29.32	17.49	53.18	11.35
Fat (I)	8.705	5.043	8.644	3.80	n.a.	9.92	52.44	37.65	5.08
Fo+F (I)	11.354	0.279	7.884	5.60	n.a.	6.68	44.36	48.95	7.83
Co+Fat (I)	11.554	3.222	9.105	3.30	0.738	12.04	41.84	46.11	6.14

In terms of lipid content, the “Co” medium exhibits the highest percentage of lipids (25.95 %), followed by the “Fo (I)” medium (19.36 %). The fatty acid profile shows that the highest SFA percentage is found in the Co medium (31.20 %), while the control medium without induction has the highest monounsaturated fatty acid percentage (60.42 %). The highest PUFA content is observed in the “Co” medium (56.98 %), which confirms that coffee oil has a positive effect on PUFA accumulation.

5.1.2.6 Rhodotorula kratochvilovae (CCY 20-2-26)

The Table 28 presents data on *Rhodotorula kratochvilovae* cultivated in various media containing frying oil, coffee oil, animal fat, and glycerol and analysing biomass properties. The highest carotenoid concentration (4.420 mg/g) is found in the control medium, while the “Fo+F+G10” medium shows the most elevated ubiquinone (9.262 mg/g) and ergosterol production (4.318 mg/g). Biomass production reaches its peak (12.50 g/l) in the “FoG25” medium, highlighting the positive effect of higher glycerol concentrations combined with frying oil. Tocopherol is detected in several media, with “CoG25” showing the highest content (0.096 mg/g).

Considering lipid accumulation, the “Fo+F+G10” medium achieves the highest lipid percentage (22.15 %), suggesting that the combination of frying oil, animal fat, and glycerol leads to enhanced lipid biosynthesis. The fatty acid composition reveals that the highest percentage of saturated fatty acids is observed in the “CoG25” medium (34.61 %), while monounsaturated fatty acids are most abundant in the “Fat+G10” medium (53.52 %). The PUFA content peaks in the “CoG10” medium (53.79 %), indicating that the yeast efficiently take up PUFA from the medium and successfully incorporate them into the cell biomass (Szotkowski M., Plhalová Ž., Sniegoňová P., Holub J. et al., 2023. *Microorganisms*. *11*(4), 1013; DOI: 10.3390/microorganisms11041013 – see attachment 1, pg. 9-11).

Table 28: Rhodotorula kratochvilovae production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with glycerol portion

	Rhodotorula kratochvilovae frying oil/coffee oil/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	4.420	1.719	2.787	9.50	n.a.	33.50	50.36	16.14	8.89
FoG10	0.202	4.754	3.594	4.90	n.a.	10.95	37.78	51.27	8.62
FoG25	0.003	6.808	3.336	12.50	n.a.	15.22	42.66	42.12	9.90
CoG10	1.933	0.720	3.771	5.30	0.092	29.61	16.60	53.79	13.60
CoG25	2.812	0.688	3.303	10.10	0.096	34.61	19.80	45.59	17.40
Fat+G10	2.821	0.159	3.381	4.30	n.a.	14.68	53.52	31.80	10.12
Fat+G25	1.912	4.738	3.804	6.10	n.a.	18.46	50.97	30.56	9.35
Fo+F+G10	2.808	9.262	4.318	12.00	n.a.	13.73	48.95	37.32	22.15
Fo+F+G25	1.468	8.000	3.417	10.40	n.a.	17.82	45.40	36.78	12.84
Co+F+G10	3.028	0.661	3.801	4.80	0.036	25.50	38.58	35.91	14.48
Co+F+G25	3.456	0.624	3.894	8.50	0.048	23.89	38.64	37.48	13.15

The Table 29 provides an analysis of *Rhodotorula kratochvilovae* grown in various media combinations. The highest carotenoid concentration (4.944 mg/g) is observed in the “Fat (I)” medium, while the “Fo+F (I)” medium shows the most elevated ubiquinone production (7.127 mg/g). Ergosterol levels peak (4.531 mg/g) in the “Co” medium, and biomass production reaches its maximum (14.30 g/l) in the “Fo+Fat” medium, indicating the positive impact of a combined lipid source on cell growth. Tocopherol is detected in several media, with the highest concentration (0.561 mg/g) in the “Co” medium.

Table 29: Rhodotorula kratochvilovae production of various lipidic antioxidants, accumulation of tocopherol and lipids, and biomass growth on different media contain oil/coffee oil/animal fat with induction by Fo in I2 media

	Rhodotorula kratochvilovae frying oil/coffee oil/animal fat+ inoculum induction								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
control	1.705	4.940	3.239	13.80	n.a.	29.56	46.77	23.67	8.06
Fo	0.399	2.030	2.987	3.80	0.457	11.05	37.15	51.80	31.74
Co	0.035	3.555	4.531	2.40	0.561	27.29	18.73	53.98	5.88
Fat	2.744	5.426	3.620	4.90	n.a.	23.32	51.16	25.52	15.81
Fo+Fat	2.183	0.934	3.607	14.30	0.111	13.05	47.98	38.98	25.48
Co+Fat	3.437	0.634	3.985	4.10	n.a.	19.38	38.60	42.02	8.40
control	2.210	1.054	3.457	12.60	n.a.	24.62	45.07	30.31	7.11
Fo (I)	0.209	2.400	2.846	6.70	0.381	7.82	40.36	51.82	10.52
Co (I)	3.058	3.481	4.462	3.50	0.184	22.86	24.90	52.24	6.68
Fat (I)	4.944	5.260	3.728	5.80	n.a.	28.26	46.46	25.28	16.37
Fo+F (I)	2.471	7.127	3.980	11.10	n.a.	10.22	47.10	42.68	14.50
Co+Fat (I)	3.334	5.504	3.990	4.50	0.005	22.81	35.01	42.18	10.78

Regarding lipid accumulation, the “Fo” medium exhibits the highest lipid content (31.74 %), indicating that frying oil combined with inoculum induction promotes lipid biosynthesis. The fatty acid composition reveals that SFA are most abundant (29.56 %) in the control medium, while monounsaturated fatty acids peak (51.16 %) in the “Fat” medium. Polyunsaturated fatty acids content is highest (53.98 %) in the “Co” medium (Szotkowski M., Plhalová Ž., Sniegoňová P., Holub J. et al., 2023. *Microorganisms*. *11*(4), 1013; DOI: 10.3390/microorganisms11041013 – see attachment 1, pg. 9-11).

5.1.2.7 Comparision of yeasts strains

The Figure 18 presents the biomass growth of six yeast strains, abbreviated as *SP*, *SM*, *CM*, *RM (19-4-6)*, *RT*, and *RK*, cultured in various media, including combinations of frying oil, coffee oil, animal fat, and glycerol. Among the *SP* strain, the “Fo (I)” medium results in the highest biomass production (14.40 g/l), followed by the “Fo+F (I)” medium (12.70 g/l), demonstrating the significant effect of frying oil in promoting growth. For the *SM* strain, the highest biomass (11.80 g/l) is observed in the “Fo+F+G25” medium, with control 3 also showing substantial growth (10.80 g/l), indicating that both combined lipid sources and a simpler medium can support robust growth. The *CM* strain achieves optimal biomass production in the “FoG25” medium (10.37 g/l), with the “Fo+F+G10” and “Co+F+G25” media also yielding high levels of growth (8.70 g/l each). For *RM (19-4-6)*, the highest biomass (11.10 g/l) is found in the control medium wit induction of inoculum.

In the *RT* strain, biomass production peaks in the control medium (12.20 g/l) although the biomass growth on pure lipidic substrates was distinguished by substantial lower growth, indicating that media consisting of glycerol supports the most growth. Finally, for *RK*, the “Fo+Fat” medium yields the highest biomass (14.30 g/l), followed closely by “FoG25” (12.50 g/l) (except the “control 2” medium with growth of 13.80 g/l), suggesting a favourable effect of glycerol and frying oil on biomass growth.

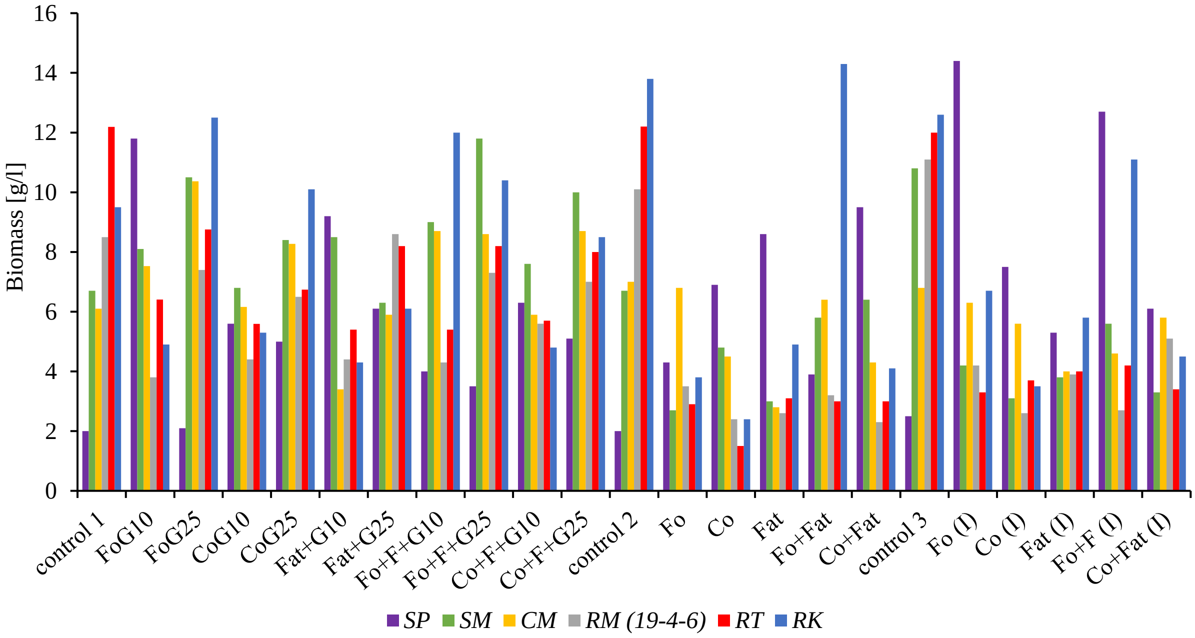


Figure 18: Comparison of biomass growth between different strains on various media

5.1.3 Cultivation on waste lipid substrates in combination with coffee hydrolysate and glycerol + cultivation on waste glycerol

This chapter focuses on waste lipidic and non-lipidic substrates, on which various genera of red yeasts were cultivated under laboratory conditions. The differences from the previous chapter lie in the use of a different nitrogenous substrate and, instead of animal fat, specifically poultry fat was used. The composition of the media and their descriptions are presented in

Table 30 and Table 31and C/N ratio is set to 25. Another difference is the addition of a microelement solution in the amount of 1 ml per liter of medium, the composition of which is found in Table 32.

Table 30: Mineral base of following experiments

Substance	Amount [g/l]
KH_2PO_4	4.000
$MgSO_4 \cdot 7\ H_2O$	0.696
KNO_3	6.090

Table 31: Carbonaceous substrate content in media. Fo – frying oil, CH – coffee hydrolysate, Pf – Poultry fat, WG- waste glycerol (in case of combination of greasy substrate with "CH", 10 % "CH" of total carbon was applied to the medium).

Medium name	Amount [g/50 ml of media]		Amount [ml/50 ml of media]	
	Glycerol	Lipidic substrate	Coffee hydrolysate	Waste glycerol
Control	2.31	-	-	-
Fo	-	1.18	-	-
Fo+G10 %	0.23	1.06	-	-
Fo+CH	-	1.06	2.67	-
Co	-	1.18	-	-
Co+G10 %	0.23	1.06	-	-
Co+CH	-	1.06	2.67	-
Pf	-	1.18	-	-
Pf+G10 %	0.23	1.06	-	-
Pf+CH	-	1.06	2.67	-
CH	-	-	26.7	-
WG	-	-	-	7.7

Table 32: Microelement solution composition added in amount of 1 ml per liter of media

Substance	Amount [g/l]
H_3BO_3	1.25
$ZnSO_4 \cdot 7\ H_2O$	1
$MnCl_2 \cdot 4\ H_2O$	0.82
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	0.5
$FeCl_3$	0.3
KI	0.25
$CuSO_4 \cdot 5\ H_2O$	0.1

5.1.3.1 *Sporidiobolus pararoseus (CCY 19-9-6)*

The Table 33 presents the production of various metabolites biomass by the red yeast *Sporidiobolus pararoseus* under different growth conditions. These conditions include the use of frying oil (Fo), coffee oil (Co), poultry fat (Pf), coffee hydrolysate (CH), and waste glycerol (WG) either alone or in combinations between themselves or with glycerol. Among the different conditions tested, the Co medium resulted into the highest production of carotenoids (4.163 mg/g) followed by the medium with the addition of coffee oil with 10 % glycerol (Co+G10 %) with value 3.906 mg/g. On the same medium, the highest ubiquinone (17.190 mg/g) and substantial ergosterol content (5.562 mg/g) was achieved. However, this condition resulted in a moderate biomass yield (6.150 g/l), indicating that while the metabolic output for certain metabolites was high, the overall biomass production was less significant compared to some other conditions. The condition with frying oil and 10 % glycerol (Fo+G10 %) led to the highest biomass yield (18.150 g/l) and a notable lipid content of 39.17 %, predominantly composed of polyunsaturated fatty acids (48.26 %), which suggests this medium is optimal for enhancing biomass and lipid accumulation.

In contrast, waste glycerol showed a lower biomass yield (5.650 g/l) but a high MUFA content (73.21 %), reflecting a lipid profile favoring monounsaturated fatty acids. Coffee hydrolysate (alone resulted in a moderate biomass yield (8.30 g/l) and a lipid profile heavily skewed towards MUFA (77.21 %), suggesting a strong influence on lipid unsaturation. The most notable PUFA and tocopherol content was observed on “Co+G10 %” medium (68.90 % and 0.093 mg/g respectively). These findings highlight the versatile metabolic responses of *Sporidiobolus pararoseus* to different carbon sources and additives.

Table 33: Results of the yeast Sporidiobolus pararoseus grown on various lipidic and non-lipidic substrates

	Sporidiobolus pararoseus Fo/ Co/poultry fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	2.107	7.522	5.420	7.050	n.a.	20.52	66.88	12.59	9.85
Fo	0.610	7.475	2.415	17.600	n.a.	12.60	42.80	44.60	49.37
Fo+G10 %	0.603	6.230	2.043	18.150	n.a.	11.33	40.41	48.26	39.17
Fo+CH	0.855	6.247	2.354	14.750	n.a.	11.27	41.14	47.60	42.06
Co	4.163	13.032	4.811	3.050	0.047	20.34	11.88	67.79	12.58
Co+G10 %	3.906	17.190	5.562	6.150	0.093	19.43	11.68	68.90	16.79
Co+CH	3.096	10.393	4.938	3.700	0.048	24.83	17.35	57.83	7.89
Pf	0.968	7.318	3.342	15.150	n.a.	20.73	52.52	26.75	32.01
Pf+G10 %	0.946	4.701	2.412	12.550	n.a.	20.11	49.60	30.29	26.00
Pf+CH	0.840	5.423	3.015	14.400	n.a.	21.83	53.27	24.90	37.43
CH	0.949	8.365	3.454	8.300	n.a.	12.79	77.21	10.00	10.93
WG	1.019	7.813	4.598	5.650	n.a.	15.69	73.21	11.09	11.97

5.1.3.2 *Rhodotorula mucilaginosa (19-4-6)*

The provided table (*Table 34*) presents the metabolic manifestation by the strain *Rhodotorula mucilaginosa* under different growth conditions. The highest carotenoid production was recorded in the control medium (4.804 mg/g), followed closely by the condition with waste glycerol at 4.710 mg/g. In contrast, the medium supplemented with coffee oil alone led to the highest ubiquinone production, reaching 11.991 mg/g, and also exhibited a notable tocopherol concentration (22.971 mg/g), which was the only medium showing significant tocopherol accumulation. The highest ergosterol content was observed in the "WG" condition (3.926 mg/g). Regarding biomass, the combination of coffee oil and coffee hydrolysate (Co+CH) resulted in the most substantial yield (15.90 g/l), suggesting a potent interaction between these carbon sources for biomass production.

Lipid accumulation varied significantly across the different media. The medium with frying oil resulted in the highest lipid content (39.15 %), with a substantial polyunsaturated fatty acid fraction (50.19 %). The combination of frying oil and coffee hydrolysate also showed a high PUFA content (50.90 %), though with a lower total lipid percentage (25.26 %). Interestingly, the highest monounsaturated fatty acid concentration (69.82 %) was observed in the medium with coffee hydrolysate alone, indicating a marked influence of this substrate on promoting MUFA-rich lipids.

Table 34: Results of the yeast Rhodotorula mucilaginosa grown on various lipidic and non-lipidic substrates

	Rhodotorula mucilaginosa (19-4-6) Fo/Co/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	4.804	4.989	3.849	8.50	n.a.	33.32	50.31	16.37	20.15
Fo	3.792	6.672	2.941	10.80	n.a.	13.37	36.44	50.20	39.15
Fo+G10 %	3.669	7.352	3.481	11.20	n.a.	15.74	37.95	46.31	36.84
Fo+CH	2.784	5.382	3.655	9.80	n.a.	13.00	36.10	50.90	25.26
Co	3.722	11.991	2.915	9.70	22.971	50.42	8.94	40.64	32.91
Co+G10 %	3.549	7.990	2.459	12.20	14.522	47.96	9.65	42.38	36.62
Co+CH	3.724	8.536	2.686	15.90	19.080	54.25	9.70	36.06	37.45
Pf	2.816	3.830	2.373	11.40	0.174	27.34	42.59	30.08	31.98
Pf+G10 %	3.465	4.800	2.793	12.90	n.a.	27.07	43.54	29.39	29.23
Pf+CH	3.031	4.442	2.926	13.70	n.a.	30.27	41.41	28.32	27.40
CH	2.526	2.688	3.729	13.50	0.061	13.07	69.82	17.11	5.09
WG	4.710	4.932	3.926	7.90	n.a.	31.77	53.39	14.84	18.49

5.1.3.3 *Rhodosporidium toruloides (CCY 62-2-4)*

The highest carotenoid content was observed in the medium containing coffee hydrolysate, reaching 3.230 mg/g, while the highest ubiquinone production (8.004 mg/g) was recorded in the waste glycerol condition. Regarding ergosterol synthesis, the "Co+CH" medium yielded the highest concentration at 6.186 mg/g. Biomass production was maximized in the control, "Pf", and "CH" media, all producing 12.40 g/l. Tocopherol accumulation, though low in general, was highest in the "Co+CH" medium (1.456 mg/g), suggesting the potential role of combined coffee oil and coffee hydrolysate in promoting tocopherol synthesis.

Table 35: Results of the yeast Rhodosporidium toruloides grown on various lipidic and non-lipidic substrates

	Rhodosporidium toruloides Fo/Co/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	1.295	5.996	2.757	12.40	n.a.	40.64	53.37	5.99	16.61
Fo	0.305	7.386	3.272	4.85	n.a.	14.07	39.96	45.96	31.54
Fo+G10 %	0.646	7.837	4.623	7.35	n.a.	14.75	42.23	43.02	29.47
Fo+CH	0.478	7.900	4.004	6.85	n.a.	13.63	40.66	45.71	28.61
Co	2.820	5.432	6.126	6.85	0.276	26.21	29.95	43.84	10.46
Co+G10 %	2.555	6.739	5.666	9.35	1.433	20.63	36.93	42.44	7.78
Co+CH	2.940	5.232	6.186	8.15	1.456	25.24	26.52	48.24	9.02
Pf	1.203	5.152	5.400	12.40	n.a.	25.00	46.68	28.33	29.85
Pf+G10 %	1.293	5.396	5.344	8.65	n.a.	24.08	45.27	30.64	19.91
Pf+CH	1.336	4.061	5.198	5.75	n.a.	21.96	38.94	39.10	10.06
CH	3.230	2.791	5.258	12.40	n.a.	17.74	58.90	23.36	5.34
WG	1.296	8.004	4.952	10.00	n.a.	38.63	53.19	8.17	15.35

Concerning lipid composition, the highest lipid content was achieved in the frying oil medium with a value of 31.54 %. Saturated fatty acid percentages peaked in the control condition (40.64 %) while the PUFA content remained low (5.99 %). The “CH” medium resulted in the highest monounsaturated fatty acid concentration (58.90 %), showcasing its strong influence on lipid unsaturation. The “Co+CH” condition had the highest polyunsaturated fatty acid content (48.24 %).

5.1.3.4 Cystofilobasidium macerans (CCY 10-1-2)

The Table 36 provides an analysis of *Cystofilobasidium macerans* cultivated under various conditions. The highest carotenoid production was observed in the Co medium (3.882 mg/g), while the most significant ubiquinone production occurred in the same medium, reaching 10.554 mg/g. Regarding ergosterol, the highest content was recorded in the “Fo+CH” medium (4.116 mg/g). The highest biomass yield was achieved in the “Co+CH” medium, producing 14.60 g/l. Tocopherol accumulation was limited but peaked in the “Co” medium, with 3.921 mg/g.

The lipid profile analysis shows that the highest lipid content was found in the Fo medium (43.13 %), accompanied by a high polyunsaturated fatty acid content of 46.32 %, while the highest PUFA content was observed on “Co+G10 %” medium (49.39 %). In terms of monounsaturated fatty acids, the “CH” medium demonstrated the highest concentration (69.68 %), while the “Co” medium exhibited the highest saturated fatty acid percentage (44.32 %), indicating distinct influences of these media on lipid composition.

Table 36: Results of the yeast Cystofilobasidium macerans grown on various lipidic and non-lipidic substrates

	Cystofilobasidium macerans Fo/Co/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	1.080	4.438	3.344	7.40	n.a.	30.29	40.03	29.69	15.00
Fo	1.972	7.402	2.679	7.70	n.a.	13.84	39.85	46.32	43.13
Fo+G10 %	1.704	8.637	2.901	8.20	n.a.	14.87	38.20	46.94	35.23
Fo+CH	3.560	6.956	4.116	7.85	n.a.	13.61	39.32	47.08	30.42
Co	3.882	10.554	3.633	7.30	3.921	44.32	9.64	46.04	27.08
Co+G10 %	3.516	9.373	3.560	9.60	3.331	38.67	11.95	49.39	26.01
Co+CH	1.972	8.778	3.164	14.60	0.253	41.33	11.07	47.61	31.52
Pf	2.019	7.107	3.654	7.60	0.130	24.86	38.15	37.00	22.99
Pf+G10 %	1.740	6.423	3.026	12.70	0.068	25.81	40.23	33.96	26.31
Pf+CH	2.107	7.153	3.481	9.85	0.066	23.78	39.15	37.08	19.55
CH	1.596	6.193	2.700	10.95	0.012	20.91	69.68	9.43	10.00
WG	2.799	4.308	3.277	6.75	n.a.	28.24	56.88	14.89	14.03

5.1.3.5 Sporidiobolus metaroseus (CCY 19-6-20)

Among the tested conditions presented in Table 37, the *Sporidiobolus metaroseus* strain exhibited on “Co” medium the highest carotenoid (3.360 mg/g) and ubiquinone (8.988 mg/g) production. The highest ergosterol content was found in the Co medium as well, reaching 3.877 mg/g, while the greatest biomass yield was observed in the “Pf” condition (9.80 g/l). Tocopherol production peaked in the “Co+CH” medium (2.467 mg/g).

Table 37: Results of the yeast Sporidiobolus metaroseus grown on various lipidic and non-lipidic substrates

	Sporidiobolus metaroseus Fo/Co/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	1.720	4.378	3.585	6.35	n.a.	27.48	32.24	40.28	16.06
Fo	0.509	6.630	1.807	4.15	n.a.	13.55	38.64	47.81	38.87
Fo+G10 %	0.712	4.284	2.651	4.50	n.a.	15.13	37.66	47.21	32.15
Fo+CH	1.783	8.523	2.850	4.90	n.a.	14.44	38.28	47.28	37.01
Co	3.360	8.988	3.877	3.20	1.249	30.38	12.32	57.30	21.27
Co+CH	2.042	7.761	2.821	6.80	2.467	33.60	10.57	55.83	31.58
Pf	1.273	7.326	3.218	9.80	0.010	24.60	40.58	34.82	31.09
Pf+G10 %	0.593	4.183	2.174	7.55	0.020	25.33	40.17	34.50	29.59
Pf+CH	1.885	6.377	3.373	7.55	n.a.	23.57	40.26	36.18	27.51
CH	2.946	5.725	2.983	7.45	n.a.	22.09	44.81	33.10	19.22
WG	1.015	3.633	3.314	5.45	n.a.	21.88	54.72	23.40	16.27

Lipid analysis reveals that the “Fo” medium resulted in the highest overall lipid content (38.87 %), with a PUFA content of 47.81 %, suggesting the strong potential of frying oil for lipid accumulation.

The “WG” medium exhibited the highest monounsaturated fatty acid content (54.72 %), whereas the “Co” medium led to the highest PUFA content (57.30 %). The saturated fatty acid content peaked in the “Co+CH” medium (33.60 %), indicating that coffee oil and coffee hydrolysate combinations affect the fatty acid composition of *Sporidiobolus metaroseus*.

5.1.3.6 Rhodotorula kratochvilovae (CCY 20-2-26)

The Table 38 presents a detailed overview of the metabolic and lipid profiles of *Rhodotorula kratochvilovae* cultivated on various lipidic and non-lipidic substrates. In terms of metabolite production, the “Co+CH” condition exhibited the highest carotenoid production (1.207 mg/g), however the production of pigments by this strain was very low. Additionally, the “Co+G10 %” condition resulted in the highest ubiquinone concentration (9.649 mg/g), while the “WG” condition led to the highest ergosterol content (3.748 mg/g). The biomass yield was maximized under the “CH” condition, producing 15.55 g/l. The “Co+G10 %” medium also led to the highest tocopherol content (1.664 mg/g). These findings suggest that coffee oil with glycerol or hydrolysate addition significantly enhances ubiquinone production and tocopherol accumulation, while waste glycerol boosts ergosterol content.

For lipid profiles, the highest overall lipid content was achieved under the “Pf” condition (47.07 %), which also showed a strong MUFA concentration (46.49 %). The highest MUFA content was observed in the “CH” medium (61.38 %), while the highest PUFA content occurred in the “Co+CH” medium (50.98 %). Saturated fatty acid percentages peaked under the “Co+G10 %” condition at 41.52 %, indicating that coffee oil and its combinations have a pronounced effect on the fatty acid composition.

Table 38: Results of the yeast Rhodotorula kratochvilovae grown on various lipidic and non-lipidic substrates

	Rhodotorula kratochvilovae Fo/Co/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	0.445	4.497	3.536	8.00	n.a.	31.79	38.44	29.77	13.30
Fo	0.206	6.214	1.940	3.25	n.a.	14.90	39.75	45.34	38.79
Fo+G10 %	0.160	7.869	2.593	4.20	n.a.	18.13	38.34	43.54	34.74
Fo+CH	0.389	6.150	2.260	4.80	n.a.	14.33	39.87	45.80	35.92
Co	0.630	7.051	3.249	7.20	0.093	39.16	10.59	50.25	36.70
Co+G10 %	0.627	9.649	3.232	7.50	1.664	41.52	12.30	46.18	35.57
Co+CH	1.207	8.105	3.014	6.10	0.219	36.35	12.66	50.98	23.80
Pf	0.388	4.175	1.702	2.90	n.a.	28.64	46.49	24.87	47.08
Pf+G10 %	0.475	4.585	2.751	4.80	n.a.	31.68	42.70	25.61	33.88
Pf+CH	0.546	4.706	1.872	3.35	n.a.	29.11	47.38	23.51	42.98
CH	0.646	3.703	2.669	15.55	n.a.	24.08	61.38	14.54	9.65
WG	0.688	5.343	3.748	9.50	n.a.	29.11	43.34	27.55	11.71

5.1.3.7 Cystofilobasidium infirmominiatum (CCY 17-18-4)

The Table 39 provides an analysis of *Cystofilobasidium infirmominiatum*. The highest carotenoid production was observed in the “Co+CH” medium (1.965 mg/g), while the highest ubiquinone content was recorded in the “Fo+CH” medium (8.109 mg/g). The “Co” medium resulted in the greatest ergosterol accumulation (4.644 mg/g), suggesting a potential role of coffee oil in promoting sterol synthesis. Regarding biomass production, the control condition led to the highest yield (8.15 g/l), surpassing other media. The highest tocopherol content was found in the “Co” medium (1.212 mg/g), suggesting that coffee oil alone may enhance antioxidant production.

In terms of lipid accumulation, the highest lipid content was observed in the “Fo” medium (25.70 %). The “Fo+CH” condition exhibited the most significant polyunsaturated fatty acid content (48.60 %), while the “Co+CH” medium resulted in the highest PUFA percentage (59.77 %). The waste glycerol condition, meanwhile, promoted the highest monounsaturated fatty acid concentration (46.99 %). Saturated fatty acids were highest in the control medium (33.41 %).

Table 39: Results of the yeast Cystofilobasidium infirmominiatum grown on various lipidic and non-lipidic substrates

	Cystofilobasidium infirmominiatum Fo/Co/animal fat + glycerol								
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	Tocopherol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	0.616	6.481	3.287	7.25	n.a.	33.41	34.63	31.95	14.81
Fo	1.022	3.769	2.414	2.45	n.a.	16.05	39.42	44.53	25.70
Fo+G10 %	0.363	6.192	3.345	4.75	n.a.	19.24	35.55	45.21	17.88
Fo+CH	1.216	8.109	2.713	5.50	n.a.	14.64	36.75	48.60	22.76
Co	1.760	5.156	4.644	2.00	1.212	28.96	13.13	57.91	8.75
Co+G10 %	1.750	5.059	4.598	3.65	0.156	27.38	23.32	49.30	8.47
Co+CH	1.965	4.954	4.296	3.40	0.435	25.80	14.42	59.77	8.82
Pf	0.825	5.601	2.575	4.15	n.a.	27.69	45.31	27.00	25.43
Pf+G10 %	1.409	5.958	3.513	5.20	0.072	28.14	40.17	31.68	18.51
Pf+CH	1.520	6.696	4.071	3.15	0.092	22.86	32.69	44.45	7.87
CH	0.785	5.351	1.940	8.15	n.a.	30.92	39.53	29.55	13.71
WG	0.501	4.281	3.344	2.35	n.a.	23.80	46.98	29.22	5.84

5.1.3.8 Sporidiobolus salmonicolor (CCY 19-6-4)

The Table 40 provides a comprehensive analysis of *Sporidiobolus salmonicolor* cultivated on various oil and fat-based media. In terms of metabolite production, the “Fo+G10 %” condition showed the highest carotenoid production (2.774 mg/g), while ubiquinone peaked in the “Fo” condition (12.115 mg/g). The greatest ergosterol content was found in the control medium (4.640 mg/g).

Biomass production was highest in the “Co+CH” condition, reaching 15.05 g/l, and the “Co+G10 %” medium exhibited the highest tocopherol concentration (4.142 mg/g) followed by accumulation on Co medium (4.100 mg/g), indicating a strong influence of coffee oil on enhancing of this antioxidant composition. Regarding lipid accumulation, the highest overall lipid content was observed in the “Fo+G10 %” condition (31.92 %), with a notable PUFA concentration of 46.93 %. The highest PUFA content overall was recorded in the “Co” medium (59.52 %), making coffee oil an effective substrate for promoting polyunsaturated lipid production. The highest MUFA content was achieved in the “CH” medium (68.85 %), while the waste glycerol condition resulted in the highest SFA content (32.95 %).

Table 40: Results of the yeast Sporidiobolus salmonicolor grown on various lipidic and non-lipidic substrates

	Sporidiobolus salmonicolor Fo/Co/animal fat + glycerol								
Media	Carote-noids [mg/g]	Ubiqui-none [mg/g]	Ergoste-rol [mg/g]	Biomass [g/l]	Tocoph-erol [mg/g]	SFA %	MUFA %	PUFA %	Lipids [%]
Control	2.680	6.938	4.640	9.20	n.a.	31.48	55.23	13.29	14.59
Fo	1.529	12.115	3.428	5.65	n.a.	14.56	39.25	46.19	21.41
Fo+G10 %	2.774	10.133	3.128	9.85	n.a.	16.20	36.88	46.93	31.92
Fo+CH	2.228	12.036	4.330	8.65	n.a.	13.04	32.36	54.59	27.63
Co	1.118	7.399	1.940	13.75	4.100	28.34	12.15	59.52	28.63
Co+G10 %	1.261	7.372	2.660	13.75	4.142	27.30	13.50	59.20	25.12
Co+CH	0.913	5.942	2.077	15.05	3.723	26.83	13.94	59.22	25.88
Pf	1.407	6.316	2.027	12.80	1.711	19.91	48.08	32.01	25.63
Pf+G10 %	1.105	6.682	2.110	13.55	n.a.	21.32	48.54	30.14	30.80
Pf+CH	1.149	5.295	1.930	12.90	n.a.	23.42	46.37	30.21	29.71
CH	1.385	3.144	4.091	11.95	0.433	14.82	68.85	16.32	6.11
WG	1.710	5.278	4.054	7.70	n.a.	32.94	60.35	6.70	12.45

5.1.3.9 Comparison biomass growths of yeasts strains

From the overall data presented in Figure 19 it is possible to recognize, that for the *SP* strain, the highest biomass yield was achieved in the "Fo+G10 %" medium (18.15 g/l), while the *RM* (19-4-6) strain reached its maximum biomass production in the "Co+CH" medium (15.90 g/l). The *RT* strain exhibited its highest growth in the control and "Pf" media, both reaching 12.40 g/l. *CM* strain growth was highest in the "Co+CH" medium (14.60 g/l). The *SM* strain saw its greatest biomass accumulation in the "Pf" medium (9.80 g/l), while the *RK* strain displayed peak growth in the "CH" medium (15.55 g/l). The *CI* strain showed its highest biomass yield in the control medium (8.15 g/l), and the SS strain exhibited maximum biomass growth in the "Co+CH" medium (15.05 g/l).

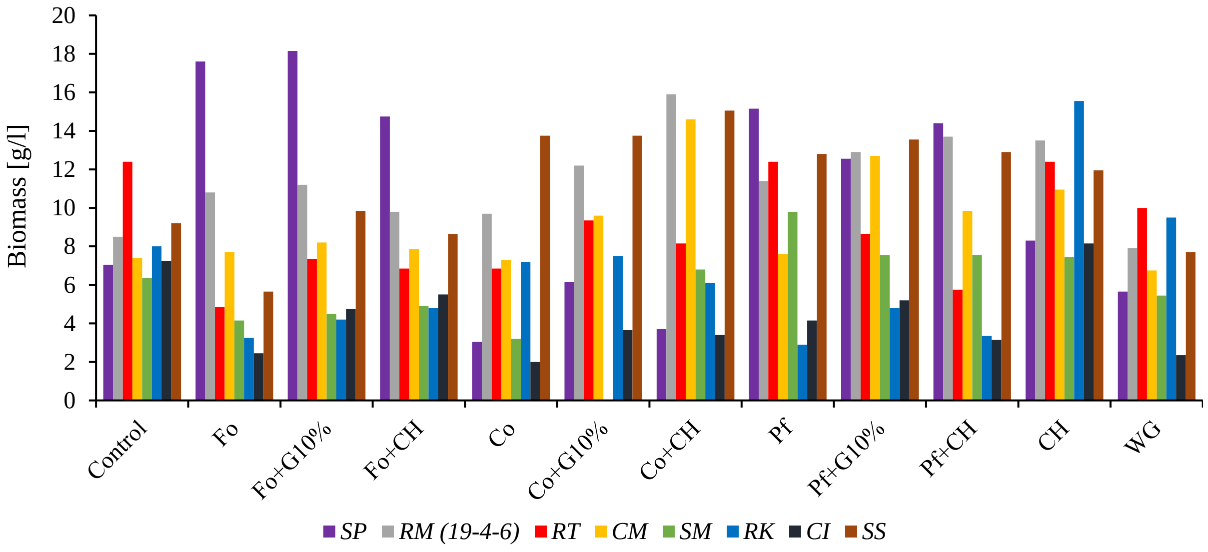


Figure 19: Comparison of biomass growths between yeast strains

A clear trend across the data is the significant impact of combining coffee hydrolysate with coffee oil or poultry fat on enhancing biomass production, particularly for the *RM* (19-4-6), *CM*, and *SS* strains. Frying oil combined with glycerol (Fo+G10 %) also notably increased biomass for the *SP*, *SS* and *RM* (19-4-6) strains. Interestingly, coffee hydrolysate alone promoted robust biomass yields in most strains, indicating its effectiveness as a growth medium. Waste glycerol, on the other hand, resulted in moderate to lower biomass growth compared to other media, suggesting its limited efficacy in promoting high biomass production across the strains tested.

The highest beta-glucan content in the *SP* strain was found in the control medium (12.30 %), followed by the "Fo" medium (9.20 %). For *RM* (19-4-6), the highest value was observed in the "WG" medium (11.01 %), while the control medium produced a beta-glucan content of 9.97 %. In the *RT* strain, the highest beta-glucan level was recorded in the control medium (19.77 %), with "WG" providing the next highest value (12.44 %). The *CM* strain exhibited its peak beta-glucan content in the control medium (16.93 %), followed by "CH" (14.18 %). For the *SM* strain, the control medium also led to the highest beta-glucan concentration (19.03 %), followed by "CH" (14.07 %). Similarly, the *RK* strain had the highest beta-glucan content in the control medium (12.55 %), with "CH" producing the second-highest value (11.850 %). In the *CI* strain, the control medium resulted in the highest beta-glucan content (18.10 %), followed by the "Co" medium (17.65 %). For the *SS* strain, the control medium again led to the highest content (10.50 %), with the next highest value found in the "Co+CH" medium (9.10 %).

A noticeable trend across the data is that the control medium consistently produced the highest or near-highest beta-glucan content across most yeast strains. However, alternative media such as waste glycerol and coffee hydrolysate slightly promoted beta-glucan production in certain strains, particularly *RM* (19-4-6) and *CM*. These findings suggest that specific media combinations can be tailored to optimize beta-glucan production in yeast strains, with the control glycerol medium remaining highly effective, but alternative substrates such as coffee hydrolysate and waste glycerol offering valuable alternatives for production of other metabolites or biomass while not significantly decreasing beta-glucan content.

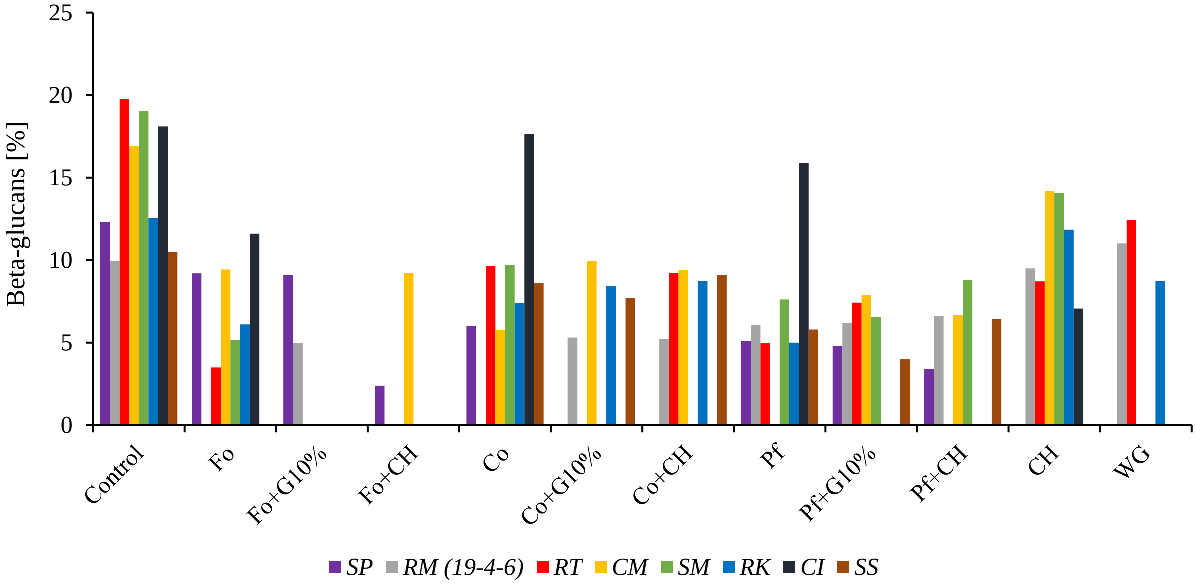


Figure 20: Beta-glucan production comparison among yeasts strains

5.1.3.10 Bioreactor cultivations on selected fat/oil substrates

The cultivations in the bioreactor were conducted using various media, with the concentration of all components in the fermenter doubled due to intensive aeration and mixing, leading to more rapid nutrient metabolism by the yeast. Ammonium sulphate was added to the media in an equimolar amount to the nitrogen in KNO_3, as nitrogen in the ammonium form is expected to be more easily utilized by the yeast, making it more suitable for growth after inoculation. The cultivations were carried out in a fermenter with a working volume of 5 liters. Table 41 outlines the composition of the media used in the bioreactors (*RT* – pure lipidic media of coffee oil; *CM* – combination of coffee hydrolysate and coffee oil in the media; *SP* – combination of coffee hydrolysate and poultry fat in the media).

Table 41: Bioreactor media composition

Substance	Amount [g/l]
KH_2PO_4	8
$MgSO_4 \cdot 7H_2O$	1.39
KNO_3	6.09
$(NH_4)_2SO_4$	3.98
Lipidic media	
Oil/fat	23.54
With CH addition	
Oil/fat	21.19
Coffee hydrolysate	10 %

***Rhodosporidium toruloides* (CCY 62-2-4)**

The Figure 21 and Figure 22 present data from a bioreactor experiment involving *Rhodosporidium toruloides* over a 119-hour period cultivation on media derived from coffee oil. Among the metabolites, carotenoids reach their highest concentration at 119 hours with 11.984 mg/g, while ubiquinone peaked at 30 hours with 14.993 mg/g. Ergosterol showed its maximum value at 119 hours (6.382 mg/g), and tocopherol reached its highest point at 24 hours with 15.623 mg/g.

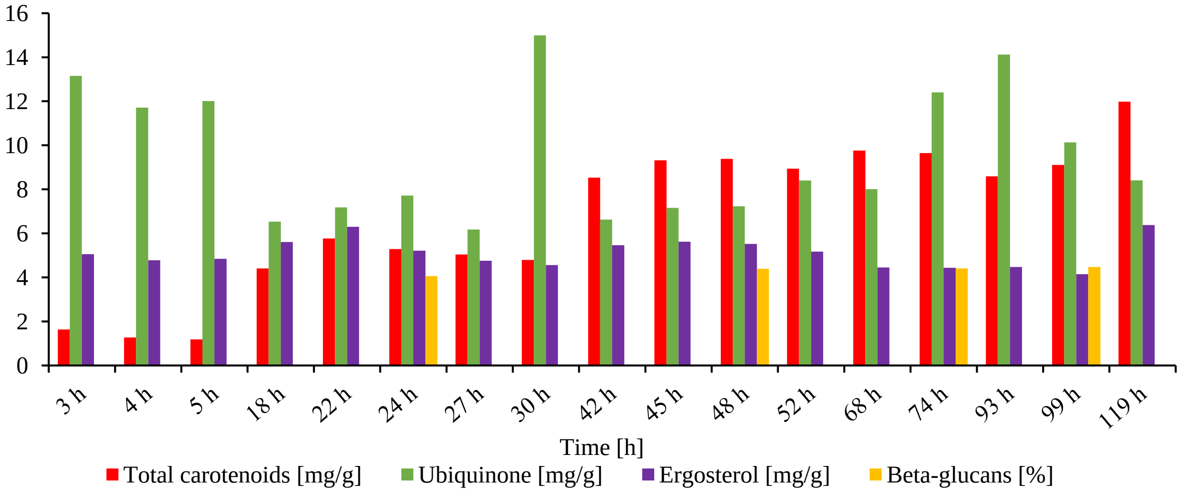


Figure 21: The production of antioxidants and beta-glucans during the bioreactor cultivation of the yeast strain Rhodosporidium toruloides using pure lipidic media derived from coffee oil

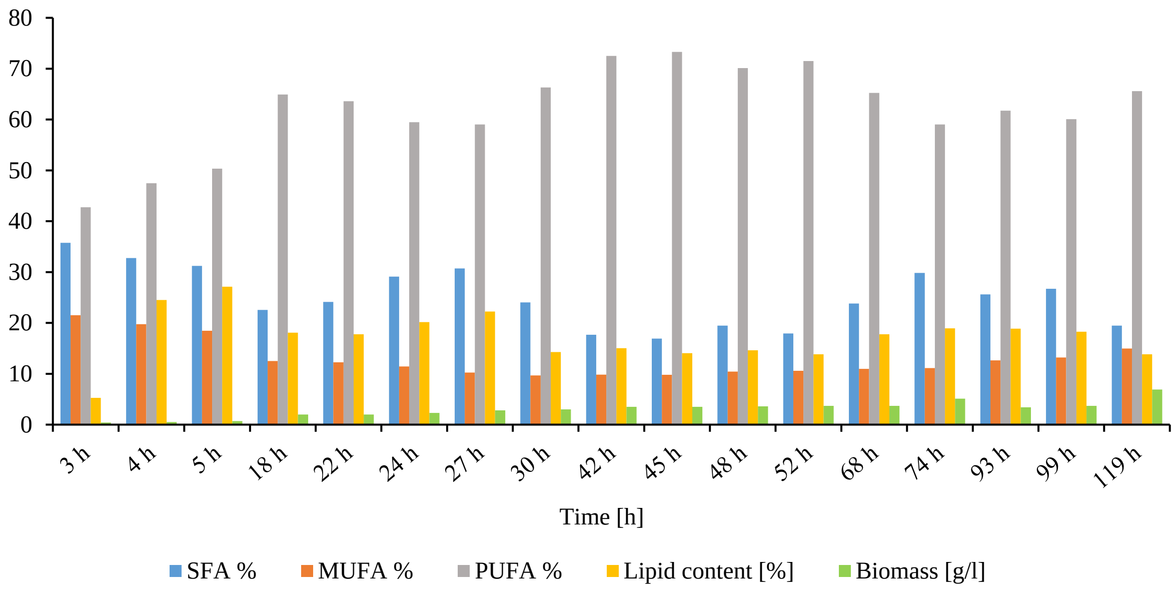


Figure 22: The accumulation of lipids and biomass growth during the bioreactor cultivation of the yeast strain Rhodosporidium toruloides using pure lipidic media derived from coffee oil

Biomass growth increased significantly throughout the cultivation, peaking at 119 hours with 6.9 g/l. Lipid content fluctuated, reaching its maximum at 45 hours with a PUFA content of 73.29 %, while the highest SFA concentration is observed at 3 hours with 35.74 %. MUFA levels peaked at 119 hours (14.95 %) except for starting condition (21.53 % at 3 hours), although they remain relatively low compared to PUFA. Beta-glucan production is tracked at specific time points, with the highest value recorded at 99 hours (4.47 %).

The trends indicate a steady rise in biomass over time, accompanied by increasing carotenoid and ergosterol production towards the later stages of the cultivation. PUFA content remains dominant across the experiment, with a peak between 42 and 45 hours (72.50-73.29 %). Interestingly, there is a decrease in SFA and MUFA as PUFA becomes the predominant lipid type during the mid-phase of the experiment. The high accumulation of tocopherol at 24 hours suggests a temporary peak in antioxidant compound accumulation, which declines in the later stages. The overall trends suggest a metabolic shift in the yeast, favouring PUFA synthesis and continued growth in biomass and metabolites such as carotenoids, especially towards the final hours of cultivation.

Cystofilobasidium macerans (CCY 10-1-2)

The Figure 23 and Figure 24 presents results of *Cystofilobasidium macerans* cultivated in a bioreactor using a medium containing 10 % coffee hydrolysate and coffee oil as carbonaceous substrates. The highest concentration of carotenoids was observed at 93 hours with a value of 3.870 mg/g, while ubiquinone peaked earlier, at 27 hours, with 12.751 mg/g. Ergosterol production reached its maximum at 21 hours (7.231 mg/g) followed by peak at 99 hours with 6.917 mg/g. In terms of biomass, the highest recorded value is 6.335 g/l at 120 hours, demonstrating significant biomass accumulation. Tocopherol showed its peak concentration at 27 hours with 9.895 mg/g, indicating a transient burst of antioxidant compound accumulation at this time.

Lipid content peaked early in the experiment, reaching 25.96 % at 3 hours, while the highest polyunsaturated fatty acid level is observed at 76 hours with 63.62 %. Saturated fatty acids show their maximum concentration at 21 hours with 40.69 %, and monounsaturated fatty acids peak also at 21 hours with 28.76 %. Beta-glucan production did not change significantly over time, with the highest value recorded at 69 hours, reaching 5.87 %.

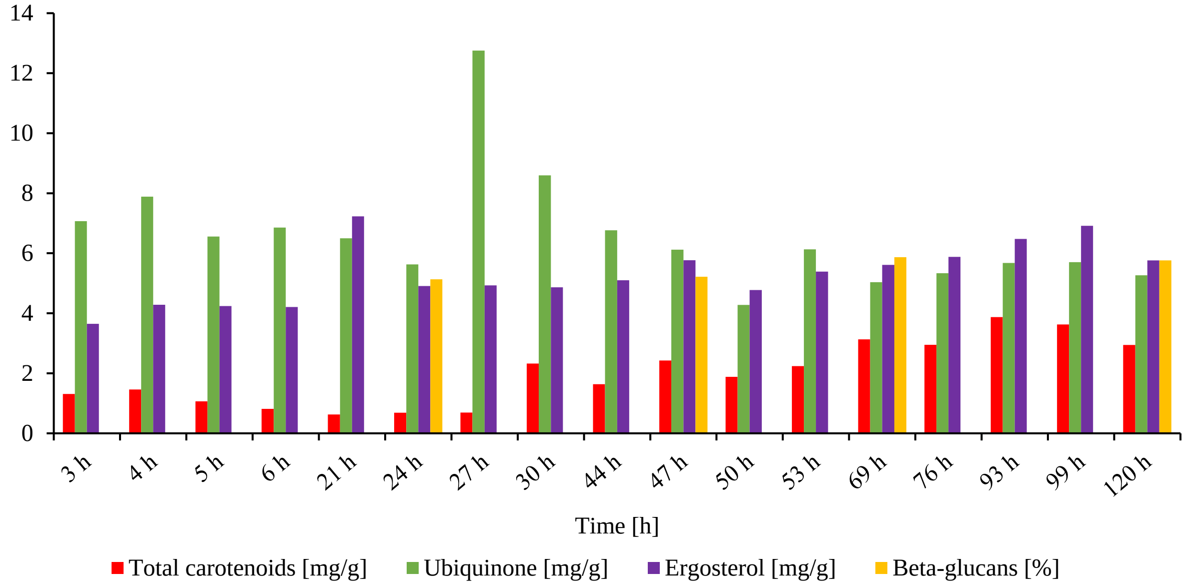


Figure 23: The production of antioxidants and beta-glucans during the bioreactor cultivation of the yeast strain Cystofilobasidium macerans using combination of 10 % coffee hydrolysate and coffee oil in media

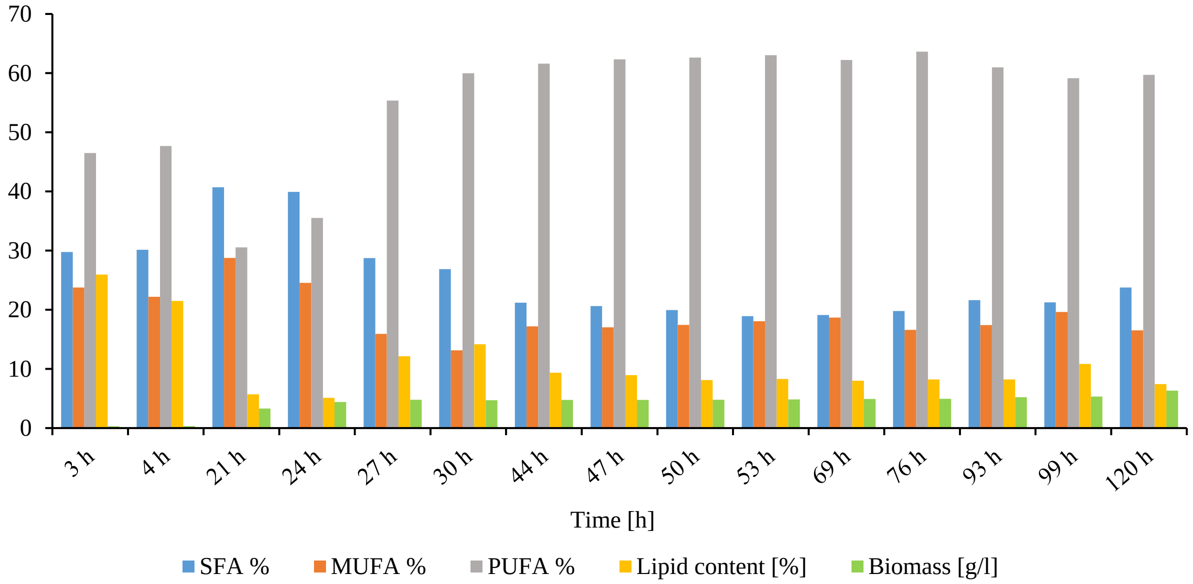


Figure 24: The accumulation of lipids and biomass growth during the bioreactor cultivation of the yeast strain Cystofilobasidium macerans using combination of 10 % coffee hydrolysate and coffee oil in media

Trends indicate dynamic shifts in metabolic processes throughout the cultivation. Early on, there was a rapid increase in ergosterol, ubiquinone, and tocopherol cumulation, followed by stabilization or decline in later phases. Lipid accumulation is highest in the early hours and then stabilizes as the experiment progresses. The fatty acid profile indicates a shift from saturated fatty acids dominating in the early hours to increased PUFA levels in the mid to late phases, suggesting membrane restructuring, metabolic adaptation and accumulation fatty acids from the substrate. Beta-glucans production showed a slight increase, indicating mild polysaccharide accumulation under the given culture conditions. These trends highlight the metabolic versatility of *C. macerans* when grown on coffee-derived substrate.

***Sporidiobolus pararoseus* (CCY 19-9-6)**

The Figure 25 and Figure 26 present results of bioreactor cultivation of the yeast *Sporidiobolus pararoseus* using a combination of 10 % coffee hydrolysate and poultry fat as a carbonaceous substrate over a period of 122 hours. Among the metabolites, the highest concentration of carotenoids is observed at 120 hours, reaching 1.330 mg/g. Ubiquinone shows its peak value at 74 hours with 9.248 mg/g, while the maximum concentration of ergosterol is achieved at 7 hours with 4.401 mg/g. In terms of biomass, the highest value of 40.12 g/l is observed at 57 hours, while till the end of cultivation slightly decreased (36.98 g/l). The lipid content was most abundant at 70 hours, with 40.56 % lipids, while betaglucan production reached its highest value at 120 hours with 4.70 %.

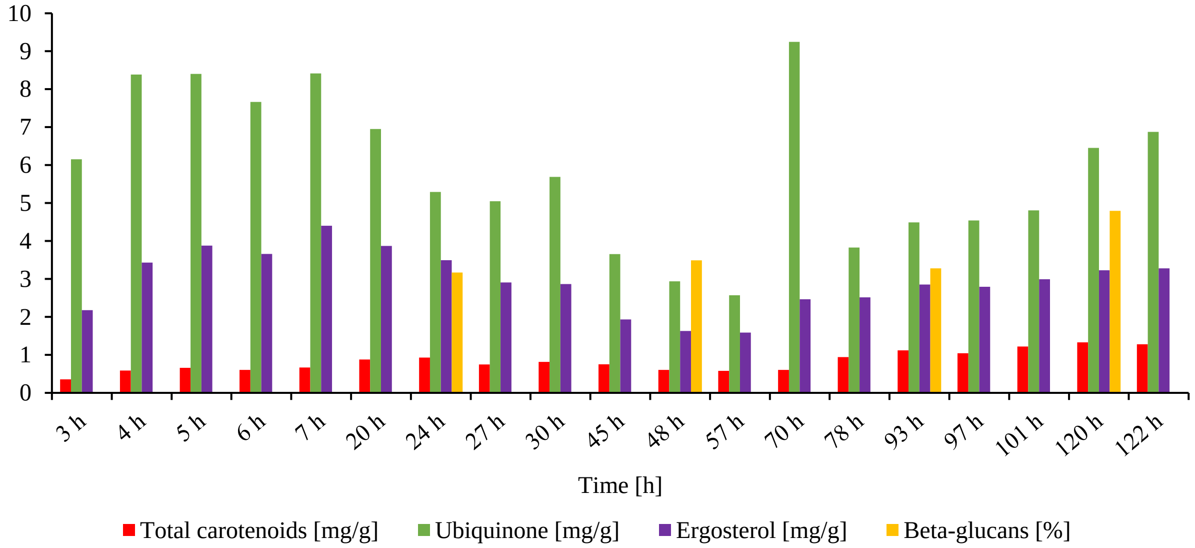


Figure 25: The production of antioxidants and beta-glucans during the bioreactor cultivation of the yeast strain Sporidiobolus pararoseus using combination of 10 % coffee hydrolysate and poultry fat in media

Regarding the fatty acid composition, saturated fatty acids peak at 4 hours with a value of 26.12 %, while monounsaturated fatty acids reach their maximum concentration at 120 hours (53.89 %). Polyunsaturated fatty acids were most abundant at 5 hours, reaching 38.88 %. These data indicate the dynamic metabolic shifts that occur during the cultivation process.

In terms of trends, the data reveals a gradual increase in biomass and lipid content over time, with notable peaks in metabolite production at various stages of the cultivation. SFA shows a decreasing trend till the ed of cultivation, peaking at 4 hours, while MUFA steadily increases towards the later stages, showing a preference for unsaturated fatty acid synthesis at the expense of SFA. PUFA levels fluctuated in the early stages but generally decrease as the experiment progresses. Beta-glucan production increases steadily, particularly in the final stages of cultivation, indicating a shift toward polysaccharide synthesis under the given culture conditions. However, beta-glucan production remained low on this substrate.

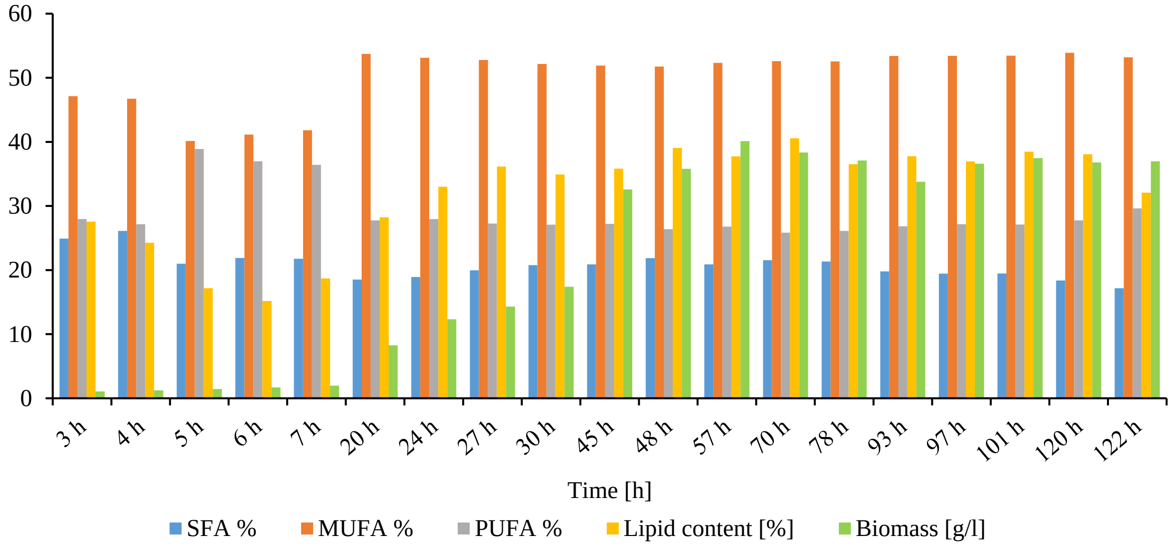


Figure 26: The accumulation of lipids and biomass growth during the bioreactor cultivation of the yeast strain Sporidiobolus pararoseus using combination of 10 % coffee hydrolysate and poultry fat in media

Cystofilobasidium macerans (CCY 10-1-2) on waste glycerol

The second bioreactor cultivation with the yeast strain *Cystofilobasidium macerans* was performed using an artificial medium with waste glycerol as the carbonaceous substrate. The composition of the medium is detailed in Table 42. Throughout the cultivation process, samples were analysed using HPLC-RI to determine the residual glycerol concentrations in the medium, allowing for the calculation of the final yield coefficient.

Table 42: Bioreactor medium with waste glycerol. Presented amounts are dedicated per 1 liter of final medium

Substance	**Amount**
KH_2PO_4	8 g
$MgSO_4 \cdot 7H_2O$	1.392 g
$NaNO_3$	10.24 g
Waste glycerol	193.4 ml

The provided figures (Figure 27 and Figure 28) display the time-dependent production of key metabolites (carotenoids, ubiquinone, and ergosterol), biomass accumulation, lipid content, and the fatty acid composition (SFA, MUFA, PUFA) in a *CM* bioreactor over a span of 98 hours. Among the metabolites, the highest concentration of carotenoids (1.601 mg/g) is observed at 74 hours, while ubiquinone reaches its peak (1.869 mg/g) at the same time point. Ergosterol shows the highest accumulation at 46 hours, with a value of 2.274 mg/g.

Biomass production steadily increases with time, starting from 0.87 g/l at 3 hours, reaching its maximum at 98 hours (8.03 g/l). The lipid content also rises progressively, peaking at 3.64 % at 98 hours. The fatty acid composition evolves significantly over time. Saturated fatty acids show the highest value at 3 hours (55.77 %), but subsequently decline, with the lowest SFA recorded at 42 and 46 hours (25.46-25.42 %). Monounsaturated fatty acids reach their maximum (38.13 %) at 70 hours, while polyunsaturated fatty acids show their highest concentration at 46 hours, peaking at 40.01 %. The overall yield coefficient for substrate-to-biomass conversion was calculated to be 0.415, based on the difference between the initial (0.44 g/l) and final (8.03 g/l) biomass concentrations, divided by the difference between the initial (44.13 g/l) and final (25.84 g/l) glycerol concentrations. This relatively high yield coefficient indicates efficient utilization of the waste glycerol substrate by the yeast for biomass production. However, biomass accumulation after 96 hours was low, likely due to the presence of inhibitory compounds within the waste glycerol mixture.

Regarding the overall trends, carotenoids, ubiquinone, and ergosterol generally increase over time, with pronounced peaks at around 70-74 hours, suggesting heightened metabolic activity during this period. Lipid content and biomass also follow an increasing trend, reflecting optimal growth conditions and metabolic productivity. The shift in fatty acid composition from SFA dominance to higher MUFA and PUFA contents as the cultivation progresses is indicative of adaptive physiological changes in response to bioreactor conditions, possibly favouring membrane fluidity and cellular stability.

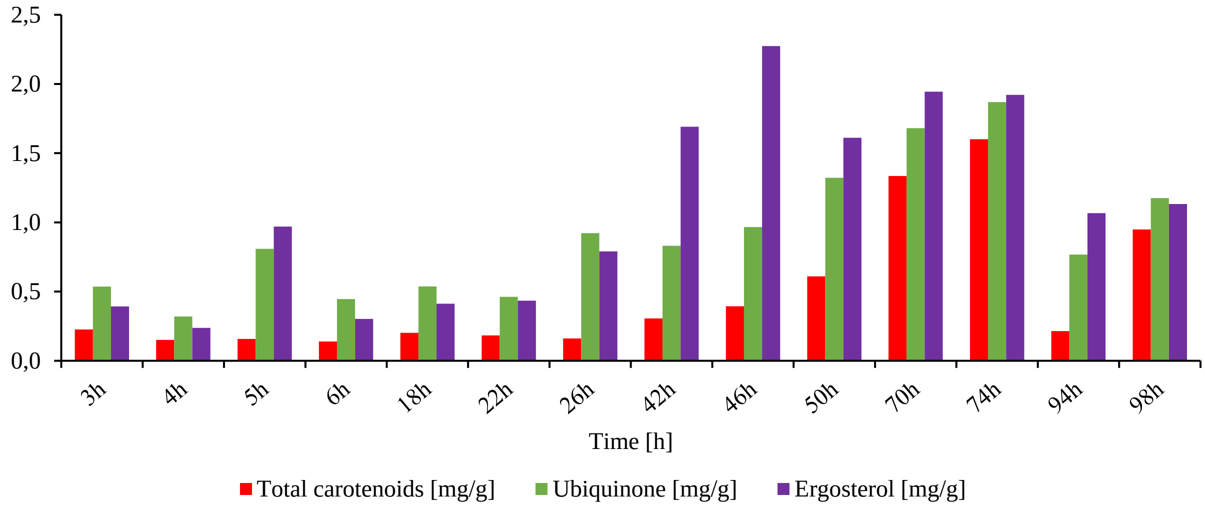


Figure 27: Metabolic progress of Cystofilobasidium macerans describing production of carotenoids, ergosterol and ubiquinone during cultivation on medium with waste glycerol

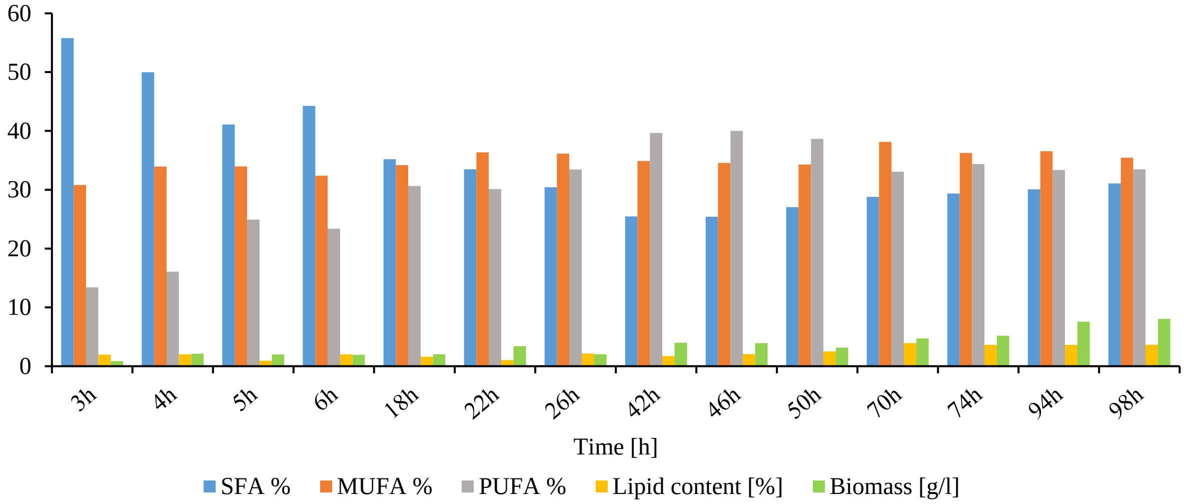


Figure 28: Accumulation of lipids and lipidic profile of accumulated lipids by Cystofilobasidium macerans also with its biomass growth on medium with waste glycerol

5.1.4 Cultivation on combined animal waste - poultry fat with glycerol and feather hydrolysate

Another experiment conducted on waste substrates involved the cultivation of red yeasts using waste poultry fat as a carbon source and feather hydrolysate as a nitrogen source. Glycerol was used as the control carbon source, and urea served as the control nitrogen source. The compositions of the experimental media are presented in Table 43, Table 44 and Table 45. The experiments were performed at two different C/N ratios – 25 and 50. The nitrogen content in the feather hydrolysate was measured using the Kjeldahl method (chapter 4.6.1). The results of chapter 5.1.4 are partially discussed in: Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2; Holub J. et al., 2022. *Proceedings of the 9th International Conference on Chemical Technology*. ISBN 978-80-88307-11-2 – see attachment 3 pg. 356-360; Holub et al., 2021. *Poster presentation; The EuroBiotech Journal*. DOI: 10.2478/ebtj-2021-0031 – see attachment 5 pg. 91.

Table 43: The mineral base of experimental media

Substance	**Amount [g/l]**
KH_2PO_4	4
$MgSO_4 \cdot 7H_2O$	0.696

Table 44: The amount of carbon and nitrogen substrates for a volume of 1000 ml and C/N ratios of 25 and 50, for a series of experiments without the addition of feather hydrolysate

C/N ratio	Substrate	GLY	Pf	Pf+G10	Pf+G25	GLY+F.H.
25	Poultry fat [g]	0	23.54	21.19	17.66	0
	Glycerol [g]	46.26	0	4.63	11.56	46.26
	Urea [g]	1.81	0	0	0	0
	Feather hydrolysate [ml]	0	0	0	0	45
50	Poultry fat [g]	0	47.09	42.38	35.31	0
	Glycerol [g]	92.51	0	9.25	23.13	92.51
	Urea [g]	1.81	0	0	0	0
	Feather hydrolysate [ml]	0	0	0	0	45

Table 45: The amount of carbon and nitrogen substrates for a volume of 1000 ml and C/N ratios of 25 and 50, for a series of experiments with the addition of feather hydrolysate

C/N ratio	Substrate	GLY (F.H.)	Pf+F.H.	Pf+G10+F.H.	Pf+G25+F.H.	GLY+F.H. (F.H.)
25	Poultry fat [g]	0	23.54	21.19	17.66	0
	Glycerol [g]	46.26	0	4.63	11.56	46.26
	Urea [g]	1.81	0	0	0	0
	Feather hydrolysate [ml]	0	45	45	45	45
50	Poultry fat [g]	0	47.09	42.38	35.31	0
	Glycerol [g]	92.51	0	9.25	23.13	92.51
	Urea [g]	1.81	0	0	0	0
	Feather hydrolysate [ml]	0	45	45	45	45

5.1.4.1 Sporidiobolus pararoseus (CCY 19-9-6)

Table 46 presents the results of the strain *Sporidiobolus pararoseus* cultivated on various combinations of poultry fat, glycerol and feather hydrolysate. The highest production of carotenoids was observed in the pure glycerol (GLY) medium, reaching 2.690 mg/g. Conversely, the maximum ubiquinone production (15.251 mg/g) was recorded in the medium containing a combination of poultry fat, feather hydrolysate, and 10 % glycerol (Pf+G10+F.H.), suggesting that this mixture enhances the synthesis of this isoprenoid lipid. The highest ergosterol production was achieved in the glycerol medium, with a concentration of 9.247 mg/g, while the most substantial biomass accumulation (16.10 g/l) was observed in the medium composed of poultry fat and feather hydrolysate (Pf+F.H.).

Concerning fatty acid profiles, the “GLY+F.H. (F.H.)” medium yielded the highest proportion of monounsaturated fatty acid, reaching 70.14 %, while the greatest polyunsaturated fatty acid content (39.16 %) was achieved with pure glycerol medium. The highest level of saturated fatty acids was found in the “Pf+G25” medium, reaching 53.22 %. Furthermore, the highest total lipid content (32.30 %) was observed in the medium containing poultry fat and feather hydrolysate (Pf+F.H.), highlighting the potential of this mixture for applications requiring enhanced lipid yield (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 15).

Table 46: Outcomes of cultivating Sporidiobolus pararoseus on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	SP C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	2.690	5.291	9.247	2.40	19.92	40.92	39.16	6.42
Pf	1.360	10.300	5.913	13.40	43.25	37.58	19.17	19.76
Pf+G10	1.873	10.485	5.806	12.50	33.92	39.88	26.20	15.41
Pf+G25	1.558	9.273	5.178	15.90	53.22	30.74	16.04	20.59
GLY+F.H.	0.987	4.429	4.072	11.30	26.57	58.36	15.07	10.26
GLY (F.H.)	0.723	6.605	8.231	1.80	21.33	42.42	36.25	5.17
Pf+F.H.	0.773	14.712	5.464	16.10	47.73	35.56	16.71	32.30
Pf+G10+F.H.	0.794	15.251	5.307	14.30	33.98	41.41	24.61	24.92
Pf+G25+F.H.	0.384	5.216	2.598	15.40	24.70	48.70	26.61	22.38
GLY+F.H. (F.H.)	0.238	8.382	5.980	9.90	20.98	70.14	8.88	10.58

Table 47 illustrates the metabolic response and biomass production of *Sporidiobolus pararoseus* cultivated on diverse media formulations at a C/N ratio of 50. The highest production of carotenoids was achieved in the "Pf+G10+F.H." medium, with a yield of 2.425 mg/g, whereas the maximal ubiquinone concentration (9.460 mg/g) and ergosterol content (14.365 mg/g) were recorded in the "GLY (F.H.)" medium. The largest biomass accumulation occurred in the "Pf+G10+F.H." medium, reaching 30.1 g/l, suggesting a strong interaction between poultry fat, glycerol and feather hydrolysate for promoting cell growth. Regarding lipid profiles, the "GLY+F.H." medium exhibited the highest monounsaturated fatty acid content, with 61.41 %, while the glycerol-based medium supported the largest polyunsaturated fatty acid concentration at 41.00 %. The greatest proportion of saturated fatty acids was found in the GLY+F.H.(F.H.). medium, reaching 47.39 %. The "Pf" medium showed the highest total lipid content (35.13 %), highlighting poultry fat as a potent substrate for lipid accumulation (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 15).

Table 47: Outcomes of cultivating Sporidiobolus pararoseus on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	SP C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	0.102	4.188	7.181	2.30	29.17	29.83	41.00	7.09
Pf	0.713	5.226	3.365	21.80	35.73	43.06	21.21	35.13
Pf+G10	0.393	6.343	4.096	20.80	35.67	41.47	22.85	34.48
Pf+G25	0.640	6.659	3.273	18.70	33.94	44.02	22.04	28.78
GLY+F.H.	0.584	5.570	2.463	11.80	29.46	61.41	9.13	19.71
GLY (F.H.)	1.460	9.460	14.365	1.60	19.46	42.02	38.52	3.68
Pf+F.H.	0.430	8.801	2.779	24.50	37.93	40.61	21.46	34.13
Pf+G10+F.H.	2.425	8.962	2.580	30.10	41.59	39.13	19.29	34.30
Pf+G25+F.H.	0.486	9.008	3.250	28.20	26.92	49.93	23.16	26.84
GLY+F.H. (F.H.)	0.398	6.709	3.590	16.60	47.39	48.49	4.12	22.38

5.1.4.2 Rhodororula mucilaginosa (19-4-6)

Table 48 presents the findings for the strain *Rhodotorula mucilaginosa* (19-4-6) cultivated at a C/N ratio of 25. The highest carotenoid production was achieved using pure glycerol with a yield of 10.483 mg/g. Regarding ubiquinone, another important antioxidant, the maximum concentration (8.932 mg/g) was observed in the medium containing a mixture of poultry fat and feather hydrolysate (Pf+F.H.). Ergosterol production peaked at 6.833 mg/g in the glycerol-based medium.

In terms of biomass accumulation, the best result was recorded in the poultry fat medium (Pf), reaching 29,50 g/l, indicating its efficiency as a substrate for microbial biomass growth. Lipid profile analysis revealed that pure glycerol supported the highest proportion of monounsaturated fatty acids, with 60.52 %, while the “Pf+G10+F.H.“ medium provided the highest content of polyunsaturated fatty acids at 34.54 %. On the other hand, the “Pf+G25+F.H.” medium yielded the highest saturated fatty acid content, with 49.45 %, suggesting its suitability for producing specific lipids based on desired fatty acid composition. The highest total lipid content was observed in the poultry fat medium (Pf), where lipids constituted 25.48 % of the total mass (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 11-12).

Table 48: Outcomes of cultivating Rhodotorula mucilaginosa (19-4-6) on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	RM (19-4-6) C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	10.483	5.726	6.833	14.70	34.93	60.52	4.55	11.44
Pf	3.280	5.144	3.616	29.50	39.87	41.54	18.60	25.48
Pf+G10	6.638	4.539	4.377	24.80	30.57	46.19	23.24	17.67
Pf+G25	7.461	5.186	5.121	21.70	32.25	46.19	21.56	20.39
GLY+F.H.	2.942	4.216	3.873	9.50	39.40	57.02	3.59	11.97
GLY (F.H.)	1.073	2.225	3.059	18.20	28.81	51.26	19.93	8.95
Pf+F.H.	2.359	8.932	2.500	16.70	27.35	39.58	33.07	19.97
Pf+G10+F.H.	1.543	4.848	2.476	16.30	27.20	38.27	34.54	23.34
Pf+G25+F.H.	0.548	2.905	2.016	17.00	49.45	29.24	21.31	24.71
GLY+F.H. (F.H.)	4.781	3.615	3.218	19.10	32.38	52.39	15.22	15.73

The Table 49 presents the findings for the strain *Rhodotorula mucilaginosa* (19-4-6) cultivated at a C/N ratio of 50. The highest production of carotenoids (6.102 mg/g) and ergosterol (6.118 mg/g) were achieved using the GLY (F.H.) substrate, indicating the efficiency of this combination for the synthesis of these antioxidants.

The maximum concentration of ubiquinone (7.838 mg/g) was recorded in the “Pf+F.H.” medium. Additionally, this medium also supported the highest biomass growth (28.30 g/l), which was similarly observed in the “Pf+G25+F.H.“ medium, indicating that these substrate combinations provide optimal conditions for yeast growth.

Regarding the lipid composition, the “Pf” substrate exhibited the highest proportion of monounsaturated fatty acids at 66.00 %, while the highest content of polyunsaturated fatty acids was found in the “Pf+F.H.” medium, with a value of 28.34 %. The maximum saturated fatty acid content was observed in the “Pf+G25+F.H.“ medium, reaching 55.97 %. Regarding total lipid content, the “Pf+G10+F.H.“ medium achieved the highest value of 38.33 %, demonstrating its potential for applications requiring higher lipid content (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 13-14).

Table 49: Results of cultivating Rhodotorula mucilaginosa (19-4-6) on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	RM (19-4-6) C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	3.317	3.714	4.594	6.90	31.37	45.94	22.69	21.18
Pf	0.649	4.228	2.749	2.00	34.00	66.00	0.00	13.17
Pf+G10	1.480	2.271	1.798	10.10	26.28	50.46	23.26	31.84
Pf+G25	3.100	4.009	3.844	12.80	33.84	49.85	16.31	16.97
GLY+F.H.	0.183	3.493	3.930	8.70	37.76	62.24	0.00	10.59
GLY (F.H.)	6.102	4.067	6.118	15.20	23.75	65.37	10.88	11.76
Pf+F.H.	4.472	7.838	3.976	28.30	31.69	39.97	28.34	30.35
Pf+G10+F.H.	4.688	6.317	3.535	27.70	54.27	28.18	17.55	38.33
Pf+G25+F.H.	5.642	7.573	4.442	28.30	55.97	27.00	17.03	35.67
GLY+F.H. (F.H.)	5.211	5.209	4.621	19.70	32.60	51.83	15.56	14.29

5.1.4.3 Rhodosporidium toruloides (CCY 62-2-4)

The Table 50 presents the results for the strain *Rhodosporidium toruloides* cultivated at a C/N ratio of 25. The highest carotenoid production was observed in the “Pf+G10” substrate, reaching 5.667 mg/g, indicating that this mixture promotes the synthesis of these bioactive compounds. In the case of ubiquinone, the highest concentration was recorded in the “Pf+G10+F.H.” substrate, with a value of 9.711 mg/g, making it an optimal choice for enhancing the production of this antioxidant. Ergosterol was most abundantly produced in the “Pf+F.H.” mixture, reaching 6.911 mg/g. The “Pf” substrate supported the highest biomass growth, with a value of 20.40 g/l, suggesting it provides favorable conditions for yeast growth. In terms of lipid composition, the GLY substrate exhibited the highest impact on monounsaturated fatty acids, with 51.89 %, while the “Pf+F.H.” substrate influenced the highest proportion of polyunsaturated fatty acids at 32.21 %. The highest saturated fatty acid content was recorded in the “GLY+F.H. (F.H.)” substrate, reaching 43.40 %. The highest total lipid content was observed in the “Pf+G10+F.H.” substrate, with a value of 25.04 % (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 6-7; Holub J. et al., 2022. *Proceedings of the 9th International Conference on Chemical Technology*. ISBN 978-80-88307-11-2 – see attachment 3 pg. 356-360).

Table 50: Results of cultivating Rhodosporidium toruloides on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	RT (19-4-6) C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	4.892	4.061	4.572	19.00	38.85	51.89	9.26	17.70
Pf	1.254	4.686	2.438	20.40	35.64	46.61	17.75	21.24
Pf+G10	5.667	7.422	5.515	18.40	36.31	49.17	14.52	21.90
Pf+G25	0.104	7.748	0.113	8.10	28.58	46.73	24.69	20.35
GLY+F.H.	0.349	5.316	2.323	3.00	41.45	39.26	19.29	6.34
GLY (F.H.)	0.675	2.891	5.544	17.80	38.01	49.84	12.14	19.05
Pf+F.H.	0.308	6.957	6.911	3.50	27.49	40.30	32.21	24.18
Pf+G10+F.H.	2.433	9.711	4.907	9.80	26.53	43.31	30.16	25.04
Pf+G25+F.H.	0.294	6.808	4.434	12.70	34.75	37.30	27.95	23.96
GLY+F.H. (F.H.)	0.272	3.384	2.300	15.30	43.40	41.72	14.88	21.75

The Table 51 presents the results for the strain *Rhodosporidium toruloides* cultivated at a C/N ratio of 50. The highest carotenoid production was observed in the Pf+G25 substrate, reaching 4.584 mg/g, which significantly exceeds production levels on other substrates. The maximum ubiquinone concentration was recorded in the "GLY+F.H. (F.H.)" substrate, with 11.611 mg/g. Ergosterol production was highest in the "Pf+G10+F.H." substrate, reaching 8.944 mg/g, demonstrating the favourable impact of combined substrates (Pf, GLY, and F.H.) on sterol biosynthesis.

Table 51: Results of cultivating Rhodosporidium toruloides on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	RT (19-4-6) C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	2.533	6.615	2.806	24.00	45.21	50.02	4.76	19.10
Pf	0.937	4.064	3.491	3.80	32.13	44.88	22.99	29.97
Pf+G10	0.446	4.057	4.134	6.30	29.95	45.39	24.65	31.14
Pf+G25	4.584	5.930	3.669	14.30	30.30	46.28	23.41	24.10
GLY+F.H.	0.591	2.456	1.423	21.00	43.41	47.20	9.39	23.01
GLY (F.H.)	1.727	8.900	2.638	19.10	42.74	51.76	5.49	20.06
Pf+F.H.	0.003	3.211	4.547	4.80	25.17	47.17	27.66	25.34
Pf+G10+F.H.	1.113	4.752	8.944	10.80	25.51	45.96	28.53	22.16
Pf+G25+F.H.	0.606	9.707	8.725	12.40	27.71	46.78	25.51	18.78
GLY+F.H. (F.H.)	0.843	11.611	4.580	20.30	37.63	52.31	10.07	16.56

In terms of biomass production, the best result was achieved using pure glycerol, reaching 24.0 g/l. Regarding lipid composition, the "GLY+F.H. (F.H.)" substrate exhibited the highest proportion of monounsaturated fatty acids at 52.31 %, while the "Pf+G10+F.H." substrate achieved the highest content of polyunsaturated fatty acids with 28.53 %.

The highest saturated fatty acid content was observed in the "GLY" substrate, reaching 45.21 %. As for total lipid content, the highest value was recorded in the "Pf+G10" substrate, with 31.14 % (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 6-9).

5.1.4.4 Cystofilobasidium macerans (CCY 10-1-2)

The Table 52 presents the results for the strain *Cystofilobasidium macerans* cultivated at a C/N ratio of 25. The highest production of carotenoids (1.967 mg/g) and ubiquinone (10.939 mg/g) were observed in the "GLY" medium, indicating its potential for the synthesis of these antioxidants. The maximum ergosterol production was recorded in the "Pf+F.H." medium, reaching 9.932 mg/g, suggesting that the addition of F.H. significantly enhances sterol biosynthesis (in comparison to the "Pf" medium, which yielded 2.209 mg/g).

In terms of biomass growth, the "GLY+F.H. (F.H.)" substrate led to the highest production, reaching 16.6 g/l, highlighting the effectiveness of F.H. in supporting microbial growth. In the lipid composition analysis, the "Pf+F.H." substrate achieved the highest saturated fatty acid content with 57.59 %. The "GLY (F.H.)" substrate exhibited the highest proportion of monounsaturated fatty acids at 43.22 %, while the "GLY" substrate supported the greatest polyunsaturated fatty acid content, reaching 46.12 %. The highest total lipid content was observed in the "Pf+F.H." substrate, with a value of 31.88 % (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 10-11; Holub J. et al., 2022. *Proceedings of the 9th International Conference on Chemical Technology*. ISBN 978-80-88307-11-2 – see attachment 3 pg. 356-360).

Table 52: Results of cultivating Cystofilobasidium macerans on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	CM C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	1.967	10.939	2.707	8.10	30.08	23.79	46.12	16.20
Pf	0.775	3.905	2.209	7.30	26.88	38.35	34.77	18.62
Pf+G10	0.360	4.205	2.315	9.10	29.18	40.68	30.14	24.18
Pf+G25	0.374	3.466	2.358	12.10	51.53	30.70	17.77	29.73
GLY+F.H.	1.325	4.104	3.383	14.60	37.45	34.57	27.98	12.58
GLY (F.H.)	0.977	7.405	3.721	10.10	33.69	43.22	23.09	12.69
Pf+F.H.	0.273	4.060	9.932	12.60	57.59	24.87	17.54	31.88
Pf+G10+F.H.	0.330	3.142	5.455	14.30	44.48	29.95	25.57	27.18
Pf+G25+F.H.	0.554	7.951	6.587	15.10	39.97	33.15	26.89	25.06
GLY+F.H. (F.H.)	0.334	6.118	3.826	16.60	41.09	41.37	17.55	20.54

The Table 53 outlines the results for *Cystofilobasidium macerans* grown at a C/N ratio of 50. The "Pf+G10" substrate yielded the highest carotenoid production at 4.916 mg/g. Ubiquinone reached its peak concentration in the "GLY+F.H. (F.H.)" medium, with 9.864 mg/g, indicating that the combination of glycerol and feather hydrolysate boosts antioxidant production. The highest ergosterol content, 8.728 mg/g, was recorded in the "Pf+G10+F.H." substrate, suggesting a strong impact of feather hydrolysate on sterol synthesis.

Regarding the biomass growth, the "GLY (F.H.)" substrate provided the highest yield at 21.30 g/l. For lipid composition, the same substrate also exhibited the highest monounsaturated fatty acid content, reaching 58.02 %. The "Pf+G10" substrate showed the greatest polyunsaturated fatty acid content at 27.84 %, while the "GLY+F.H." medium had the highest saturated fatty acid content at 45.46 %. The highest total lipid content was found in the Pf+F.H. medium, with 26.46 % (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 11).

Table 53: Results of cultivating Cystofilobasidium macerans on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	CM C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	1.950	4.701	2.992	15.50	42.64	51.71	5.66	13.38
Pf	4.056	6.123	4.544	3.10	22.69	49.47	27.83	17.45
Pf+G10	4.916	5.819	4.746	9.70	24.13	48.03	27.84	16.40
Pf+G25	1.881	5.107	2.880	13.50	31.73	45.39	22.88	20.97
GLY+F.H.	1.726	7.815	3.737	14.20	45.46	42.83	11.71	14.85
GLY (F.H.)	0.593	8.228	3.602	21.30	36.03	58.02	5.95	22.62
Pf+F.H.	0.217	4.202	2.516	5.70	27.73	48.71	23.56	26.46
Pf+G10+F.H.	0.532	6.490	8.728	14.00	25.76	48.57	25.67	20.59
Pf+G25+F.H.	0.432	7.630	6.456	14.30	26.42	49.22	24.36	23.92
GLY+F.H. (F.H.)	0.412	9.864	3.142	17.50	38.84	53.39	7.77	18.95

5.1.4.5 Sporidiobolus metaroseus (CCY 19-6-20)

The Table 54 showcases the metabolic profiles, biomass, and lipid production of *Sporidiobolus metaroseus* cultured on various substrates with a C/N ratio of 25. The highest carotenoid production was observed in the "GLY+F.H. (F.H.)" medium, achieving 1.675 mg/g, while the maximum ubiquinone content was recorded in the "Pf+G25+F.H." substrate at 5.610 mg/g. The greatest ergosterol concentration, 4.295 mg/g, was produced in the "GLY" medium. In terms of biomass, the "Pf+G25+F.H." substrate led to the highest yield, reaching 15.20 g/l.

Examining the fatty acid profiles, the "GLY+F.H. (F.H.)" substrate exhibited the highest percentage of saturated fatty acids at 67.59 %, while the Pf substrate had the largest proportion of monounsaturated fatty acids at 42.44 %. The "GLY" substrate supported the highest concentration of polyunsaturated fatty acids, with 49.30 %. In terms of total lipid content, the "Pf+G25+F.H." substrate displayed the maximum lipid percentage at 36.41 %, making it the most effective medium for lipid accumulation (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 14-15).

Table 54: Results of cultivating Sporidiobolus metaroseus on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	SM C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	0.507	1.834	4.295	2.20	32.65	18.05	49.30	8.35
Pf	0.329	4.222	2.985	5.80	21.38	42.44	36.18	35.02
Pf+G10	0.428	3.269	3.065	7.70	26.65	40.82	32.53	19.99
Pf+G25	0.304	5.408	2.701	6.80	26.69	39.45	33.86	30.34
GLY+F.H.	0.953	4.507	3.757	1.40	45.48	17.94	36.59	12.22
GLY (F.H.)	1.247	5.490	3.084	7.20	34.32	28.99	36.69	11.99
Pf+F.H.	0.927	4.579	2.216	11.20	36.76	34.87	28.37	23.70
Pf+G10+F.H.	0.824	4.254	1.880	14.30	47.41	28.39	24.20	33.67
Pf+G25+F.H.	0.883	5.610	2.454	15.20	50.69	30.66	18.65	36.41
GLY+F.H. (F.H.)	1.675	3.208	3.346	13.50	67.59	19.05	13.35	23.56

The Table 55 presents the metabolic outcomes, biomass production, and lipid profiles for *Sporidiobolus metaroseus* cultured on various substrates with a C/N ratio of 50. The highest carotenoid (1.260 mg/g), ubiquinone (7.845 mg/g) and ergosterol (3.755 mg/g) contents were observed in the "GLY+F.H. (F.H.)" medium, highlighting the enhanced metabolic production on this substrate. In terms of biomass production, the "Pf+G25+F.H." substrate supported the highest growth, yielding 15.70 g/l.

In the lipid composition analysis, the "GLY" substrate exhibited the highest percentage of saturated fatty acids, at 67.55 %. The "GLY+F.H." substrate showed the greatest concentration of monounsaturated fatty acids, reaching 51.64 %, while the "GLY+F.H. (F.H.)" medium demonstrated the largest proportion of polyunsaturated fatty acids, with 41.70 %. In the "Pf+F.H." medium, the highest total lipid content was produced (44.23 %,) highlighting its efficiency for lipid accumulation (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 15).

Table 55: Results of cultivating Sporidiobolus metaroseus on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	SM C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	0.629	2.290	1.905	3.40	67.55	24.76	7.69	8.47
Pf	0.384	4.945	2.356	4.50	30.03	47.07	22.89	24.06
Pf+G10	0.469	4.855	0.326	8.30	36.69	38.39	24.91	23.85
Pf+G25	0.512	3.921	1.455	5.00	31.93	45.90	22.17	20.16
GLY+F.H.	0.587	3.103	0.509	4.00	24.58	51.64	23.78	10.98
GLY (F.H.)	0.931	6.130	2.810	4.20	34.09	26.10	39.81	13.28
Pf+F.H.	0.882	7.023	1.923	7.00	21.45	46.34	32.21	44.23
Pf+G10+F.H.	0.682	7.088	2.003	11.00	21.17	43.19	35.64	38.60
Pf+G25+F.H.	0.901	7.482	2.373	15.70	23.50	43.37	33.13	31.91
GLY+F.H. (F.H.)	1.260	7.845	3.755	7.20	27.55	30.75	41.70	14.87

5.1.4.6 *Rhodororula mucilaginosa (20-9-7)*

The Table 56 details the metabolic and biomass production results of *Rhodotorula mucilaginosa* (20-9-7) grown on various substrates at a C/N ratio of 25. The highest carotenoid content was observed in the “GLY” medium, reaching 2.256 mg/g, while the maximum ubiquinone concentration (5.988 mg/g) was recorded in the “GLY+F.H. (F.H.)” medium and also ergosterol production peaked in the “GLY+F.H. (F.H.)” substrate as well, at 4.969 mg/g. The “GLY (F.H.)” substrate led to the highest biomass yield of 17.80 g/l.

In the lipid profile, the highest proportion of saturated fatty acids was found in the “GLY (F.H.)” medium, reaching 68.90 %, while the “GLY+F.H.” substrate displayed the highest monounsaturated fatty acid content, at 42.35 %. The “GLY” substrate exhibited the highest concentration of polyunsaturated fatty acids, at 39.78 %. Additionally, the “Pf” medium demonstrated the highest total lipid content, with 46.79 % (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 14).

Table 56: Results of cultivating Rhodotorula mucilaginosa (20-9-7) on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	RM (20-9-7) C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	2.256	2.129	3.237	3.20	22.86	37.36	39.78	10.31
Pf	0.475	1.561	0.695	7.10	34.87	35.26	29.88	46.79
Pf+G10	1.324	3.379	2.152	6.80	31.02	39.08	29.90	31.56
Pf+G25	1.085	3.894	2.334	6.00	35.11	36.01	28.88	37.28
GLY+F.H.	1.297	3.574	3.649	11.20	52.15	42.35	5.50	20.55
GLY (F.H.)	0.323	3.662	4.945	17.80	68.90	18.43	12.67	24.24
Pf+F.H.	0.235	5.418	4.153	15.10	51.56	26.80	21.64	23.98
Pf+G10+F.H.	0.245	4.069	3.771	15.90	33.59	33.42	32.99	23.25
Pf+G25+F.H.	0.182	4.356	3.900	17.20	44.97	25.99	29.04	24.20
GLY+F.H. (F.H.)	0.227	5.988	4.969	15.70	32.19	38.06	29.75	15.18

The Table 57 provides an overview of the metabolic activities, biomass production, and lipid composition of *Rhodotorula mucilaginosa* (20-9-7) cultivated on various media with a C/N ratio of 50. The highest carotenoid content, 1.240 mg/g, was achieved in the “Pf+G10” medium, while the greatest ubiquinone concentration was observed in the “Pf+F.H.” medium, reaching 7.894 mg/g. The “Pf+F.H.” substrate also showed the highest ergosterol production, at 3.895 mg/g. Biomass growth was maximized in the “Pf+F.H.” medium as well, with 26.10 g/l, demonstrating its superiority for supporting cell proliferation and antioxidant accumulation.

In terms of lipid profiles, the highest percentage of saturated fatty acids was found in the “Pf+G25+F.H.” medium, with 54.29 %, while the “GLY” substrate displayed the highest monounsaturated fatty acid content, at 66.20 %. The “Pf+G25” medium showed the greatest polyunsaturated fatty acid content, with 30.12 %, indicating a balanced distribution of fatty acids in this medium. In addition, the “Pf+G25+F.H.” medium exhibited the highest total lipid content, reaching 34.59 % Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 14).

Table 57: Results of cultivating Rhodotorula mucilaginosa (20-9-7) on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	RM (20-9-7) C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	1.201	1.792	2.472	5.90	33.80	66.20	0.00	6.81
Pf	1.113	5.763	2.402	8.50	30.45	43.40	26.15	30.20
Pf+G10	1.240	6.084	2.195	8.30	30.97	43.19	25.84	28.34
Pf+G25	1.121	5.371	2.147	8.70	27.40	42.48	30.12	32.85
GLY+F.H.	0.943	4.772	3.328	6.60	35.32	64.68	0.00	6.76
GLY (F.H.)	0.212	1.572	3.441	16.80	25.39	55.64	18.97	12.67
Pf+F.H.	0.407	7.894	3.895	26.10	36.78	39.28	23.94	28.06
Pf+G10+F.H.	0.339	5.450	3.704	22.00	40.18	33.76	26.06	33.34
Pf+G25+F.H.	0.905	5.516	3.104	24.80	54.29	25.78	19.93	34.59
GLY+F.H. (F.H.)	0.813	3.674	2.993	12.30	26.40	59.06	14.53	10.10

5.1.4.7 Rhodotorula kratochvilovae (CCY 20-2-26)

The Table 58 presents the metabolic activities, biomass growth, and lipid composition of *Rhodotorula kratochvilovae* cultivated on various substrates at a C/N ratio of 25. The highest carotenoid production was observed in the “GLY” substrate, yielding 1.602 mg/g, while the same medium also supported the most significant ubiquinone production at 10.495 mg/g. The maximum ergosterol content, 3.778 mg/g, was recorded in the “GLY+F.H.” substrate. The highest biomass accumulation was observed in the “Pf+G25” medium, producing 19.20 g/l, highlighting its efficiency for microbial growth.

Table 58: Results of cultivating Rhodotorula kratochvilovae on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 25

	RK C/N 25 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	1.602	10.495	3.443	8.10	43.56	28.89	27.55	15.42
Pf	0.258	5.669	0.796	5.30	33.31	43.14	23.55	21.06
Pf+G10	0.558	5.161	2.025	17.70	27.85	46.40	25.75	21.11
Pf+G25	0.338	4.559	1.163	19.20	35.23	41.85	22.92	21.66
GLY+F.H.	1.442	3.672	3.778	3.10	24.61	39.03	36.37	7.42
GLY (F.H.)	0.287	5.011	3.416	13.70	39.29	27.87	32.83	9.81
Pf+F.H.	0.169	2.960	1.373	14.10	39.32	37.89	22.79	40.82
Pf+G10+F.H.	0.331	4.843	2.383	13.90	26.96	44.81	28.23	32.07
Pf+G25+F.H.	0.313	6.238	2.455	15.30	27.71	45.28	27.01	28.10
GLY+F.H. (F.H.)	0.427	5.342	2.958	12.20	48.08	33.36	18.56	15.64

In terms of fatty acid composition, the “GLY+F.H. (F.H.)” medium exhibited the highest proportion of saturated fatty acids, reaching 48.08 %. The “Pf+G10” medium showed the greatest monounsaturated fatty acid content at 46.40 %, while the “GLY+F.H.” substrate displayed the highest level of polyunsaturated fatty acids, with 36.37 %. Regarding total lipid content, the “Pf+F.H.” substrate reached the highest value, at 40.82 %, demonstrating its potential for maximizing lipid yields (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 9; Holub J. et al., 2022. *Proceedings of the 9th International Conference on Chemical Technology*. ISBN 978-80-88307-11-2 – see attachment 3 pg. 356-360).

The Table 59 provides a comprehensive overview of the metabolic activity, biomass production, and lipid composition of *Rhodotorula kratochvilovae* cultivated on various substrates at a C/N ratio of 50. The highest carotenoid content, 1.324 mg/g, was achieved in the "GLY (F.H.)" medium, while the most significant ubiquinone concentration, 15.505 mg/g, was recorded in the "GLY+F.H. (F.H.)" substrate. The highest ergosterol content was observed in the "Pf+G10+F.H." substrate, reaching 11.584 mg/g. In terms of biomass production, the "Pf+G25+F.H." medium supported the highest yield, producing 21.50 g/l, indicating its superior ability to promote growth.

For the lipid profiles, the "GLY" substrate exhibited the highest proportion of saturated fatty acids, at 47.36 %. The "GLY (F.H.)" medium contained the largest proportion of monounsaturated fatty acids, with 58.71 %, whereas the "Pf+G10+F.H." substrate showed the highest polyunsaturated fatty acid content, at 33.99 %. Additionally, the "Pf+G25+F.H." medium had the highest total lipid content, reaching 37.87 %, making it the most effective substrate for lipid accumulation (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 10).

Table 59: Results of cultivating Rhodotorula kratochvilovae on substrates consisting of poultry fat, glycerol, and feather hydrolysate, with C/N ratio of 50

	RK C/N 50 Pf/F.H. + glycerol							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
GLY	1.197	5.702	3.705	11.60	47.36	29.82	22.82	17.43
Pf	0.391	3.959	1.481	18.70	34.29	46.24	19.47	23.14
Pf+G10	0.430	4.749	1.628	18.60	29.45	45.95	24.60	23.90
Pf+G25	0.579	4.742	1.891	21.20	30.52	43.87	25.61	23.68
GLY+F.H.	0.811	4.211	3.294	12.70	45.81	35.92	18.27	17.80
GLY (F.H.)	1.324	13.149	5.442	17.00	35.94	58.71	5.35	14.23
Pf+F.H.	0.506	4.831	5.634	11.50	27.61	49.50	22.89	26.59
Pf+G10+F.H.	0.511	8.306	11.584	16.70	22.44	43.57	33.99	15.45
Pf+G25+F.H.	0.572	8.332	4.937	21.50	25.70	47.88	26.41	37.87
GLY+F.H. (F.H.)	0.488	15.505	5.057	15.50	36.77	56.62	6.62	17.77

5.1.4.8 Biomass comparison

Figure 29 illustrates the biomass growth (g/l) of various yeast strains, denoted as *SP*, *RM (19-4-6)*, *RT*, *CM*, *SM*, *RK*, and *RM (20-9-7)*, cultured at a C/N ratio of 25 on different media. For the *SP* strain, the highest biomass accumulation was observed on the "Pf+F.H." medium, yielding 16.10 g/l, while the *RM (19-4-6)* strain demonstrated its maximum biomass of 29.50 g/l on the "Pf" medium, indicating that poultry fat (Pf) serves as an optimal substrate for this strain. The *RT* strain also achieved its highest biomass on the "Pf" medium, with 20.40 g/l, reflecting similar trends in these yeast strains.

For the *CM* strain, the most significant biomass growth was observed on the “GLY+F.H. (F.H.)” substrate, yielding 16.60 g/l, suggesting a strong influence of feather hydrolysate. The amount of carbon and nitrogen substrates for a volume of 1000 ml and C/N ratios of 25 and 50, for a series of experiments without the addition of feather hydrolysate on biomass production for this strain. The *SM* strain exhibited the highest growth on the “Pf+G25+F.H.” medium, with 15.20 g/l, while the *RK* strain demonstrated maximum biomass production of 19.20 g/l on the “Pf+G25” medium. Finally, the *RM (20-9-7)* strain achieved the highest biomass of 17.80 g/l on the “GLY (F.H.)” medium, underscoring glycerol’s role in promoting biomass growth when combined with feather hydrolysate.

A notable trend across the strains is the consistent positive impact of media containing combinations of poultry fat (Pf) and feather hydrolysate (F.H.) on biomass production. Substrates such as “Pf+F.H.”, “Pf+G10+F.H” and “Pf+G25+F.H.” frequently led to higher biomass yields, particularly in strains such as *SM*, *CM*, *RM (20-9-7)*, and *SP*. Additionally, glycerol-based substrates like “GLY (F.H.)” also demonstrated favorable results, especially for the *RM (20-9-7)* and *CM* strains. These findings highlight the importance of optimizing substrate composition for maximizing biomass production across different yeast strains (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2).

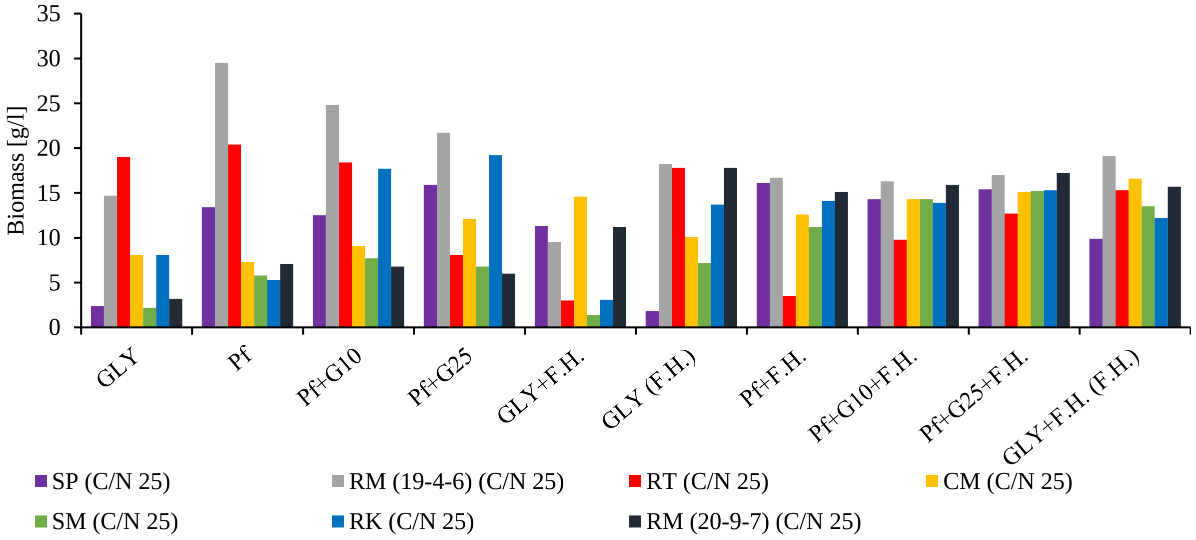


Figure 29: Comparison of biomass growths between yeast strains, grown on media with C/N 25

Figure 30 presents biomass growth data (g/l) for various yeast strains, including *SP*, *RM* (19-4-6), *RT*, *CM*, *SM*, *RK*, and *RM* (20-9-7), cultured under a C/N ratio of 50 on different media. Among the *SP* strain, the highest biomass production was observed on the “Pf+G10+F.H.” medium, yielding 30.10 g/l. For the *RM* (19-4-6) strain, the “Pf+F.H.” and “Pf+G25+F.H.” substrates achieved the highest values, both reaching 28.30 g/l, suggesting an optimal growth environment for this strain in these media. In the *RT* strain, the “GLY” medium led to the greatest biomass accumulation at 24.00 g/l, indicating glycerol’s role as a highly effective substrate for this strain. For the *CM* strain, biomass peaked at 21.30 g/l on the “GLY (F.H.)” medium, while the *SM* strain showed its highest growth on the “Pf+G25+F.H.” substrate, yielding 15.70 g/l. The *RK* strain demonstrated maximum biomass production of 21.50 g/l in the “Pf+G25+F.H.” medium, highlighting the efficiency of this combination for this specific strain. Finally, the *RM* (20-9-7) strain produced the highest biomass in the “Pf+F.H.” medium, reaching 26.10 g/l.

A notable trend observed in this dataset is the consistently high biomass yields for multiple strains when grown on media containing a combination of poultry fat (Pf) and feather hydrolysate (F.H.), particularly the “Pf+G25+F.H.” and “Pf+F.H.” substrates. These results indicate that the inclusion of both poultry fat and feather hydrolysate synergistically enhances biomass production across various yeast strains. Additionally, glycerol-based media such as “GLY” and “GLY (F.H.)” also show significant biomass-promoting effects, especially for strains like *RT* and *CM*, highlighting glycerol’s versatility as a substrate for microbial growth (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2).

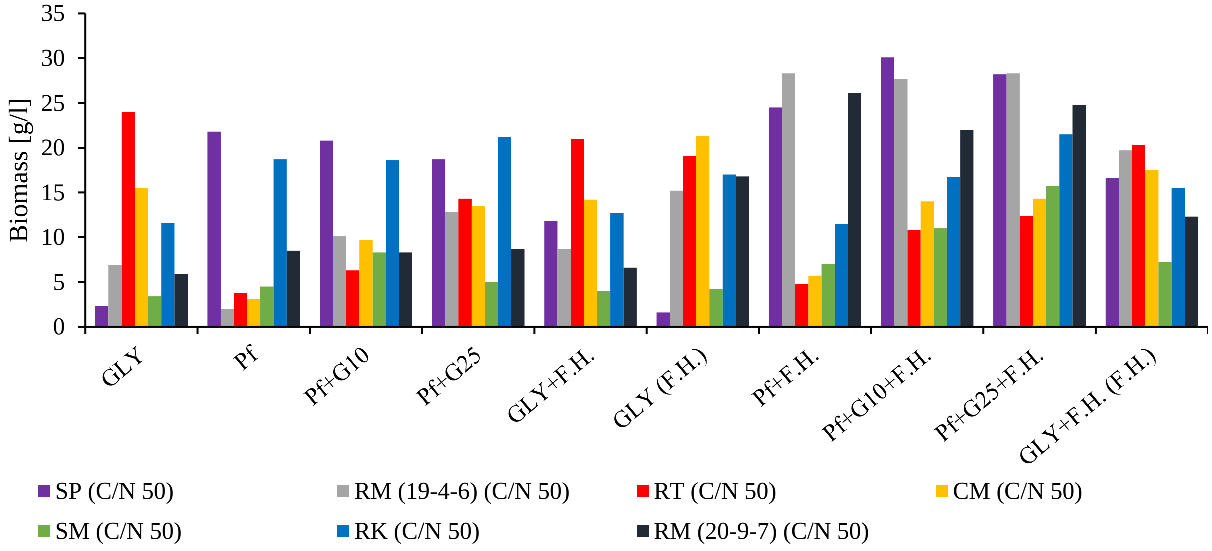


Figure 30: Comparison of biomass growths between yeast strains, grown on media with C/N 50

5.1.4.9 Rhodosporidium toruloides (CCY 62-2-4) – cultivation in bioreactor

The cultivation in the bioreactor was carried out with an operational volume of 2.2 liters, including the inoculum (with an inoculation ratio of 1:10). The composition of the media including feather hydrolysate and glycerol is shown in Table 60. The yeast strain tested was *Rhodosporidium toruloides*. During this cultivation, neither the carbon nor nitrogen concentrations were doubled, as was typically the case in other bioreactor cultivations.

Table 60: Composition of bioreactor medium (per 1000 ml) including feather hydrolysate

Substance	**Amount**
KH_2PO_4	4 g
$MgSO_4 \cdot 7H_2O$	0.696 g
Glycerol (g)	46.26 g
Feather hydrolysate	45 ml

Figure 31 and Figure 32 summarize the bioreactor cultivation of *Rhodosporidium toruloides* using glycerol and feather hydrolysate as carbon and nitrogen sources. The data tracks the production of key metabolites, biomass, lipid content, and fatty acid composition over a cultivation period of 144 hours. The maximum carotenoid content of 1.602 mg/g was observed after the inoculation (0, while the highest ubiquinone concentration of 12.059 mg/g occurred after 144 hours of cultivation. The peak of ergosterol production was recorded at 96 hours, reaching 5.184 mg/g. Biomass accumulation steadily increased throughout the cultivation, reaching a maximum of 34.92 g/l at 144 hours. Regarding lipid profiles, the highest saturated fatty acid content was observed at 72 hours, with 48.08 %. The maximum monounsaturated fatty acid content of 46.40 % was recorded at 5 hours, while polyunsaturated fatty acids peaked at 36.37 % after 24 hours. Total lipid content exhibited fluctuations during the cultivation, with the highest value of 40.82 % at 44 hours, followed closely by 36.93 % at 96 hours.

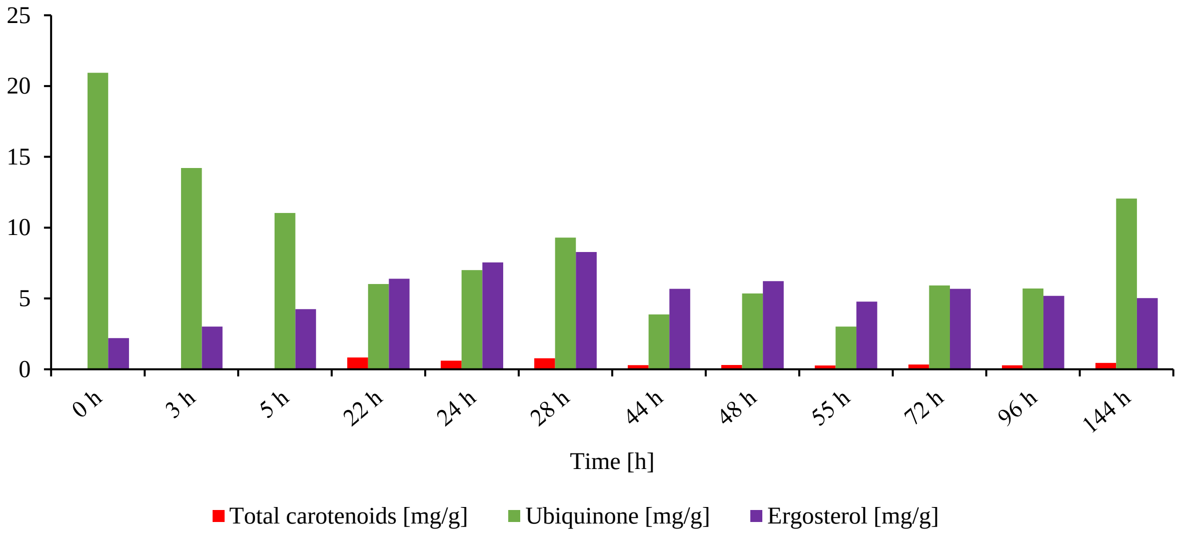


Figure 31: The production of antioxidants during the bioreactor cultivation of the yeast strain Rhodosporidium toruloides using glycerol with combination feather hydrolysate media

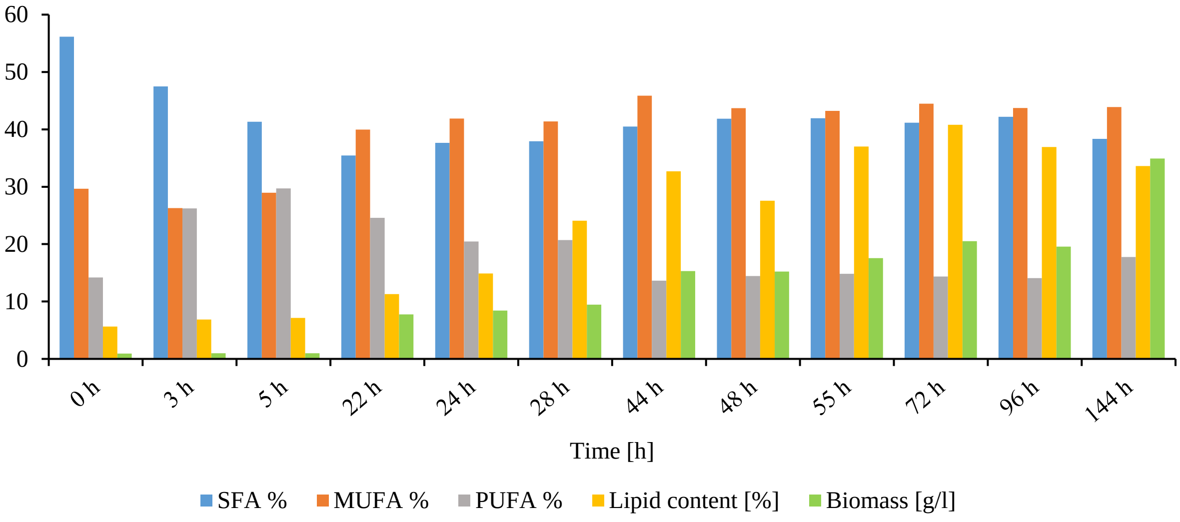


Figure 32: The accumulation of lipids and biomass growth during the bioreactor cultivation of the yeast strain Rhodosporidium toruloides using glycerol with combination feather hydrolysate media

A clear trend observed in the data is the steady increase in biomass as cultivation progressed, peaking after 144 hours. This biomass increase corresponds with rising ubiquinone and ergosterol concentrations during later stages of growth. Lipid accumulation showed a distinct pattern, with a shift toward higher SFA and MUFA content as cultivation time progressed. Initially, the medium supported higher MUFA levels, with balanced levels of SFA and PUFA contents, which gradually gave way to increased SFA proportions over time. These trends highlight the dynamic metabolic shifts in *Rhodosporidium toruloides* as it adapts to the glycerol and feather hydrolysate substrates throughout the bioreactor cultivation (Holub J. et al., 2023. *Microorganisms*. *11*(2), 321; DOI: 10.3390/microorganisms11020321 – see attachment 2 pg. 8-10).

5.1.5 Comparison of whey and hydrolysed poultry feathers as nitrogen sources

The following experiment investigated the use of various waste-derived nitrogen and carbon sources and their combinations in the cultivation of carotenoid-producing yeasts, while maintaining a constant carbon-to-nitrogen (C/N) ratio of 25. The mineral base of the media, along with the dosages of individual substrates, are presented in Table 61, Table 62, Table 63, with Table 64 outlining the nomenclature used for the individual media. Based on nitrogen determination using the Kjeldahl method (chapter 4.6.1) in combination with elemental CHNS analysis (as employed in[139]), the elemental ratios of key macronutrients in the waste nitrogen substrates were determined. Using this data, the appropriate amounts of carbon substrates were calculated and added to achieve the desired C/N ratio of 25. For the control medium using urea as the nitrogen source, the precise quantities of carbon substrates were weighed according to the specifications listed Table 63. The results of Chapter 5.1.5 are partially discussed in: Holub et al., Cultivation of carotenogenic yeast on various combination of waste nitrogenous and carbonaceous substrates. *Poster presentation; Eurobiotech Congress 2024*, 3.10.-5.10.2024, Istanbul - Turkey; Holub et al., 2024. *Book of abstracts of the 11th International Conference on Chemical Technology*. ISBN 978-80-88214-48-9.

Table 61: Mineral base of media of following experiments

Substance	**Amount**
KH_2PO_4	4 g
$MgSO_4 \cdot 7H_2O$	0.696 g
Tap Water	1000 ml

Table 62: Nitrogen and partly carbon substrates used in following experiments

Substance	**Amount**
Urea	1.81 g
Whey	745.6 ml
Feather hydrolysate	45 ml

Table 63: Carbon substrates used in control experiments

Substance	**Amount [g]**
Glycerol	46.26
Poultry fat	6.2
Frying oil	6.2
Glucose	45.24

Table 64: Descriptions of the individual combinations of carbon and nitrogen substrates in the media

Nitrogen source	Medium	Shortcut
Urea	Glycerol (control)	Gly(Ur)
	Poultry fat	Pf(Ur)
	Friying oil (waste)	Fo(Ur)
	Glucose	Glu(Ur)
Whey	Glycerol (control)	Gly(Wh)
	Fat	Pf(Wh)
	Friying oil (waste)	Fo(Wh)
	Glucose	Glu(Wh)
Hydrolysed poultry feathers	Glycerol (control)	Gly(F.H.)
	Poultry fat	Pf(F.H.)
	Friying oil (waste)	Fo(F.H.)
	Glucose	Glu(F.H.)

5.1.5.1 Rhodosporidium toruloides (CCY 62-2-4)

The Table 65 presents a detailed analysis of various media and nitrogen source combinations on the growth and metabolic outputs of *Rhodosporidium toruloides*. The highest carotenoid production is observed in the medium Glucose with Urea (Glu(Ur)), reaching a concentration of 5.701 mg/g. For ubiquinone, glycerol with urea (Gly(Ur)) produces the highest value (12.984 mg/g), while glucose with whey (Fo(Ur)) yields the highest ergosterol concentration (10.429 mg/g). These findings highlight the impact of medium composition on antioxidant yields.

Table 65: Results of the yeast strain Rhodosporidium toruloides on various nitrogenous and carbonaceous substrates

	Rhodosporidium toruloides							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
Gly(Ur)	3.603	12.984	3.640	14.35	31.31	57.74	10.95	16.65
Pf(Ur)	2.119	5.820	6.368	1.65	22.37	49.80	27.83	13.66
Fo(Ur)	4.475	3.855	10.429	1.85	7.83	64.62	27.55	6.16
Glu(Ur)	5.701	9.409	4.035	15.00	26.43	62.00	11.58	13.26
Gly(Wh)	3.636	5.456	4.788	10.75	47.65	38.86	13.50	21.82
Pf(Wh)	4.245	3.379	5.979	4.95	28.76	45.34	25.90	20.72
Fo(Wh)	2.289	10.476	5.710	8.70	13.72	60.52	25.77	19.81
Glu(Wh)	5.109	4.666	7.142	8.10	33.84	32.72	33.44	9.51
Gly(F.H.)	4.325	5.594	4.622	8.40	41.06	44.15	14.78	20.47
Pf(F.H.)	0.820	9.430	3.119	3.80	26.79	46.35	26.86	16.36
Fo(F.H.)	3.779	6.612	5.600	2.65	12.95	56.75	30.30	13.64
Glu(F.H.)	3.561	8.912	5.402	12.25	37.99	38.61	23.40	15.15

For biomass production, the combination of Glucose with Urea (Glu(Ur)) is the most effective, resulting in 15.00 g/l. Regarding lipid composition, the highest monounsaturated fatty acids are found in the Frying Oil with Urea (Fo(Ur)) medium, accounting for 64.62 % of the total fatty acids. In contrast, the highest polyunsaturated fatty acids content is observed in the Glucose with Whey (Glu(Wh)) medium at 33.44 %. Saturated fatty acids are most prevalent in the Glycerol with Whey (Gly(Wh)) medium, constituting 47.65 % of the total fatty acids. The lipid percentage is maximized in the Glycerol with Whey (Gly(Wh)) medium, reaching 21.82 % (Holub et al., 2024. *Book of abstracts of the 11th International Conference on Chemical Technology*. ISBN 978-80-88214-48-9 – see attachment 4 pg. 201).

5.1.5.2 Cystofilobasidium macerans (CCY 10-1-2)

The Table 66 outlines the effects of different nitrogen sources and media on the metabolic activity of *Cystofilobasidium macerans*. Notably, the highest carotenoid production is recorded in the medium Frying Oil with Urea (Fo(Ur)), achieving 2.954 mg/g, while the maximum ubiquinone concentration is found in the Frying Oil with Hydrolysed Poultry Feathers (Fo(F.H.)), reaching 14.317 mg/g. For ergosterol, the most significant yield is observed in Frying Oil with Urea (Fo(Ur)), with a concentration of 7.285 mg/g. These results indicate a strong influence of specific medium components on antioxidant production.

When considering biomass production, the Poultry Fat with Whey (Pf(Wh) medium leads with 18.80 g/l, closely followed by the Glucose with Urea (Glu(Ur)) and Glucose with Whey (Glu(Wh)) media, producing 18.00 g/l and 18.20 g/l, respectively. Regarding lipid composition, the highest monounsaturated fatty acids content is noted in Frying Oil with Urea (Fo(Ur)), constituting 60.94 % of the total fatty acids. Polyunsaturated fatty acids are most abundant in the Glycerol with Urea (Gly(Ur)) medium at 44.67 %. Saturated fatty acids are maximized in Poultry Fat with Whey (Pf(Wh)) at 41.16 %. The percentage of lipids is greatest in the Poultry Fat with Hydrolysed Poultry Feathers (Pf(F.H.)) medium, reaching 24.34 %.

Table 66: Results of the yeast strain Cystofilobasidium macerans on various nitrogenous and carbonaceous substrates

	Cystofilobasidium macerans							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
Gly(Ur)	0.960	4.440	4.572	7.25	25.42	29.91	44.67	11.78
Pf(Ur)	1.738	6.922	5.838	7.15	37.43	38.22	24.35	17.33
Fo(Ur)	2.954	7.870	7.285	7.25	10.06	60.94	29.00	9.35
Glu(Ur)	1.694	5.435	4.664	18.00	29.76	53.95	16.29	11.76
Gly(Wh)	2.683	11.803	3.207	7.85	24.82	31.32	43.87	14.19
Pf(Wh)	1.266	6.904	3.640	18.80	41.16	32.86	25.98	18.38
Fo(Wh)	1.559	6.873	3.044	17.60	13.88	53.46	32.66	13.29
Glu(Wh)	0.674	6.295	3.359	18.20	23.29	46.98	29.74	14.19
Gly(F.H.)	1.821	10.036	4.041	6.65	22.82	34.45	42.73	14.13
Pf(F.H.)	1.026	10.626	3.892	3.10	30.14	43.65	26.22	24.34
Fo(F.H.)	1.028	14.317	4.335	2.80	12.32	56.77	30.91	14.47
Glu(F.H.)	1.928	9.818	4.581	15.70	37.12	43.94	18.94	19.70

5.1.5.3 Phaffia rhodozyma (CCY 77-1-1)

The Table 67 provides an overview of the metabolic performance of *Phaffia rhodozyma* under various nitrogen sources and media conditions. The highest carotenoid content is achieved in the Glycerol with Urea (Gly(Ur)) medium, measuring 6.366 mg/g, indicating the potential of this medium for carotenoid biosynthesis. The maximum ubiquinone concentration is observed in Frying Oil with Hydrolysed Poultry Feathers (Fo(F.H.)), reaching a significant 25.416 mg/g. In terms of ergosterol, the highest value of 6.290 mg/g is also recorded in the Frying Oil with Hydrolysed Poultry Feathers (Fo(F.H.)) medium, demonstrating the enhanced sterol production under these conditions. For biomass production, the Glucose with Hydrolysed Poultry Feathers (Glu(F.H.)) medium is the most effective, yielding 19.30 g/l, closely followed by Glycerol with Urea (Gly(Ur)) and Glycerol with Whey (Gly(Wh)), both showing substantial biomass yields of 19.10 g/l and 19.00 g/l, respectively.

Examining the lipid composition, the highest monounsaturated fatty acids content is found in the Glycerol with Whey (Gly(Wh)) medium at 62.94 %, suggesting an optimal condition for MUFA synthesis. The greatest proportion of polyunsaturated fatty acids is detected in the Poultry Fat with whey (Pf(Wh)) medium, accounting for 39.42 % of the total fatty acids, while the saturated fatty acids are most abundant in the Glycerol with Urea (Gly(Ur)) medium at 40.08 %. The highest total lipid content is found in the Poultry Fat with Hydrolysed Poultry Feathers (Pf(F.H.)) medium, with a lipid percentage of 24.02 %.

Table 67: Results of the yeast strain Phaffia rhodozyma on various nitrogenous and carbonaceous substrates

	Phaffia rhodozyma C/N 25							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
Gly(Ur)	6.366	3.191	3.960	19.10	40.08	42.79	17.14	13.75
Pf(Ur)	1.378	4.856	5.403	4.70	39.28	39.81	20.91	23.25
Fo(Ur)	0.510	5.608	5.183	4.50	15.06	60.74	24.20	18.44
Glu(Ur)	1.871	5.584	4.056	15.90	36.28	38.08	25.64	10.29
Gly(Wh)	4.570	6.230	3.533	19.00	18.46	62.94	18.60	8.81
Pf(Wh)	2.175	12.732	3.378	9.85	25.03	35.55	39.42	13.18
Fo(Wh)	1.381	5.599	4.963	10.00	10.84	51.28	37.88	11.05
Glu(Wh)	1.679	11.475	3.526	10.75	23.24	45.25	31.52	9.00
Gly(F.H.)	2.686	3.431	2.933	17.30	16.68	57.59	25.74	10.01
Pf(F.H.)	3.379	6.945	4.139	17.30	24.18	41.28	34.54	24.02
Fo(F.H.)	3.984	25.416	6.290	1.40	10.91	61.11	27.97	14.38
Glu(F.H.)	3.598	5.668	3.301	19.30	23.87	56.31	19.81	17.60

5.1.5.4 Biomass comparison

The Figure 33 presents the biomass growth across three species (*RT*, *CM*, *PR*) in different media combinations, highlighting the varying effectiveness of each nitrogen source and medium in promoting cellular proliferation. For *RT*, the highest biomass growth is observed in the Glucose with Urea (Glu(Ur)) medium, achieving 15.00 g/l, indicating a slight growth-promoting effect under these conditions.

For *CM*, the combination of poultry fat with whey (Pf(Wh)) produces the most substantial biomass growth, reaching 18.80 g/l. This suggests that this specific nitrogen source and medium combination is highly conducive to the growth of *CM*. Similarly, *PR* shows the highest biomass accumulation in the glucose with hydrolysed poultry feathers (Glu(F.H.)) medium at 19.30 g/l.

The trends across the figure suggest that glucose-based media (Glu) generally supports higher biomass accumulation, especially in combination with Urea (Ur) or Hydrolysed poultry feathers (F.H.) as nitrogen sources. Additionally, the *CM* strain demonstrates significantly higher biomass growth in poultry fat (Pf) medium combined with whey (Wh), indicating a preference for lipid-richer substrates. Conversely, the Fo(Ur) and Fo(F.H.) media show the lowest biomass production for all strains, indicating that frying oil might be less suitable as a lipidic substrate for these yeast strains.

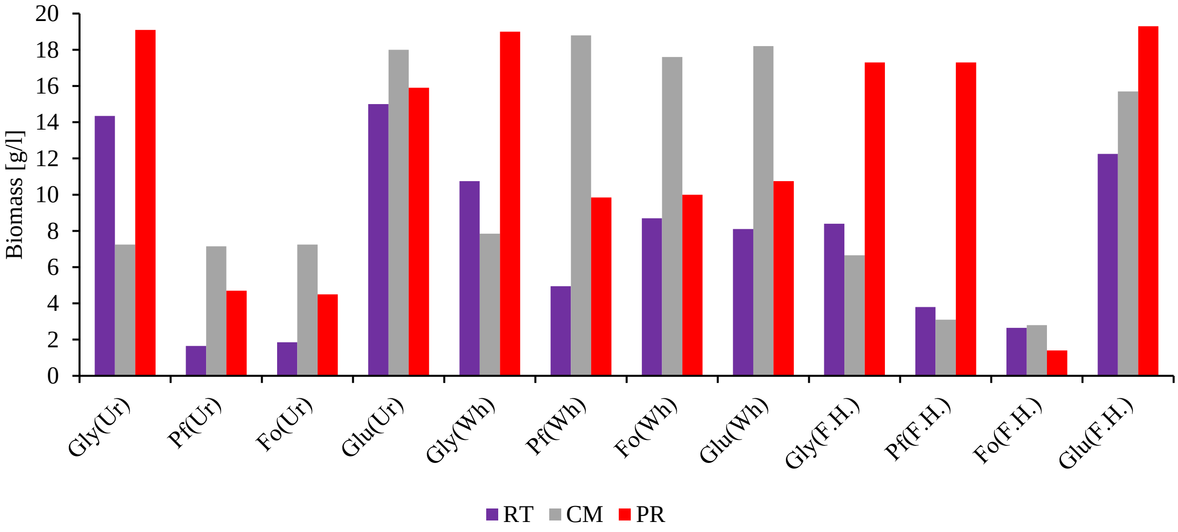


Figure 33: Comparison of biomass growths between yeast strains, grown on media with various nitrogen sources

5.1.6 Brewer’s yeast and spent grain

In the following experiments, the cultivation of carotenoid-producing yeasts was tested using hydrolysate from spent grain mash as a carbon substrate, in comparison to glucose and glycerol as controls (see Table 68), under conditions with a threefold increase in nitrogen concentration in the medium. Subsequently, the effect of adding yeast hydrolysate to the medium, replacing commercially added nitrogen, was evaluated on glycerol and glucose (see Table 69) Additionally, yeast hydrolysate was tested as a complete substitute for nitrogen in the medium.

Furthermore, two types of yeast autolysates, produced from commercial granulated yeast and fresh waste yeast from a local brewery, were tested on *Rhodosporidium toruloides (CCY 62-2-4)*. The impact of a threefold increase in nitrogen compared to standard media was also investigated, using a combination of nitrogen salts – potassium nitrate and ammonium sulphate (see Table 70). The comparative analysis focused on the potential of these alternative nitrogen sources to enhance yeast growth and metabolite production.

Table 68: Carbon substrates used in experiments, C/N ratios in brackets refers to standard media composition

No. and Label	**Substance**	**Amount [g]**
1GLY	Glycerol (C/N 50)	92.51
2GLY	Glycerol (C/N 25)	46.26
3GLU	Glucose (C/N 50)	90.48
4GLU	Glucose (C/N 25)	45.26
5HM	Hydrolysed grain mash (C/N 50)	90.48
6HM	Hydrolysed grain mash (C/N 25)	45.26

Table 69: Basic composition of production media used for carbonaceous substrate comparison

Substance	**Amount**
Tap water	1000 ml
KH_2PO_4	4 g
$MgSO_4 \cdot 7H_2O$	1.392 g
$(NH_4)_2SO_4$	3.98 g
$NaNO_3$	10.37 g
Yeast autolysate	2 g
Microelement solution	10 ml

Table 70: Inorganic and nitrogen composition of media per 1000 ml used for comparison cultivations on different nitrogenous substrates.(1N – standard nitrogen amount, 3N – triple nitrogen amount, Add – addition of autolysate -as is in Table 69, CA – commercial autolysate, SY – Spent brewery yeast autolysate, GR – granulated yeast autolysate, Cont – control media)

Label	**Amount [g]**					**Amount [ml]**	
	KH_2PO_4	$MgSO_4 \cdot 7H_2O$	$(NH_4)_2SO_4$	$NaNO_3$	CA	gran. CA	STB CA
Cont (1N+Add)	4	1.392	1.33	3.42	2	-	-
GR(Add)	4	1.392	1.33	3.42	-	130.334	-
SY(1N+Add)	4	1.392	1.33	3.42	-	-	33.318
SY(1N)	4	1.392	-	-	-	-	166.646
GR(1N)	4	1.392	-	-	-	672.832	-
SY(3N+Add)	4	1.392	3.98	10.37	-	-	33.318
SY-3N	4	1.392	-	-	-	-	433.3
Cont (3N+Add)	4	1.392	3.98	10.37	2	-	-

5.1.6.1 Sporidiobolus pararoseus (CCY 19-9-6)

Based on Table 71, among the metabolites, the highest carotenoid concentration is found in the SP-5HM condition (1.039 mg/g), while SP-5HM also shows the highest ubiquinone level (12.833 mg/g). The largest ergosterol accumulation occurs in SP-1GLY (7.208 mg/g). Biomass yield is maximized in SP-5GLU (9.00 g/l), indicating that this glucose-based medium supports higher biomass productivity compared to other media. In terms of lipid content, SP-3GLU exhibits the highest lipid percentage (11.70 %). Regarding the fatty acid composition, the highest SFA content is observed in SP-2GLY

(45.17 %), while SP-3GLU displays the greatest MUFA content at 87.19 %. The highest PUFA proportion is found in SP-1GLY (25.08 %), highlighting this condition as the richest in PUFA.

Table 71: Results of cultivations of Sporidiobolus pararoseus on substrates consisting of hydrolysed mash, glycerol, glucose and triple amount of nitrogenous source with commercially available autolysate addition

	Sporidiobolus pararoseus							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
SP-1GLY	0.874	7.235	7.208	6.800	6.464	68.460	25.076	5.632
SP-2GLY	0.987	7.547	6.255	6.775	45.174	37.270	17.556	11.014
SP-3GLU	0.772	5.782	3.867	8.675	6.122	87.187	6.691	11.701
SP-5GLU	0.179	3.103	2.833	9.000	7.107	84.718	8.175	9.524
SP-5HM	1.039	12.833	5.138	5.800	4.649	72.782	22.570	6.014
SP-6HM	0.465	7.466	3.406	5.750	7.733	80.572	11.695	10.827

A noticeable trend emerges when comparing the different media types and C/N ratios. Media based on glucose, particularly SP-3GLU and SP-5GLU, tend to yield higher lipid content and MUFA percentages, while glycerol-based media such as SP-1GLY show higher PUFA levels. Hydrolysed mash media (SP-5HM and SP-6HM) generally produce lower biomass and lipid content, but SP-5HM has a notable carotenoid and ubiquinone concentration compared to other media. This suggests that media composition and the C/N ratio significantly influence the metabolic and lipid profiles of the samples.

5.1.6.2 Rhodosporidium toruloides (CCY 62-2-4)

Based on the analysis of the biomass of the yeast strain *Rhodosporidium toruloides*, presented in the Table 72, it is evident that different substrates significantly influence the production of antioxidants, biomass, and lipid composition. The highest concentration of carotenoids was observed in sample RT-1GLY, with a value of 2.636 mg/g, suggesting that glycerol is particularly effective in enhancing carotenoid synthesis as in other types of media carotenoid production was beyond limit of detection. The ubiquinone concentration peaked in the RT-1GLY sample at 4.100 mg/g, indicating that glycerol also promotes production of this antioxidant. The highest ergosterol content was found in the RT-5HM sample, reaching 3.857 mg/g.

Table 72: Results of cultivations of Rhodosporidium toruloides on substrates consisting of hydrolysed mash, glycerol, glucose and triple amount of nitrogenous source with commercially available autolysate addition

	Rhodosporidium toruloides							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
RT-1GLY	2.636	4.100	2.903	8.90	35.08	58.74	6.18	12.17
RT-2GLY	1.011	2.272	2.732	10.50	39.23	56.05	4.73	13.80
RT-3GLU	n.a.	2.714	3.100	7.65	33.93	58.13	7.94	9.65
RT-5GLU	n.a.	1.877	2.827	8.65	42.46	51.89	5.66	11.44
RT-5HM	n.a.	2.963	3.857	7.45	57.59	35.26	7.15	10.74
RT-6HM	0.100	3.493	3.636	9.10	25.37	64.62	10.01	7.80

Regarding biomass production, the RT-2GLY sample achieved the highest yield with 10.50 g/l, demonstrating the potential of glycerol to foster substantial biomass accumulation under the tested conditions. In terms of lipid composition, the sample RT-5HM exhibited the highest percentage of saturated fatty acids at 57.59 %. Monounsaturated fatty acids were most prevalent in the RT-6HM sample, which achieved a concentration of 64.62 %, while polyunsaturated fatty acids reached their highest percentage in the RT-6HM sample at 10.01 %. The total lipid content was highest in the RT-2GLY sample, measuring 13.80 %.

5.1.6.3 Rhodotorula mucilaginosa (CCY 20-9-7)

Table 73 presents data for the yeast *Rhodotorula mucilaginosa* (20-9-7). Among the metabolites, the highest carotenoid concentration is found in the RM-4GLU condition (2.306 mg/g), while RM-4GLU also shows the highest ubiquinone level (5.293 mg/g). The maximum ergosterol content is present in RM-1GLY (4.978 mg/g). For biomass production, the highest yield (10.25 g/l) is recoded in medium RM-6HM, where the highest lipid content is also observed (7.84 %).

In terms of fatty acid composition, RM-6HM exhibits the highest SFA proportion (20.65 %), while RM-3GLU displays the greatest MUFA level at 74.14 %. The highest polyunsaturated fatty acids concentration is seen in RM-1GLY (28.30 %). Overall, hydrolysed mash media (RM-5HM and RM-6HM) result in comparatively lower biomass, ergosterol and carotenoid levels.

Interestingly, the RM-3GLU condition stands out for its very high MUFA content (74.14 %) but relatively low PUFA (10.39 %) and SFA (15.48 %) levels, suggesting that glucose at a C/N ratio of 50 favors MUFA accumulation. Conversely, RM-1GLY and RM-2GLY, using glycerol, show a more balanced fatty acid profile, with significant PUFA levels but lower MUFA. These trends indicate that both the carbon source and C/N ratio have a profound influence on metabolite and lipid synthesis in *Rhodotorula mucilaginosa*.

Table 73: Results of cultivations of Rhodotorula mucilaginosa (20-9-7) on substrates consisting of hydrolysed mash, glycerol, glucose and triple amount of nitrogenous source with commercially available autolysate addition

	Rhodotorula mucilaginosa (20-9-7)							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
RM-1GLY	1.061	4.824	4.978	10.05	16.80	54.90	28.30	4.75
RM-2GLY	0.416	4.009	3.930	9.00	16.42	59.27	24.32	4.99
RM-3GLU	0.833	2.620	2.049	10.20	15.48	74.14	10.39	6.51
RM-5GLU	2.306	5.293	3.358	9.45	20.42	70.63	8.95	6.66
RM-5HM	n.a.	5.262	2.621	8.00	10.25	62.99	26.75	5.21
RM-6HM	1.066	3.145	2.428	10.25	20.65	69.94	9.41	7.84

5.1.6.4 Rhodotorula kratochvilovae (CCY 20-2-26)

The dataset provided in Table 74 for *Rhodotorula kratochvilovae* illustrates the effects of various substrates on the production of antioxidants, biomass, and lipid composition. Among the metabolites, the highest carotenoid concentration is found in RK-5HM (2,049 mg/g), while RK-3GLU displays the highest ubiquinone level (3.345 mg/g). The maximum ergosterol content is observed in RK-5HM

(2.920 mg/g). The highest biomass yield is recorded in RK-3GLU (12.45 g/l). In terms of lipid content, RK-4GLU stands out with the highest percentage (10.67 %).

Regarding the fatty acid profiles, RK-4GLU and RK-3GLU exhibits the highest SFA (34.16 %) and MUFA (59.35 %) levels. The highest PUFA proportion is observed in RK-2GLY (33.61 %), indicating a more balanced fatty acid profile in this condition.

Table 74: Results of cultivations of Rhodotorula kratochvilovae on substrates consisting of hydrolysed mash, glycerol, glucose and triple amount of nitrogenous source with commercially available autolysate addition

	Rhodotorula kratochvilovae							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
RK-1GLY	1.175	3.193	2.891	7.65	22.39	46.95	30.66	6.95
RK-2GLY	0.806	2.142	2.726	6.90	19.99	46.40	33.61	5.74
RK-3GLU	1.342	3.345	1.642	12.45	33.58	59.73	6.68	10.13
RK-5GLU	1.290	3.024	1.514	11.80	34.16	59.35	6.49	10.67
RK-5HM	2.049	2.635	2.920	8.50	23.99	59.48	16.52	7.82
RK-6HM	1.613	2.334	1.762	11.35	33.27	59.65	7.08	10.62

Trends observed in the data suggest that glucose-based media (RK-3GLU and RK-4GLU) tend to support higher biomass and lipid content, as well as elevated levels of SFA and MUFA, while glycerol-based media such as RK-2GLY show a significantly higher PUFA proportion. The hydrolysed mash condition (RK-5HM) is notable for its higher carotenoid and ergosterol production, despite having relatively modest biomass and lipid yields compared to glucose-based media. Additionally, the extremely low PUFA levels in glucose-based media (RK-3GLU and RK-4GLU) contrast sharply with their high SFA and MUFA percentages, indicating a shift toward more saturated and monounsaturated fatty acids under these conditions. This trend highlights the impact of both the carbon source and the C/N ratio on fatty acid composition in *Rhodotorula kratochvilovae.*

5.1.6.5 Cystofilobasidium infirmominiatum (CCY 17-18-4)

Regarding metabolites (see Table 75), the highest carotenoid concentration is found in CI-4GLU (1.340 mg/g), however carotenoid production remains low among all tested condition. The highest ubiquinone level is recorded in CI-6HM (15.301 mg/g). The maximum ergosterol accumulation occurs in CI-5HM (2.826 mg/g). For biomass production, CI-6HM shows the highest yield (11.90 g/l), suggesting that hydrolysed mash with a C/N ratio of 25 promotes optimal biomass growth. CI-6HM also exhibits the highest lipid content (15.42 %).

In terms of fatty acid composition, the highest SFA percentage is found in CI-1GLY (56.46 %), while the greatest MUFA proportion is seen in CI-6HM (37.25 %). The highest PUFA content is observed in CI-5HM (45.47 %), indicating a diverse fatty acid profile in this condition.

A clear trend emerges in the data showing that hydrolysed mash media, especially CI-6HM, significantly enhances the production of ubiquinone, biomass, and lipid content, alongside favourable MUFA accumulation. In contrast, glycerol-based conditions like CI-1GLY result in a very high SFA percentage but comparatively low biomass, lipid, and carotenoid production.

Table 75: Results of cultivations of Cystofilobasidium infirmominiatum on substrates consisting of hydrolysed mash, glycerol, glucose and triple amount of nitrogenous source with commercially available autolysate addition

	Cystofilobasidium infirmominiatum							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
CI-1GLY	n.a.	0.785	0.984	2.15	56.46	14.53	29.01	8.24
CI-2GLY	0.096	1.195	0.845	2.05	41.03	17.93	41.03	4.99
CI-3GLU	0.310	8.719	2.620	6.00	30.92	31.46	37.62	9.52
CI-4GLU	1.340	13.310	2.606	9.30	33.84	36.18	29.98	14.07
CI-5HM	0.287	9.883	2.826	6.48	27.65	26.88	45.47	6.94
CI-6HM	0.347	15.301	2.577	11.90	34.70	37.25	28.04	15.42

5.1.6.6 *Sporidiobolus salmonicolor (CCY 19-6-4)*

As it can be seen in Table 76, the highest carotenoid concentration is found in SSA-3GLU (1.056 mg/g), while SSA-2GLY shows the highest ubiquinone content (7.173 mg/g) and ergosterol level (5.600 mg/g). Biomass production is highest in SSA-3GLU (14.88 g/l). In terms of lipid content, SSA-2GLY shows the highest lipid percentage at 8.98 %.

Looking at fatty acid composition, SSA-1GLY has the highest SFA content (15.63 %), while SSA-3GLU stands out for its extremely high MUFA proportion (90.21 %), which is significantly higher than in other conditions. The highest PUFA percentage is observed in SSA-2GLY (41.76 %).

Glucose-based media, especially SSA-3GLU, favour higher carotenoid and biomass production, as well as an extreme shift toward MUFA content, with much lower PUFA levels compared to glycerol-based media. On the other hand, glycerol-based media (SSA-2GLY) show a more balanced fatty acid profile with high levels of both MUFA and PUFA. Hydrolysed mash media generally result in lower metabolite production, with SSA-6HM showing the lowest carotenoid and ubiquinone levels but maintaining very high MUFA content (80.42 %). These patterns suggest that carbon source and media composition significantly influence lipid metabolism and fatty acid profiles in *Sporidiobolus salmonicolor*.

Table 76: Results of cultivations of Sporidiobolus salmonicolor on substrates consisting of hydrolysed mash, glycerol, glucose and triple amount of nitrogenous source with commercially available autolysate addition

	Sporidiobolus salmonicolor							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
SSA-1GLY	0.086	6.639	5.546	4.73	15.63	46.91	37.46	7.79
SSA-2GLY	0.164	7.173	5.600	3.20	14.68	43.55	41.76	8.98
SSA-3GLU	1.056	2.519	3.966	14.88	5.96	90.21	3.83	8.51
SSA-4GLU	0.664	2.842	4.071	10.48	10.46	69.31	20.23	7.07
SSA-5HM	0.155	1.584	4.140	6.70	9.18	70.40	20.42	5.64
SSA-6HM	0.042	1.679	3.039	6.95	6.30	80.42	13.28	7.21

5.1.6.7 Comparison of biomass growths

The Figure 34 illustrates the biomass production (g/l) of various yeast strains across different culture conditions, highlighting their metabolic efficiency and adaptability. Among the strains, *Sporidiobolus salmonicolor* achieved the highest biomass yield of 14.88 g/l when grown with 3GLU, demonstrating its better ability to thrive in this medium.

Rhodotorula kratochvilovae and *Rhodosporidium toruloides* also exhibited substantial biomass production under different conditions, with *Rhodotorula kratochvilovae* reaching a peak of 12.45 g/l with 3GLU and *Rhodosporidium toruloides* achieving 10.50 g/l with 2GLY. This indicates that these strains are well-adapted to their respective environments, likely due to their efficient nutrient assimilation and conversion capabilities. Additionally, *Cystofilobasidium infirmominiatum* displayed a notable biomass increase of 11.9 g/l with 6HM, suggesting it can effectively utilize the more complex carbon sources.

The data reveals that glucose-based media (particularly 3GLU and 4GLU) generally support strong biomass growth across multiple yeast strains, suggesting glucose's effectiveness as a carbon source. Several strains, such as *Rhodotorula kratochvilovae* and *Sporidiobolus salmonicolor*, exhibit enhanced growth in these conditions, indicating their metabolic adaptability to glucose. Glycerol-based media, while effective for certain strains like *Rhodosporidium toruloides,* do not seem to foster the same level of biomass growth across all yeasts as glucose does.

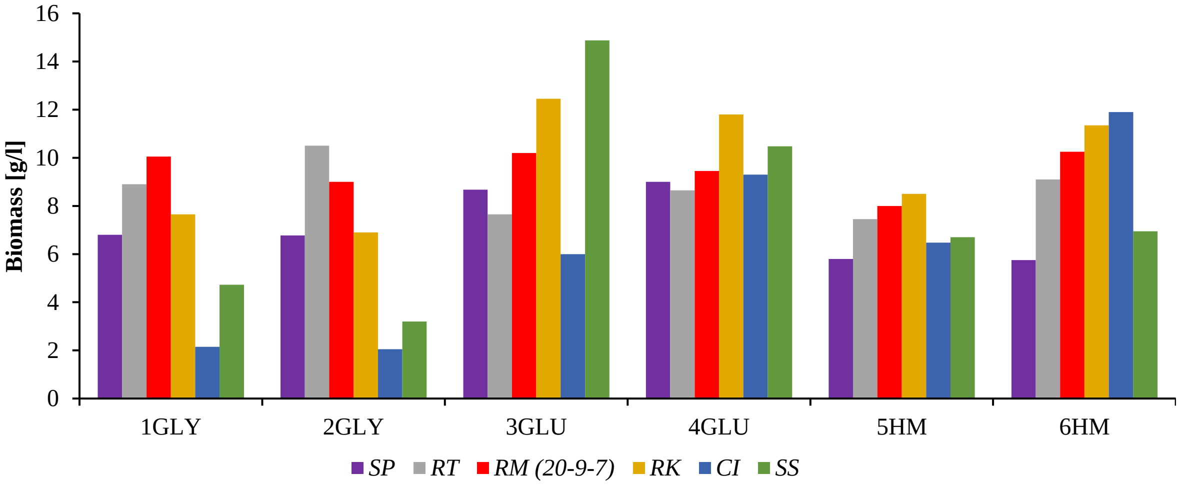


Figure 34: Comparison of biomass growths between yeast strains, grown on media with various carbonaceous substrate

5.1.6.8 Rhodosporidium toruloides (CCY 62-2-4) – cultivation on various nitrogen sources

Among the antioxidants, the highest carotenoid production was observed in GR(1N)2 with a value of 2.475 mg/g, indicating that granular yeast under a classical nitrogen amount boosts carotenoid synthesis. The peak ergosterol content was recorded in SY(1N)4 at 9.196 mg/g, suggesting that spent yeast with a classical nitrogen level enhances ergosterol production. Ubiquinone levels were highest in GR(Add)1, reaching 10.929 mg/g, showing the effectiveness of granular yeast addition with autolysate in promoting ubiquinone accumulation. These findings highlight how varying combinations of nitrogen levels and yeast treatments can distinctly influence antioxidant biosynthesis in *Rhodosporidium toruloides*.

In terms of biomass production, GR(Add)3 yielded the highest at 33.28 g/l, indicating that granular yeast addition, under specific conditions, favours substantial cell growth. Other notable biomass results include SY(1N)3 with 33.23 g/l and Cont (1N+Add)3 with 29.63 g/l, demonstrating the influence of different nitrogen concentrations and yeast treatments on growth dynamics. This variation in biomass yield suggests the adaptability of *Rhodosporidium toruloides* to diverse nutritional and environmental settings. A notable trend is that biomass production tends to be higher in conditions with lower nitrogen availability (e.g., 1N), particularly in combinations with additional carbon sources, such as SY and GR. This is evident in samples like SY(1N+Add)3, SY(1N)3, and GR(1N)3, where biomass consistently surpasses 28 g/l, suggesting that nitrogen limitation encourages biomass accumulation in this strain.

Analysing the lipid profile, the highest proportion of saturated fatty acids was found in GR(Add)3 with a value of 66.84 %. Monounsaturated fatty acids were most prevalent in SY(3N)4 at 72.75 %, and polyunsaturated fatty acids peaked in Cont (1N+Add)4 at 23.24 %. Additionally, the highest total lipid content was recorded in Cont (3N+Add)1 at 27.55 %. An observable trend is the shift in fatty acid profiles across conditions. Under nitrogen limitation, monounsaturated fatty acids tend to dominate, especially in GR and SY conditions. Polyunsaturated fatty acids are less prominent under these conditions, generally showing lower percentages. Lipid content, on the other hand, seems to increase with nitrogen limitation and certain additives, particularly in 1N conditions, such as SY(1N+Add)3, where lipids reach nearly 26 %. This suggests that *Rhodosporidium toruloides* efficiently redirects resources toward lipid production when nitrogen is restricted, further influenced by the presence of additional carbon sources. Overall, this dataset highlights the metabolic flexibility of *Rhodosporidium toruloides* in response to nutrient availability, particularly nitrogen, and the effect this has on biomass and lipid biosynthesis.

Table 77: The impact of various cultivation conditions of inorganic and organic nitrogenous sources on yeast strain Rhodosporidium toruloides (1N – standard nitrogen amount, 3N – triple nitrogen amount, Add – addition of autolysate -as is in Table 69, CA – commercial autolysate, SY – Spent brewery yeast autolysate, GR – granulated yeast autolysate, Cont – control media)

	Rhodosporidium toruloides							
Media	Carotenoids [mg/g]	Ubiquinone [mg/g]	Ergosterol [mg/g]	Biomass [g/l]	SFA %	MUFA %	PUFA %	Lipids [%]
Cont (3N+Add)1	n.a.	2.084	1.045	6.85	51.86	39.42	8.72	27.55
Cont (3N+Add)2	n.a.	3.274	2.210	7.00	45.22	37.87	16.91	18.88
Cont (3N+Add)3	n.a.	2.489	0.697	7.45	39.30	52.66	8.03	12.03
Cont (3N+Add)4	n.a.	2.645	0.693	7.20	50.32	42.38	7.30	12.17
Cont (1N+Add)1	0.799	5.089	2.339	25.45	44.08	50.38	5.54	19.39
Cont (1N+Add)2	0.794	3.135	3.398	13.45	32.83	48.48	18.69	15.77
Cont (1N+Add)3	1.330	5.901	3.611	29.63	41.26	53.01	5.73	23.46
Cont (1N+Add)4	1.513	2.876	6.112	18.25	23.31	53.45	23.24	6.52
GR(Add)1	1.602	10.929	5.509	19.00	36.03	58.45	5.52	11.60
GR(Add)2	1.942	5.054	5.591	20.08	42.79	46.19	11.02	14.30
GR(Add)3	1.245	3.824	4.340	33.28	66.84	24.07	9.09	10.30
GR(Add)4	1.221	3.383	6.386	21.48	30.98	52.72	16.30	14.30
SY(1N+Add)1	2.257	4.648	2.437	28.15	38.67	55.78	5.54	18.78
SY(1N+Add)2	1.741	2.361	7.214	20.10	18.33	64.20	17.47	9.38
SY(1N+Add)3	1.299	4.668	3.236	28.53	44.30	52.02	3.68	25.68
SY(1N+Add)4	1.883	4.351	5.334	20.28	24.48	56.80	18.72	6.04
SY(1N)1	1.731	3.758	2.906	30.70	35.54	56.82	7.64	15.80
SY(1N)2	0.624	2.787	7.153	23.25	26.49	60.59	12.91	12.81
SY(1N)3	1.797	3.490	2.946	33.23	37.68	59.37	2.95	24.69
SY(1N)4	0.701	2.270	9.196	22.48	22.03	59.55	18.42	9.76
GR(1N)1	1.159	4.493	2.720	28.25	38.42	56.29	5.29	15.71
GR(1N)2	2.475	5.462	4.235	27.40	34.89	56.35	8.76	15.63
GR(1N)3	0.870	8.331	2.709	31.95	42.70	54.12	3.18	21.56
GR(1N)4	2.442	3.373	4.945	27.50	35.74	53.51	10.75	16.53
SY(3N+Add)1	n.a.	1.333	2.900	13.08	35.24	58.40	6.35	18.19
SY(3N+Add)2	n.a.	2.144	3.681	12.60	34.21	57.20	8.58	18.09
SY(3N+Add)3	n.a.	1.837	3.732	9.85	27.63	65.71	6.66	11.53
SY(3N+Add)4	0.030	1.345	4.230	10.28	26.72	64.30	8.99	8.06
SY(3N)1	2.299	3.215	1.852	28.10	41.86	54.99	3.15	11.45
SY(3N)2	2.393	2.236	3.075	28.43	36.44	59.97	3.59	11.02
SY(3N)3	0.993	4.503	2.044	28.18	26.34	68.48	5.19	6.90
SY(3N)4	1.673	1.230	3.478	27.85	10.46	72.75	16.79	4.49

5.1.7 Comparison of different nitrogen substrates

The results obtained from numerous cultivation experiments provide insight into the comparison of various nitrogen substrates and their combinations. To evaluate the impact of different nitrogen sources, media containing glycerol as the primary carbon source were selected, in order to eliminate any unwanted effects from components that may be present in different waste substrates. The data were obtained and combined from chapters above (5.1.1, 5.1.2, 5.1.3, 5.1.4, 5.1.6) as well as concentration of glycerol and nitrogen sources. The graphs presented below offer an overview of how the selected yeast strains respond to different organic and inorganic nitrogen substrates under varying C/N ratios. For this comparison, four representatives from different yeast genera were chosen: *Sporidiobolus*, *Rhodotorula*, *Rhodosporidium*, and *Cystofilobasidium*.

5.1.7.1 Sporidiobolus pararoseus (CCY 19-9-6)

The Figure 35 presents a detailed analysis of the impact of different nitrogen sources and carbon-to-nitrogen (C/N) ratios on the antioxidant production, biomass yield, and lipid composition in *Sporidiobolus pararoseus*. At a C/N ratio of 25, ammonium sulphate demonstrated the highest total carotenoid concentration at 3.664 mg/g and the highest ergosterol content at 10.946 mg/g. However, the maximum ubiquinone concentration was achieved with feather hydrolysate, reaching 8.382 mg/g. In terms of biomass production, feather hydrolysate also led with a yield of 9.90 g/l under the same C/N ratio. The lipid composition favored ammonium sulphate for monounsaturated fatty acids with 78.46 %, while urea supported the highest polyunsaturated fatty acid percentage of 39.16 %. For saturated fatty acids, N-triple provided the highest content at 45.17 %. N-triple nitrogen also showed the highest lipid content at 11.01 %.

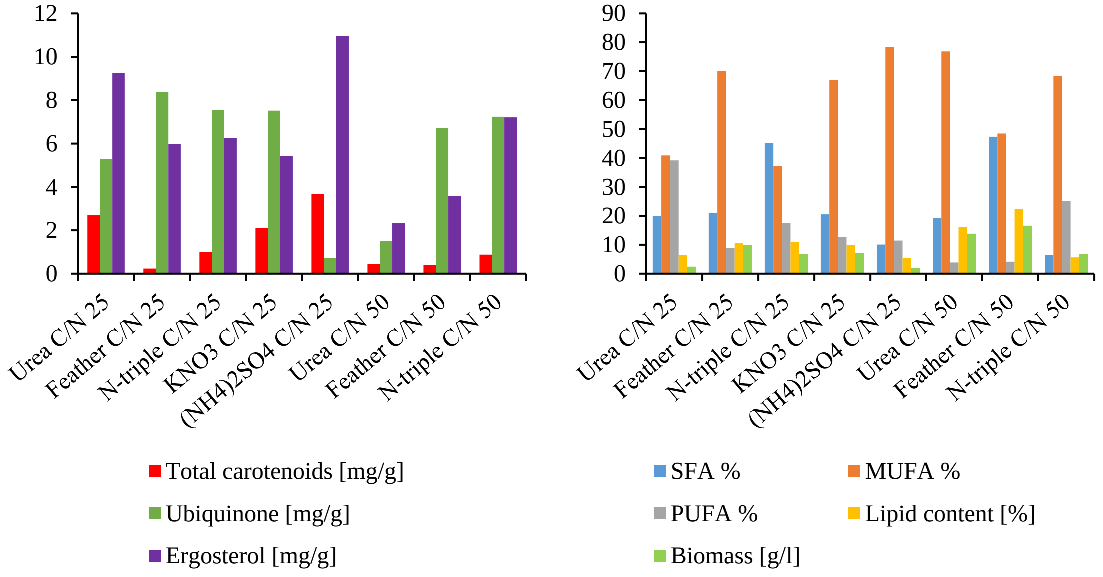


Figure 35: A comprehensive metabolic and growth analysis of the yeast strain Sporidiobolus pararoseus cultivated on diverse nitrogen substrates under varying C/N ratios

For the C/N ratio of 50, the most notable results were different. The highest biomass yield was observed with feather hydrolysate, producing 16.60 g/l, which also resulted in the highest lipid content at 22.38 %. In terms of antioxidant production, N-triple nitrogen showed a significant ergosterol concentration of 7.208 mg/g, and also the highest ubiquinone concentration at 7.235 mg/g. Regarding lipid composition, urea excelled in producing monounsaturated fatty acids at 76.85 %, and N-triple showed the highest polyunsaturated fatty acid content at 25.08 %. Additionally, feather hydrolysate recorded the highest saturated fatty acid content at 47.39 %. These findings emphasize the variability in metabolic responses depending on both the nitrogen source and the C/N ratio, highlighting the potential for tailored nutrient management to optimize specific biochemical outputs in *Sporidiobolus pararoseus*.

5.1.7.2 Rhodotorula kratochvilovae (CCY 20-2-26)

The data for *Rhodotorula kratochvilovae* (Figure 36) highlights the effect of different nitrogen sources and C/N ratios on antioxidant production, biomass yield, and lipid composition. At a C/N ratio of 25, ammonium sulphate stands out with the highest total carotenoid concentration of 3.062 mg/g and biomass yield of 11.65 g/l. Urea also demonstrated significant results, showing the highest ubiquinone concentration at 10.495 mg/g. The highest ergosterol content was observed with potassium nitrate at 3.536 mg/g. In terms of lipid profiles, ammonium sulphate promoted the highest monounsaturated fatty acids at 48.57 %, while N-triple nitrogen resulted in the highest PUFA at 33.61 %. Urea showed the highest saturated fatty acids at 43.56 % and the maximum lipid content at 15.42 %.

At a C/N ratio of 50, urea again proved to be highly effective, providing the highest ubiquinone concentration of 5.702 mg/g and biomass yield of 11.600 g/l. It also had the highest ergosterol content at 3.705 mg/g. The maximum total carotenoid content at this C/N ratio was achieved with urea at 1.197 mg/g and also SFA with value 47.36 %. Regarding lipid composition, N-triple nitrogen stood out with the highest monounsaturated fatty acids at 46.96 % and polyunsaturated fatty acids at 30.66 %. The highest lipid content was again found with urea at 17.43 %. These findings underscore the importance of selecting appropriate nitrogen sources and C/N ratios to optimize antioxidant production, biomass, and lipid composition in *Rhodotorula kratochvilovae*, allowing for precise adjustments based on desired outcomes.

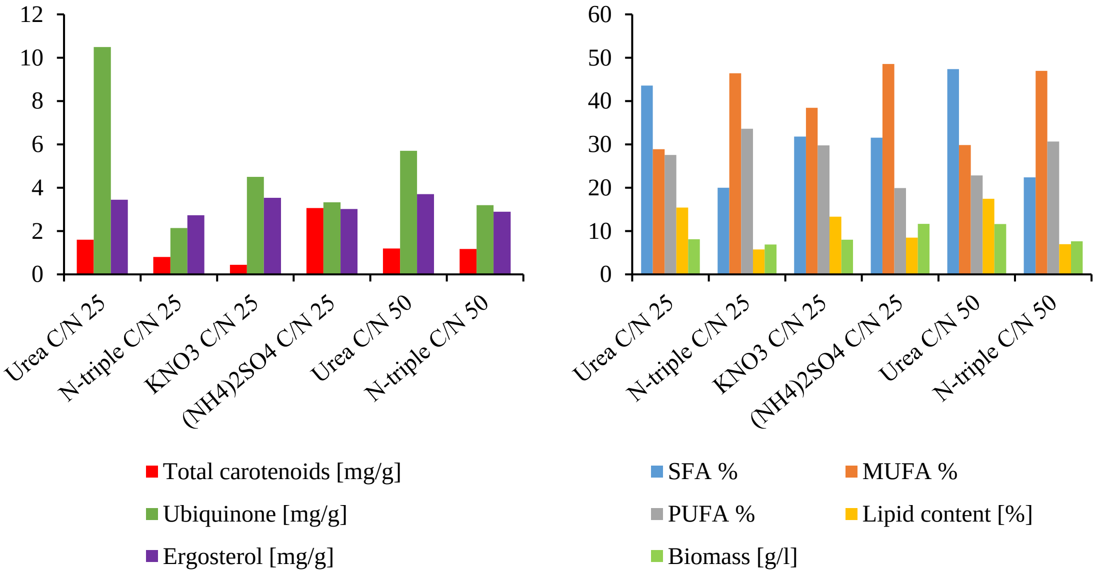


Figure 36: A comprehensive metabolic and growth analysis of the yeast strain Rhodotorula kratochvilovae cultivated on diverse nitrogen substrates under varying C/N ratios

5.1.7.3 *Rhodosporidium toruloides (CCY 62-2-4)*

The data (Figure 37) for *Rhodosporidium toruloides* provides valuable insights into how different nitrogen sources and carbon-to-nitrogen (C/N) ratios influence the production of antioxidants, biomass yield, and lipid composition. At a C/N ratio of 25, ammonium sulphate led to the highest total carotenoid concentration at 5.986 mg/g. The most notable ubiquinone content was observed with potassium nitrate, which reached 5.996 mg/g, while urea yielded the highest ergosterol concentration at 4.572 mg/g. In terms of biomass production, urea again stood out with the greatest yield of 19.00 g/l. For lipid profiles, ammonium sulphate showed the highest monounsaturated fatty acid percentage at 56.09 %, while feather hydrolysate demonstrated the highest PUFA content at 14.88 %. The highest saturated fatty acids were observed with feather hydrolysate at 43.40 %, and the maximum lipid content was also achieved with feather hydrolysate at 21.75 %.

At a C/N ratio of 50, the nitrogen source dynamics shifted. Feather hydrolysate provided the highest ubiquinone concentration at 11.611 mg/g and also achieved the highest biomass yield at 20.30 g/l, along with the greatest ergosterol content at 4.580 mg/g, however carotenoid content was significantly decreased. Urea produced the highest total carotenoid content, recording 4.111 mg/g. Regarding lipid composition, urea excelled with the highest monounsaturated fatty acid content at 71.93 %, while feather hydrolysate had the highest polyunsaturated fatty acid content at 10.07 % and also showed the highest saturated fatty acids content at 37.63 %. The overall highest lipid content at a C/N ratio of 50 was observed with feather hydrolysate, which recorded 16.56 %. These results underscore the significant impact of nitrogen source and C/N ratio in optimizing specific biochemical pathways and metabolite yields in *Rhodosporidium toruloides*.

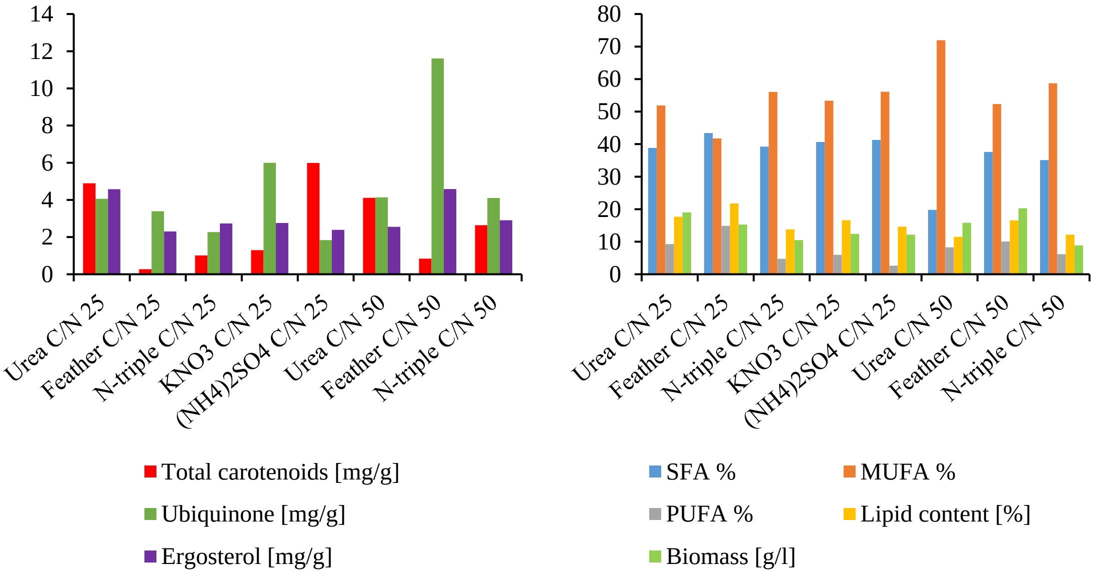


Figure 37: A comprehensive metabolic and growth analysis of the yeast strain Rhodosporidium toruloides cultivated on diverse nitrogen substrates under varying C/N ratios

5.1.7.4 *Cystofilobasidium macerans (CCY 10-1-2)*

The data (Figure 38) for *Cystofilobasidium macerans* illustrates the effects of various nitrogen sources and C/N ratios on the production of antioxidants, biomass yield, and lipid composition. At a C/N ratio of 25, urea led to the highest total carotenoid concentration of 1.967 mg/g and the highest ubiquinone concentration at 10.939 mg/g. The highest ergosterol content was observed with feather hydrolysate, reaching 3.826 mg/g, which also resulted in the greatest biomass yield at 16.60 g/l. In terms of lipid composition, ammonium sulphate achieved the highest monounsaturated fatty acids at 61.48 %, while urea produced the highest polyunsaturated fatty acids content at 46.12 %. Feather hydrolysate exhibited the highest saturated fatty acids at 41.09 % and the maximum lipid content at 20.54 %.

At a C/N ratio of 50, urea showed the highest biomass yield, achieving 23.20 g/l. It also had the highest polyunsaturated fatty acids content at 12.39 %. Feather hydrolysate displayed a significant ubiquinone concentration of 9.864 mg/g and the highest ergosterol content at 3.142 mg/g. Urea produced the most carotenoids, with a concentration of 1.622 mg/g. Regarding lipid composition, feather hydrolysate led with the highest monounsaturated fatty acids at 53.39 % and saturated fatty acids at 38.84 %. The highest lipid content at this C/N ratio was also observed with feather hydrolysate, which recorded 18.95 %. These findings underscore the importance of selecting the appropriate nitrogen source and C/N ratio to optimize the production of specific metabolites in *Cystofilobasidium macerans*, allowing for targeted enhancements in biochemical outputs based on desired applications.

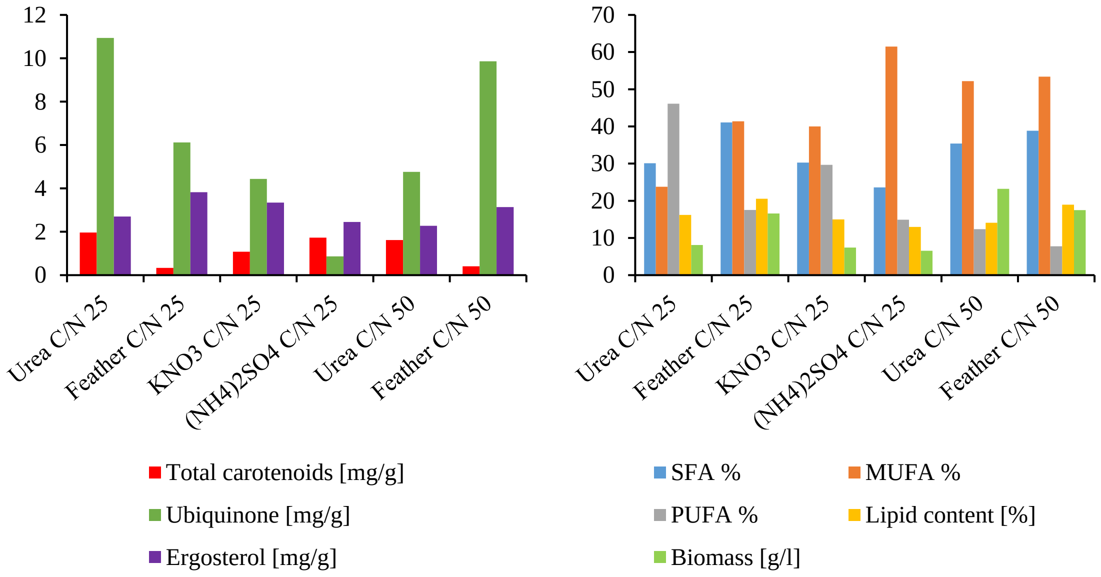


Figure 38: A comprehensive metabolic and growth analysis of the yeast strain Cystofilobasidium macerans cultivated on diverse nitrogen substrates under varying C/N ratios

5.1.8 Data obtained from literature and their comparison with presented study

The Table 78 and Table 79 describe data obtained from various scientific sources and publications, providing information on the testing and application of yeast strains for cultivation purposes on waste substrates, including waste glycerol, waste animal fat, spent coffee grounds hydrolysate (SCG hydrolysate), molasses, whey, chicken feather peptone, and olive mill waste.

Table 78: Several sources describing carotenogenic yeasts usage for waste materials utilization and their final metabolic and growth characterization. Data obtained from graph are marked by abbreviation „approx.”; (a) means only beta-carotene content not all carotenoids; (b) means bioreactor cultivation.

Source	Yeast strain	Culture collection	C/N ratio	Cultivation length [h]	Substrate	Biom. [g/l]	Lipids [%]	Carotenoids
[140]	*R. toruloides*	DSM 4444	n.a.	96	SCG hydrolysate+ yeast extract	28.1	39.4	n.a.
[141]	*R. glutinis*	NCIM	80	96	Molasses + yeast extract+ $(NH_4)_2SO_4$	10.38	45	0.014 %
[142]	*R. mucilaginosa*	n.a.	n.a.	96	20 % Olive mill waste	approx. 7.5	n.a.	5.5 mg/l
[143]	*R. glutinis*	TISTR 5159	85	72	Waste crude glycerol+ $(NH_4)_2SO_4$	5.49	41.78	117.38 mg/l
[144]	*R. mucilaginosa*	BCRC23454	n.a.	216	molasses+NH_4Cl	9.72	n.a.	268.6 μg/g
[108]	*R. mucilaginosa*	CCY 20-7-31	n.a.	80	Deprotein. Whey+glucose+ $(NH_4)_2SO_4$	6.93	n.a.	228.6 μg/g (a)
	R. glutinis	CCY 20-2-26				9.16	n.a.	1268.5 μg/g (a)
	S. roseus	CCY 19-4-8				5.25	n.a.	2780.5 μg/g (a)
	R. mucilaginosa (b)	CCY 20-7-31	n.a.	n.a.	Deprotein. Whey+glucose + $(NH_4)_2SO_4$	27.06	n.a.	2436.0 μg/g (a)
	R. glutinis (b)	CCY 20-2-26				44.56	n.a.	325.16 μg/g (a)
	S. roseus (b)	CCY 19-4-8				9.59	n.a.	1025.12 μg/g (a)

In comparing the cultivation of the yeast *Rhodosporidium toruloides* (DSM 4444) on spent coffee grounds (SCG), the study by [140] demonstrated a 126.6 % higher biomass yield compared to this study *R. toruloides* (CCY 62-2-4) (12.4 g/l). Lipid production in that publication was also 34.10 % higher in terms of biomass yield compared to the results in this work (5.34 %). This discrepancy could be attributed to differences in nitrogen sources, as KNO_3 was used in this study, while the previous work employed an organic nitrogen source – yeast extract. Additionally, a variation in the C/N ratio may contribute to the difference, as this study utilized a C/N ratio of 25, whereas the ratio in the publication was not specified.

Table 79: More sources describing carotenogenic yeasts usage for waste materials utilization and their final metabolic and growth characterization. Data obtained from graph are marked by „approx."

Source	Yeast strain	Culture collection	C/N ratio	Cultivation length [h]	Substrate	Biom. [g/l]	Lipids [%]	Carotenoids
[100]	*C. macerans*	CCY 17-9-3	approx. 30	96	Animal crude fat+$(NH_4)_2SO_4$	10.75	n.a.	approx. 100 µg/g
	R. mucilaginosa	CCY 20-2-28				3.3	n.a.	n.a.
	C. infirmominiatum	CCY 18-9-3				1.64	n.a.	n.a.
	S. pararoseus	CCY 19-9-6				2.9	n.a.	n.a.
	S. shibatanus	CCY 19-20-3				5.86	n.a.	approx. <100 µg/g
[145]	*R. glutinis*	n.a.	n.a.	120-144	Glucose+chicken feather peptone+ yeast extract	14.2	n.a.	92 mg/l
[33]	*R. glutinis*	CCY 20-2-26	13	96	Animal crude fat+$(NH_4)_2SO_4$	approx. 4.5	approx. 11	approx. 3.8 mg/l
			25			approx. 5.5	approx. 11	approx. 1.8 mg/l
			50			approx. 6.0	approx. 12.5	approx. 2.5 mg/l
			100			approx. 7.5	approx. 15	approx. 0.4 mg/l
	C. macerans	CCY 10-1-2	13			approx. 1.0	approx. 17.5	approx. 1.7 mg/l
			25			approx. 5.0	approx. 28.5	approx. 1 mg/l
			50			approx. 4.5	approx. 22.5	approx. 3.8 mg/l
			100			approx. 11.5	approx. 25	approx. 2.2 mg/l
	R. mucilaginosa	CCY 19-4-6	13			approx. 1.0	approx. 8	approx. 1.8 mg/l
			25			approx. 6.5	approx. 25	approx. 3.2 mg/l
			50			approx. 5.5	approx. 25	approx. 4.2 mg/l
			100			approx. 6.0	approx. 26	approx. 5 mg/l
	S. pararoseus	CCY 19-9-6	13			approx. 4.0	approx. 11	approx. 9.2 mg/l
			25			approx. 3.0	approx. 52.5	approx. 2.6 mg/l
			50			approx. 4.0	approx. 40	approx. 3.8 mg/l
			100			approx. 7.0	approx. 37.5	approx. 5.2 mg/l
[37]	*R. glutinis*	CCY 20-2-26	n.a.	96	Waste glycerol + $(NH_4)_2SO_4$+yeast extract	17.25	approx. 17.5	578.3 µg/g
	R. aurantiaca	CCY 20-9-7				19.92	approx. 20	375.6 µg/g
	R. mucilaginosa	CCY 20-7-28				15.97	approx. 16	239.8 µg/g
	C. capitatum	CCY 10-1-1				21.76	approx. 22	645.3 µg/g
	S. roseus	CCY 19-6-4				16.71	approx. 17	687.0 µg/g
	S. shibatanus	CCY 19-20-3				20.52	approx. 21	402.8 µg/g
	S. salmonicolor	CCY 19-4-6				16.71	approx. 17	905.2 µg/g
[146]	*R. glutinis*	RCMB 028001	n.a.	120	salted whey	12.93	n.a.	438 µg/g

When comparing with the results of [143], the reference strain *Rhodotorula kratochvilovae* (CCY 20-2-26) from this work was compared to *Rhodotorula glutinis* (NCIM), cultivated on waste glycerol in the publication. The strain in the publication showed a 42.20 % lower biomass yield (9.50 g/l in this study) but a nearly 20 times higher carotenoid production (0.688 mg/g in this study). The difference in lipid production was also significant, with *R. glutinis* producing 30.10 % more. The notable variation in metabolite production may be due to the different C/N ratios (25 in this study versus 85 in the publication), the cultivation duration (96 hours in this work versus 72 hours in the publication), and the nitrogen source, as ammonium sulphate is more easily utilized compared to potassium nitrate, which was used in this study.

In another comparison of this study (chapter 5.1.3) with publication [37] regarding waste glycerol cultivation, the biomass yield in the publication surpassed most of the strains cultivated here by over 100 %. Higher lipid production was also observed in most cases, although all strains from the publication exhibited lower carotenoid production compared to this study. For instance, *R. glutinis* (CCY 20-2-26) (compared to *R. kratochvilovae* CCY 20-2-26) exhibited 81 % higher biomass growth and 5.8 % greater lipid accumulation but had 94.9 % lower carotenoid levels. Similarly, *R. mucilaginosa* (CCY 20-7-28) (compared to *R. mucilaginosa* CCY 19-4-6) showed 101 % greater biomass but 2.5 % lower lipid accumulation and 76.9 % less carotenoid production. *C. capitatum* (CCY 10-1-1) in the publication exceeded *C. macerans* (CCY 10-1-2) from this work in biomass yield by 222.4 % and lipid accumulation by 8 %, yet its carotenoid production was 76.9 % lower. The strain *S. roseus* (CCY 19-6-4) from the publication, compared to *S. metaroseus* (CCY 19-6-20) in this work, showed a 206.6 % higher biomass yield and a 0.7 % increase in lipid accumulation, but a 32.3 % lower carotenoid yield. Likewise, *S. shibatanus* (CCY 19-20-3) (compared to the *S.pararoseus* CCY 19-9-6) exhibited a 263.2 % higher biomass growth and a 9.0 % greater lipid yield, while carotenoid production was 60.5 % lower. Finally, *S. salmonicolor* (CCY 19-4-6) (compared to the *S.pararoseus* CCY 19-9-6) demonstrated 195.8 % higher biomass growth and 5.0 % greater lipid yield but 11.2 % lower carotenoid production. The large differences in biomass and lipid production could be due to the nitrogen substrates, with ammonium sulphate and yeast autolysate used in the publication, which are more easily utilized than potassium nitrate, as well as differences in waste glycerol composition, which may contain toxic compounds for yeast (e.g., methanol, ethanol, isopropanol) or the C/N ratio, which was not provided in the publication. The higher carotenoid production in this study is likely due to increased oxidative stress, possibly from glycerol components or potassium nitrate.

When animal fat was used as a carbon source [33] with a C/N ratio of 25, four strains were compared. The *R. glutinis* (CCY 20-2-26) strain from the publication demonstrated 12.2 % higher biomass yield than the *R. kratochvilovae* (CCY 20-2-26) reference strain from this work, though lipid production was 4.8 % lower, and carotenoid production was 88.1 % lower. *C. macerans* (CCY 10-1-2) (the same strain as used in this work) exhibited a 78.6 % higher biomass yield and a 19 % higher lipid yield, though carotenoid production was 94 % lower. *R. mucilaginosa* (CCY 19-4-6) (the same strain as used in this work) also had a higher biomass yield (150 % higher) and 18.7 % higher lipid yield, though carotenoid levels were 55.7 % lower. *S. pararoseus* (CCY 19-9-6) (the same strain as used in this work) had a 65 % lower biomass yield but 12.8 % higher lipid content and 661 % higher carotenoid production than this study. These differences could be due to variations in the nitrogen source or inoculum density prior to cultivation, suggesting the need for standardized inoculum density (e.g., cell count or CFU) in future studies.

In a comparison with publication [100], it was found that *C. macerans* (CCY 17-9-3) exhibited a 283.9 % higher biomass yield in the publication (compared to *C. macerans* CCY 10-1-2), and *R. mucilaginosa* (CCY 20-2-28) also showed 26.9 % higher growth (compared to the strain *R. mucilaginosa* CCY 19-4-6). In contrast, *C. infirmominiatum* (CCY 18-9-3) (reference strain: *C. macerans* CCY 10-1-2) exhibited a 41.4 % reduction in biomass production. Similarly, *S. pararoseus* (CCY 19-9-6) (same strain as reference) had 66.3 % lower biomass yield in the publication compared to this work. *S. shibatanus* (CCY 19-20-3) showed a 31.9 % decrease in biomass production compared to this study's *S. pararoseus* (CCY 19-9-6) reference. These comparisons indicate that *S. pararoseus* may prefer a combination of animal fat and urea (as used in this work), based on findings from both [100] and [33].

Although waste whey was only used as a carbon source in the publications [108; 146], in contrast to this study where it was used as both a carbon and nitrogen source, a rough comparison can be made for cultivation on whey supplemented with glucose to achieve a C/N ratio of 25. In [108], strains of *Rhodotorula* were compared to *R. toruloides* (CCY 62-2-4), which served as the reference strain in this study. *R. mucilaginosa* (CCY 20-7-31) exhibited 14.4 % lower biomass production compared to the reference (8.1 g/l) and 95.5 % lower carotenoid production (reference: 5.109 mg/g). *R. glutinis* (CCY 20-2-26) showed 13.1 % higher biomass production and 75.2 % lower carotenoid production than the reference. In [146], *R. glutinis* (RCMB 028001) demonstrated 59.6 % higher biomass production and 91.4 % lower carotenoid production compared to the reference *R. kratochvilovae* (CCY 20-2-26). The variation between cultivations could be attributed to differences in the C/N ratio, which was not provided in the publications, or the nitrogen source and osmotic stress, as the whey in [146] contained 8 g/l salt.

Reference [145] discusses the use of chicken feathers, specifically peptone derived from them, as a supplement in cultivation media for *R. glutinis.* In this work, feather hydrolysate was used as a complete nitrogen source replacement, but this comparison illustrates the difference between supplementation and complete substitution of nitrogen substrates with products from poultry feathers. The reference strain *R. kratochvilovae* (CCY 20-2-26) from this work was compared at C/N ratios of 25 and 50. *R. glutinis* at C/N 25 showed 24.7 % higher biomass growth (reference: 12.2 g/l) and 1418 % higher carotenoid production compared to the reference (0.427 mg/g). At C/N 50, *R. glutinis* exhibited 20.1 % lower biomass growth (reference: 15.5 g/l) but 1228.1 % higher carotenoid accumulation (reference: 0.488 mg/g). Although the C/N ratio in the publication was not reported, a higher glycerol concentration in the reference medium likely increased biomass yield. Furthermore, feather peptone supplementation greatly enhanced carotenoid yield, which could be of interest for future experiments.

5.2 Lag phases experiments

Carotenogenic yeasts demonstrate significant potential for industrial applications due to their ability to utilize a wide variety of substrates. For this reason, lag phases were tested to monitor the initial adaptation of cells to new media and the stress induced by these conditions. The accumulation of various compounds, such as antioxidants or lipids, is part of the stress response to changes in environmental conditions. Understanding this process could provide valuable insights for future research, especially for industrial purposes where reducing the duration of the lag phase to the minimum necessary time is crucial. Standard glycerol medium with urea as a nitrogen source was used for cultivation, maintaining a C/N ratio of 50 (see Table 80). The experimental procedure is described in Chapter 4.5.4.

Table 80: Composition of the basic production medium used for cultivation

Component	**Amount**
KH_2PO_4	4 g
$MgSO_4 \cdot 7H_2O$	0.696 g
Urea	1.81 g
Distilled water	1000 ml
Glycerol	92.51 g

5.2.1.1 Rhodosporidium toruloides (CCY 62-2-4)

The Figure 39, Figure 40 and Figure 41 provide a comprehensive overview and visualisation of the lag phase of the yeast *Rhodosporidium toruloides*, examining various biochemical and physiological parameters to understand the dynamics of its cultivation. Initially, the yeast inoculum contains baseline levels of carotenoids, ubiquinone, ergosterol, biomass, and lipid profile percentages, which serve as reference points for observing changes during the cultivation period. As the yeast progresses through various hours of cultivation, there is a notable fluctuation in these parameters, suggesting dynamic metabolic shifts occurring within the yeast cells. For instance, the carotenoid concentration starts at 3.753 mg/g at 0 hours and decreases to 1.568 mg/g by 4 hours, which may indicate a response to the stress of inoculation and a potential shift in metabolic pathways towards adaptation. However, the carotenoid levels increase significantly by 24 hours (4.568 mg/g), indicating an initial stress response followed by a possible adaptation or increased synthesis phase. Similarly, ubiquinone levels rise within the first few hours, peaking at around 2.5 hours (4.706 mg/g), before gradually declining to 2.448 mg/g at 24 hours, suggesting its consumption in cellular processes such as energy production or oxidative stress mitigation.

Over the course of the 24-hour period, there are marked trends in lipid composition and biomass accumulation. The biomass concentration steadily increases from 1.70 g/l at 0 hours to a significant 6.70 g/l at 24 hours, which correlates with the increase in total lipid content from around 4.5 % at the start to approximately 6.8 % at 24 hours. This suggests that lipid biosynthesis is closely linked to biomass accumulation in this yeast. The yeast's lipid profile also changes significantly; initially, the saturated fatty acids are at 7.18 % at 0 hours, which then increase progressively to 35.0 % by 24 hours, indicating a shift towards more saturated lipid synthesis, potentially for membrane stability under new growth conditions. Correspondingly, the monounsaturated and polyunsaturated fatty acids percentages decrease over time, reflecting a metabolic shift away from unsaturated lipid synthesis. This shift could indicate an adaptation mechanism where increased saturation of lipids may provide structural membrane stability under cultivation conditions.

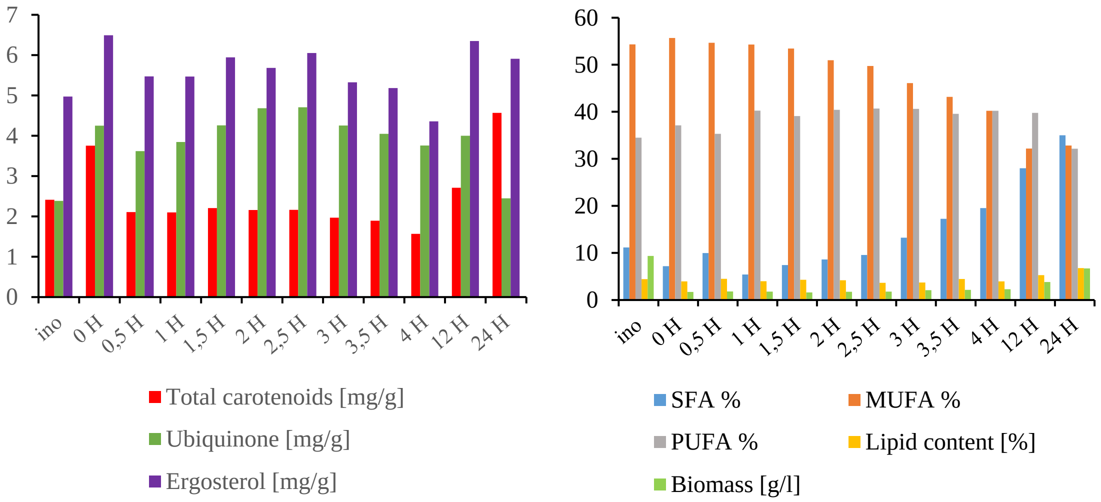


Figure 39: Metabolic and growth progress of R. toruloides in early stages of adaptation on artificial medium

Analysing the flow cytometry data (Figure 40), the cell count dynamics provides further insights into the yeast's adaptation mechanisms. The total count of non-stained cells decreases initially from 1164.14 cells/µl at 0 hours to 898.47 cells/µl by 4 hours, indicating cell adaptation and possible cell death or lysis of less viable cells. In contrast, the Nile red-stained cells, which indicate intracellular lipid content, show an increase in high-intensity staining (NR-HI) from 181.59 cells/µl at 0 hours to 433.31 cells/µl by 4 hours. This increase suggests an accumulation of intracellular lipids, possibly as a stress response or preparation for rapid growth. By 24 hours, there is a significant increase in both biomass (6.70 g/l) and high-intensity Nile red-stained cells (1507.35 cells/µl), indicating that the yeast has entered an exponential growth phase with substantial lipid accumulation, likely for energy storage and membrane synthesis needed for cell division. These observed trends suggest a complex interplay of metabolic processes that *Rhodosporidium toruloides* undergoes to adapt to its environment during the lag phase, transitioning from stress response to active growth and biosynthesis.

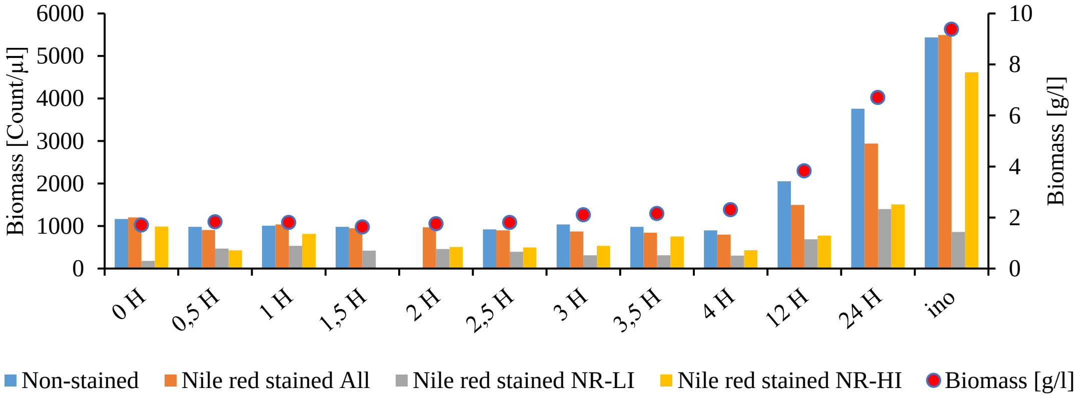


Figure 40: Data regarding growth of R. toruloides; cytometric data on major axis and biomass growth on minor axis; NR - Nile red stained; LI- low fluorescence intensity; HI- high fluorescence intensity

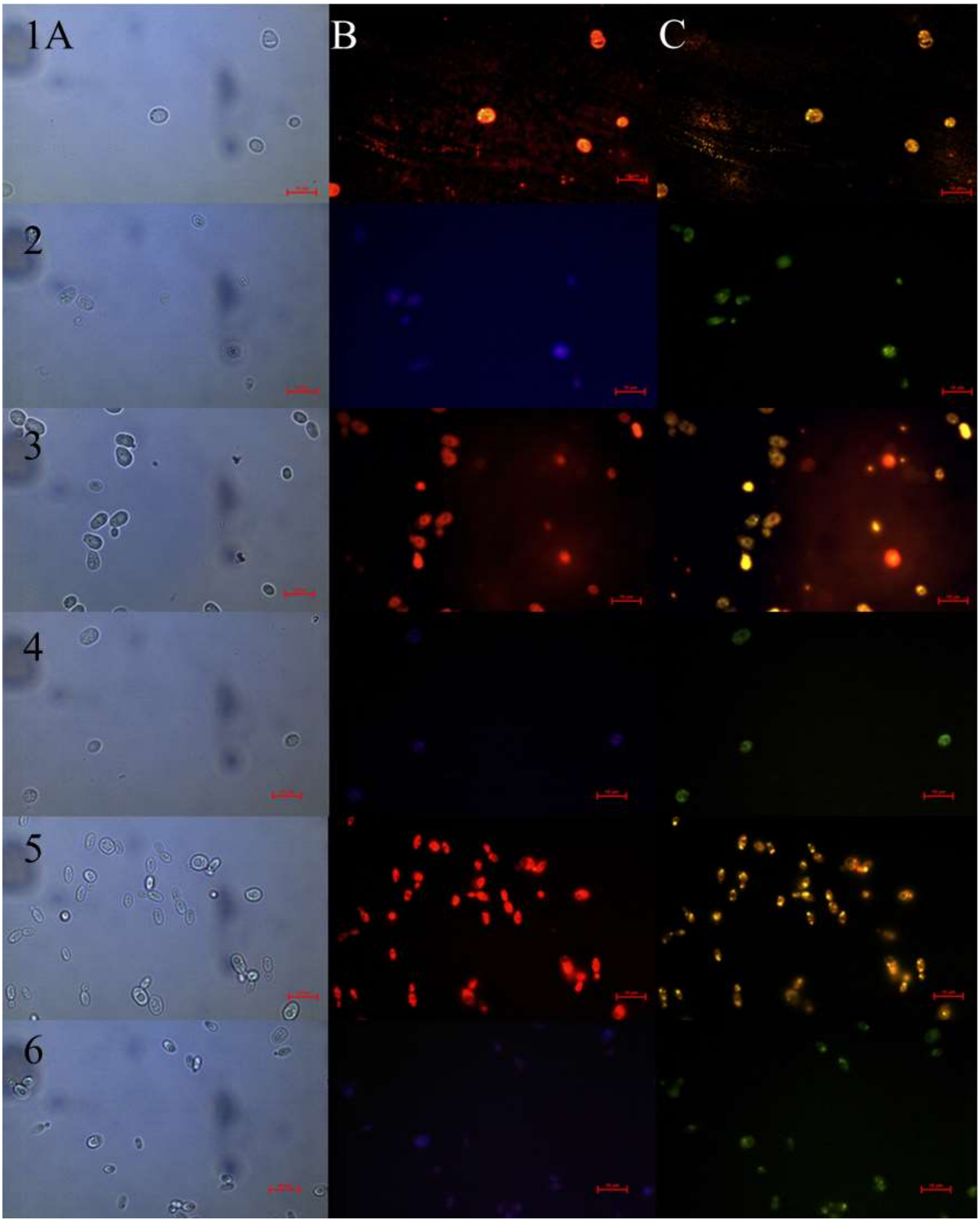


Figure 41: Microscope photo of Rhodosporidium toruloides illuminated under fluorescent light. 1;2 – 0 hour after inoculation, 3;4 – 4 hours after inoculation, 5;6 – 24 hours after inoculation. 1;3;5 – nile red stained, 2;4;6 – Nile red non-stained. A – classic light, B – green excitation filter, C – blue fluorescence filter. Scale presents length of 10 μm.

5.2.1.2 *Rhodotorula kratochvilovae (CCY 20-2-26)*

The Figure 42, Figure 43 and Figure 44 present a detailed analysis and visualisation of the lag phase of the yeast *Rhodotorula kratochvilovae*, examining both biochemical changes and cell population dynamics. The biochemical data indicate that the yeast undergoes significant metabolic shifts in response to the cultivation environment. For instance, carotenoid levels initially increase slightly from 0.546 mg/g in the inoculum to 0.699 mg/g at 0 hours and then fluctuate, reaching a peak of 1.348 mg/g at 24 hours. This suggests a possible role in antioxidant defence or cellular adaptation to the cultivation conditions. Similarly, ubiquinone concentrations show notable fluctuations; they rise sharply to 4.441 mg/g at 0 hours, decrease to 1.699 mg/g at 1 hour, and then peak again at 4.786 mg/g at 2.5 hours before gradually declining to 4.105 mg/g by 24 hours. This pattern indicates dynamic adjustments in cellular respiration and oxidative stress responses.

Ergosterol levels, which are critical for maintaining cell membrane integrity and fluidity, also change throughout the cultivation period, increasing from 4.217 mg/g at 0 hours to a maximum of 5.231 mg/g at 12 hours before declining to 4.584 mg/g at 24 hours. This fluctuation suggests periods of active cell membrane synthesis and remodelling, possibly in response to changes in environmental conditions. The lipid composition further supports these observations, with saturated fatty acids showing an initial decrease from 17.54 % in the inoculum to 12.56 % at 0 hours and then increasing to 17.30 % at 24 hours. Conversely, monounsaturated and polyunsaturated fatty acids show varying trends, with PUFAs peaking at 53.36 % at 12 hours, indicating a temporary increase in membrane fluidity before stabilizing.

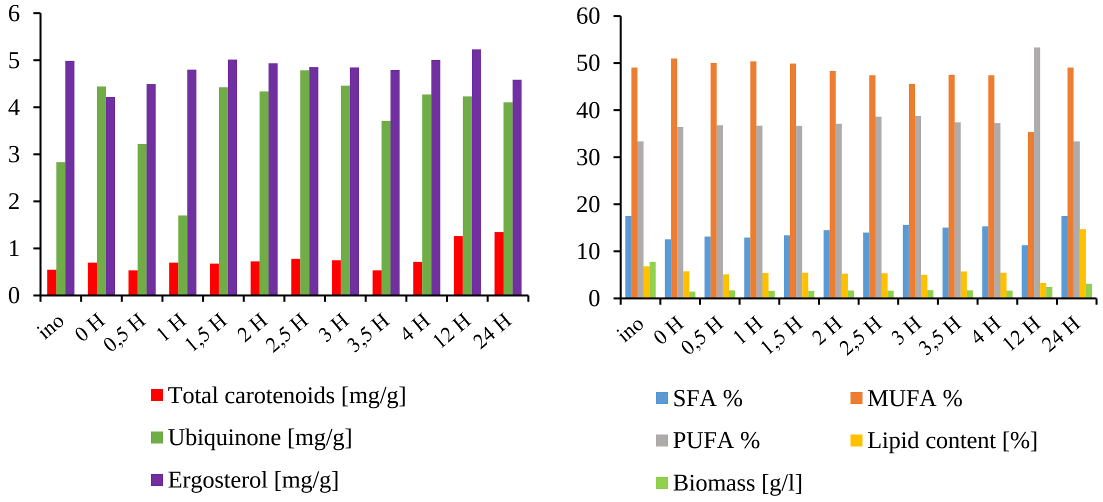


Figure 42: Metabolic and growth progress of R. kratochvilovae in early stages of adaptation on artificial medium

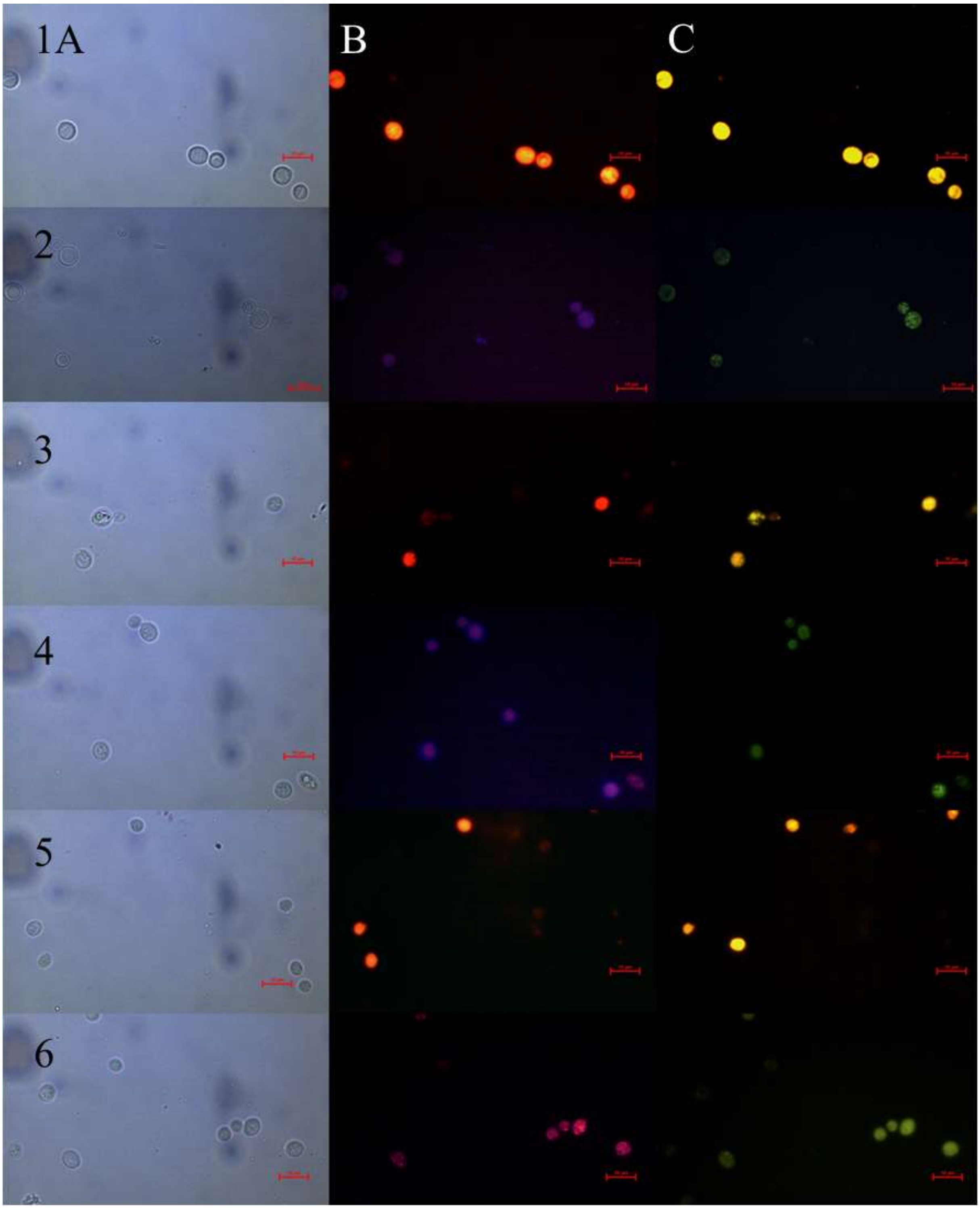


Figure 43: Microscope photo of Rhodotorula kratochvilovae illuminated under fluorescent light. 1;2 – 0 hour after inoculation, 3;4 – 4 hours after inoculation, 5;6 – 24 hours after inoculation. 1;3;5 – Nile red stained, 2;4;6 – Nile red non-stained. A – classic light, B – green excitation filter, C – blue excitation filter. Scale presents length of 10 μm.

The flow cytometry data (Figure 44) provide additional insights into the yeast's response to cultivation. The total number of non-stained cells increases from 434.03 cells/µl at 0 hours to 977.52 cells/µl by 24 hours, reflecting cell growth and division. Meanwhile, the Nile red-stained cells, which indicate intracellular lipid content, show dynamic changes. Low-intensity Nile red-stained cells (NR-LI) increase from 28.48 cells/µl at 0 hours to 247.86 cells/µl by 24 hours, suggesting gradual lipid accumulation. High-intensity Nile red-stained cells (NR-HI) initially decrease, reflecting a consumption or redistribution of lipid stores, before increasing to 710.7 cells/µl at 12 hours and stabilizing at 642.22 cells/µl by 24 hours. This suggests a phase of lipid mobilization followed by accumulation as the cells adapt to the environment. The biomass data correlate with these trends, showing an increase from 1.450 g/l at 0 hours to 3.10 g/l by 24 hours, indicating overall cellular growth. These findings highlight the complex metabolic adjustments *Rhodotorula kratochvilovae* undergoes during the lag phase to optimize survival and prepare for subsequent growth phases.

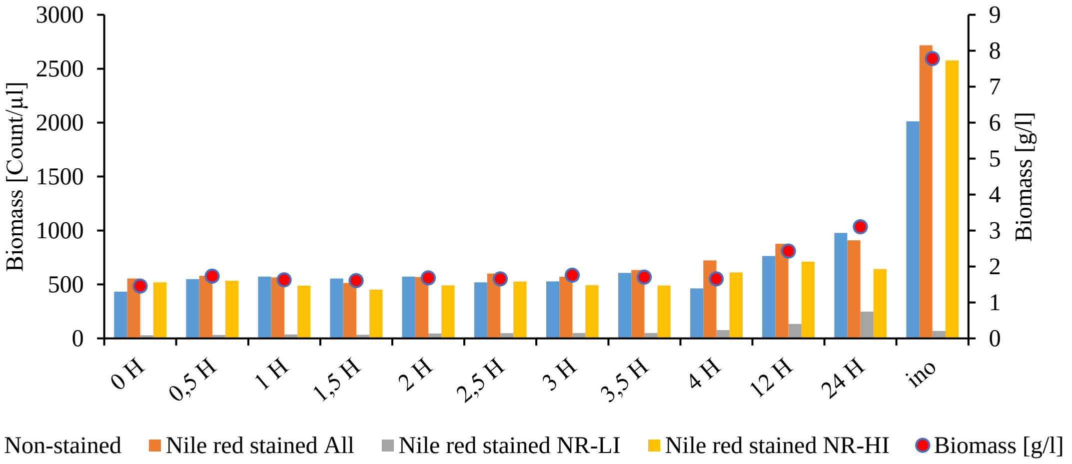


Figure 44: Data regarding growth of R. kratochvilovae; cytometric data on major axis and biomass growth on minor axis; NR - Nile red; LI- low fluorescence intensity; HI- high fluorescence intensity

5.2.1.3 *Phaffia rhodozyma (CCY 77-1-1)*

The Figure 45, Figure 46 and Figure 47 provide an extensive analysis and visualisation of the lag phase in the yeast *Phaffia rhodozyma*, focusing on various biochemical parameters and cell population dynamics over time. During the initial stages of cultivation, several significant changes occur in the yeast's metabolism and cellular composition. The carotenoid content~~,~~ increases from 1.634 mg/g in the inoculum to 2.176 mg/g at 0 hours, peaking at 3.013 mg/g by 24 hours. This substantial rise indicates an adaptive response to oxidative stress and a preparation for prolonged growth phases. Similarly, ubiquinone levels, which play a crucial role in cellular respiration and protecting against oxidative damage, fluctuate from 2.906 mg/g in the inoculum to 2.015 mg/g at 24 hours, suggesting dynamic adjustments in mitochondrial function and energy metabolism.

Ergosterol, essential for maintaining cell membrane integrity, shows a general increasing trend, from 3.570 mg/g in the inoculum to a peak of 5.206 mg/g at 12 hours before slightly decreasing to 4.324 mg/g at 24 hours. This pattern likely reflects periods of active membrane synthesis and stabilization as the yeast adapts to the cultivation environment. The biomass concentration also increases significantly from 2.075 g/l at 0 hours to 8.600 g/l by 24 hours, demonstrating substantial cell growth and proliferation. In parallel, changes in lipid composition are observed; the proportion of saturated fatty acids rises dramatically from 8.44 % in the inoculum to 35.07 % at 24 hours, indicating a shift towards more stable membrane structures in response to environmental stress. Meanwhile, the levels of monounsaturated fatty acids and polyunsaturated fatty acids decrease, reflecting metabolic shifts towards saturated lipid synthesis for membrane rigidity.

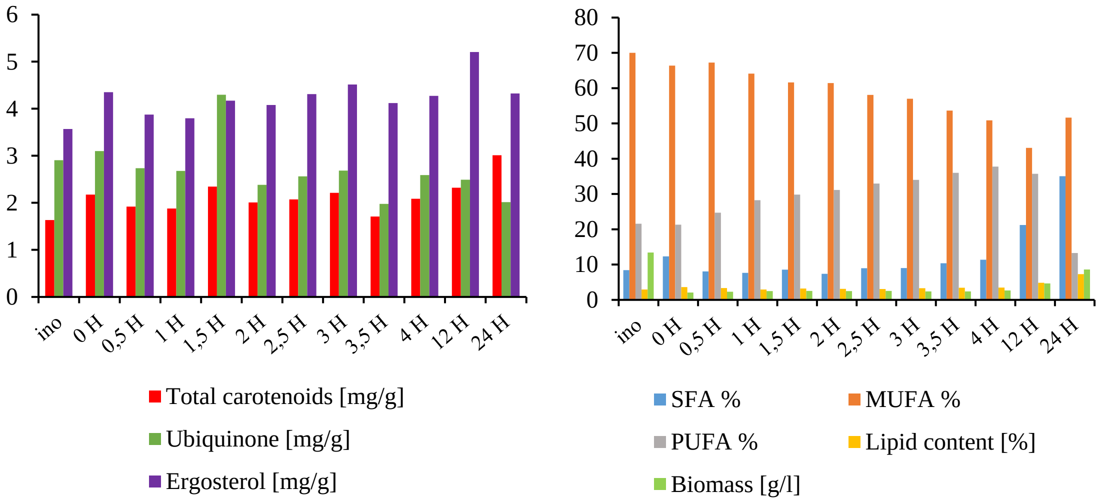


Figure 45: Metabolic and growth progress of P. rhodozyma in early stages of adaptation on artificial medium

The flow cytometry data presented in Figure 46 describe further insights into the cellular responses of *Phaffia rhodozyma* during the lag phase. Initially, the total count of non-stained cells is 951.47 cells/µl at 0 hours, which increases significantly to 6666.67 cells/µl by 24 hours, indicating robust cell proliferation. The Nile red-stained cells, which reveal intracellular lipid content, show distinct patterns; low-intensity Nile red-stained cells (NR-LI) increase from 457.75 cells/µl at 0 hours to 2137.69 cells/µl by 24 hours, suggesting lipid accumulation within the cells. High-intensity Nile red-stained cells (NR-HI), indicative of high lipid content, also increase substantially, from 278.72 cells/µl at 0 hours to 1656.54 cells/µl by 24 hours, reflecting enhanced lipid storage. These trends suggest that *Phaffia rhodozyma* undergoes significant metabolic reprogramming during the lag phase, characterized by increased carotenoid and ergosterol synthesis, lipid accumulation, and biomass growth, all indicative of adaptation to the cultivation environment and preparation for subsequent growth phases.

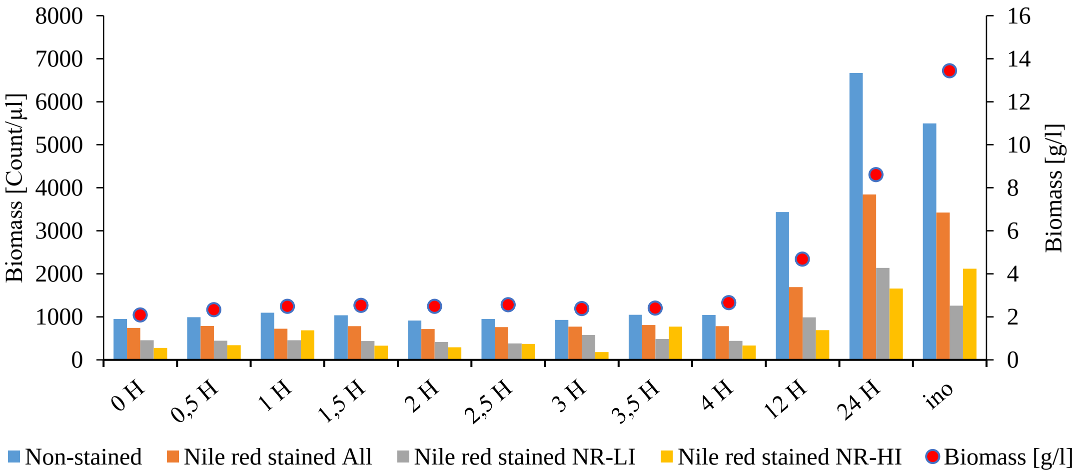


Figure 46: Data regarding growth of P. rhodozyma; cytometric data on major axis and biomass growth on minor axis; NR -Nile red; LI- low fluorescence intensity; HI- high fluorescence intensity

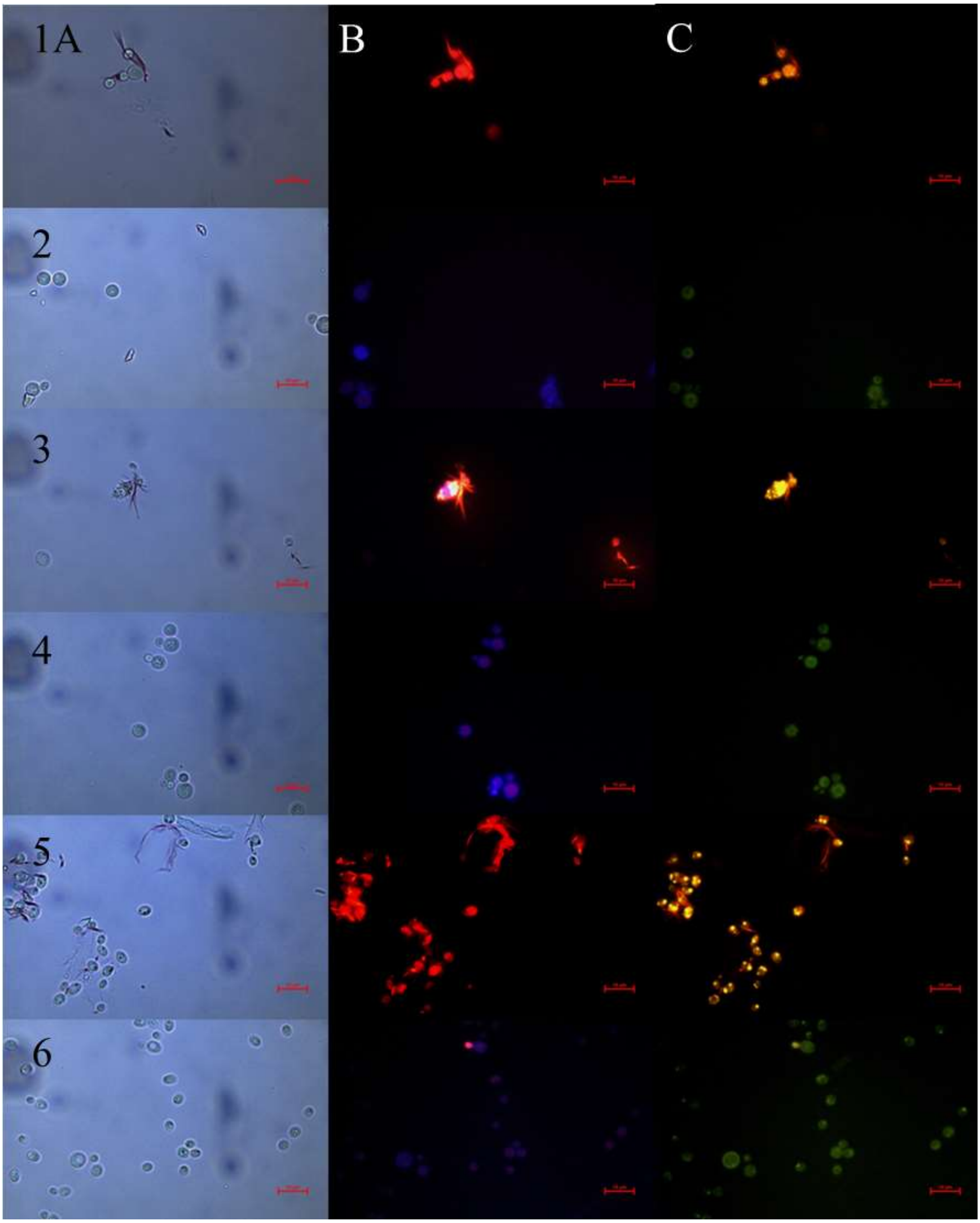


Figure 47: Microscope photo of Phaffia rhodozyma illuminated under fluorescent light. 1;2 – 0 hour after inoculation, 3;4 – 4 hours after inoculation, 5;6 – 24 hours after inoculation. 1;3;5 – Nile red stained, 2;4;6 – Nile red non-stained. A – classic light, B – green excitation filter, C – blue excitation filter. Scale presents length of 10 μm.

5.2.1.4 Cystofilobasidium macerans (CCY 10-1-2)

The Figure 48, Figure 49 and Figure 50 provide a comprehensive analysis of the lag phase of the yeast *Cystofilobasidium macerans*, focusing on various biochemical changes and cell population dynamics over time. During the initial hours of cultivation, several metabolites and cell counts exhibit significant variations, reflecting the yeast's metabolic adaptation to its new environment. Carotenoids, which serve as antioxidants, decrease from 3.402 mg/g in the inoculum to 2.259 mg/g at 4 hours, but then slightly increase to 2.743 mg/g by 24 hours. This fluctuation suggests a dynamic response to oxidative stress during the early phase, followed by stabilization as the cells adapt. Ubiquinone, essential for mitochondrial function and oxidative stress defence, initially increases from 1.899 mg/g in the inoculum to a peak of 5.501 mg/g at 3 hours, then gradually decreases to 2.870 mg/g by 24 hours, indicating a similar pattern of initial upregulation and metabolic burn followed by a stabilization phase.

Ergosterol levels, crucial for maintaining cell membrane integrity, fluctuate throughout the cultivation period. The concentration starts at 5.656 mg/g in the inoculum, peaks at 6.981 mg/g at 12 hours, and slightly decreases to 5.382 mg/g at 24 hours. This trend suggests active membrane synthesis and adaptation to changing environmental conditions. Biomass shows significant growth, from 1.675 g/l at 0 hours to 7.500 g/l at 24 hours, indicating robust cell proliferation.

Lipid composition undergoes notable changes, with saturated fatty acids increasing from 15.10 % at 0 hours to 37.89 % at 24 hours. This increase indicates a shift towards more saturated lipid synthesis, possibly to enhance membrane stability under stressful conditions. Conversely, monounsaturated and polyunsaturated fatty acids display varying trends, with MUFA decreasing and PUFA peaking at 41.67 % at 12 hours before decreasing, suggesting a temporary increase in membrane fluidity to facilitate adaptation.

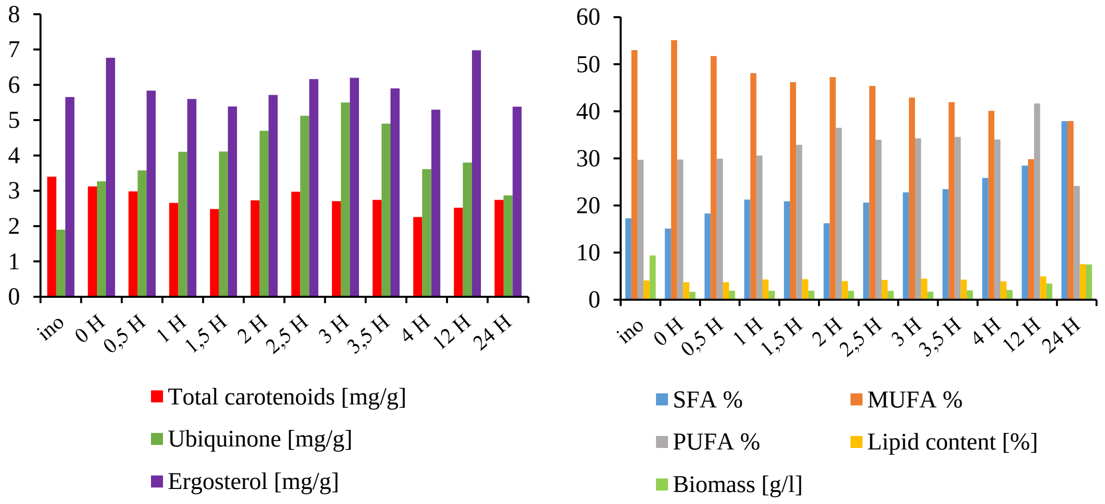


Figure 48: Metabolic and growth progress of C. macerans in early stages of adaptation on artificial medium

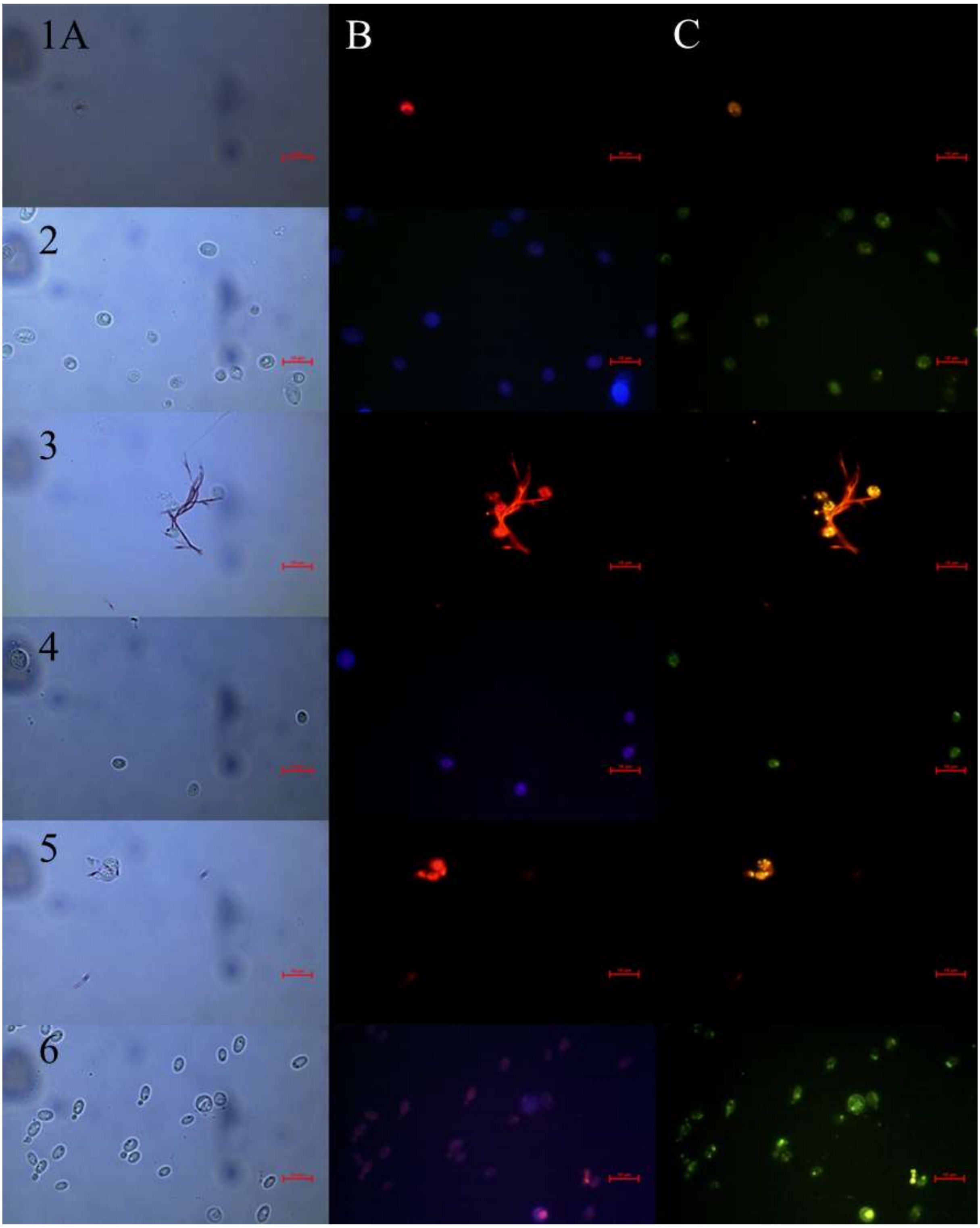


Figure 49: Microscope photo of Cystofilobasidium macerans illuminated under fluorescent light. 1;2 – 0 hour after inoculation, 3;4 – 4 hours after inoculation, 5;6 – 24 hours after inoculation. 1;3;5 – Nile red stained, 2;4;6 – Nile red non-stained. A – classic light, B – green excitation filter, C – blue excitation filter. Scale presents length of 10 µm.

The flow cytometry data (Figure 50) further elucidate these trends, showing an increase in non-stained cell counts from 831.95 cells/µl at 0 hours to 4065.04 cells/µl by 24 hours, indicating significant cell growth. Nile red-stained cells, which indicate intracellular lipid content, also show distinct patterns;

low-intensity stained cells (NR-LI) increase from 88.59 cells/µl at 0 hours to 719.34 cells/µl by 24 hours, suggesting gradual lipid accumulation. High-intensity stained cells (NR-HI) rise significantly from 656.28 cells/µl at 0 hours to 2030.11 cells/µl at 24 hours, indicating enhanced lipid storage, potentially as a stress response or preparation for rapid growth. These observations suggest that *Cystofilobasidium macerans* undergoes complex metabolic adjustments during the lag phase, balancing between oxidative stress management, membrane adaptation, and lipid accumulation to optimize growth and survival. Example of cytometric analysis scattergrams (Nile red – stained *C. macerans* at 24th hour of cultivation) are presented on the Figure 51.

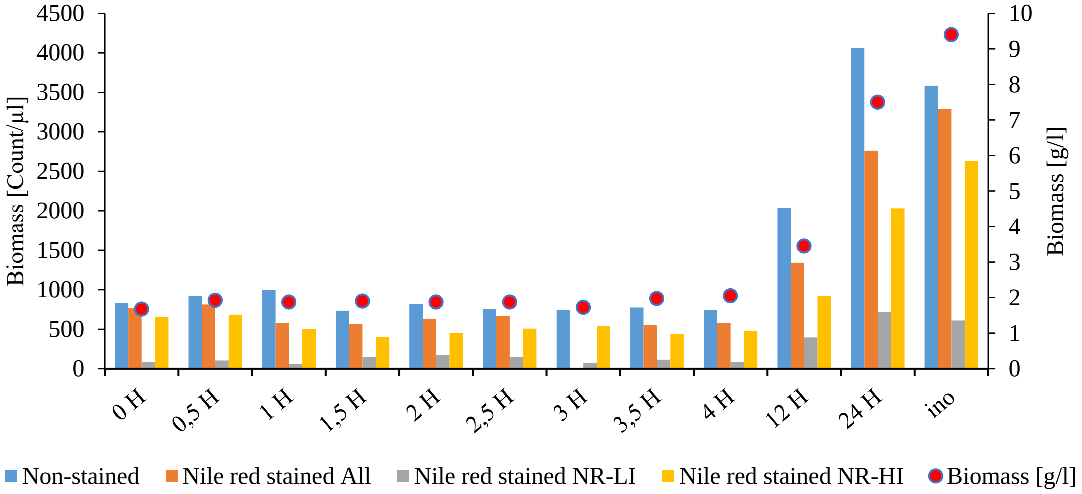


Figure 50: Data regarding growth of C. macerans; cytometric data on major axis and biomass growth on minor axis; NR - Nile red; LI- low fluorescence intensity; HI- high fluorescence intensity

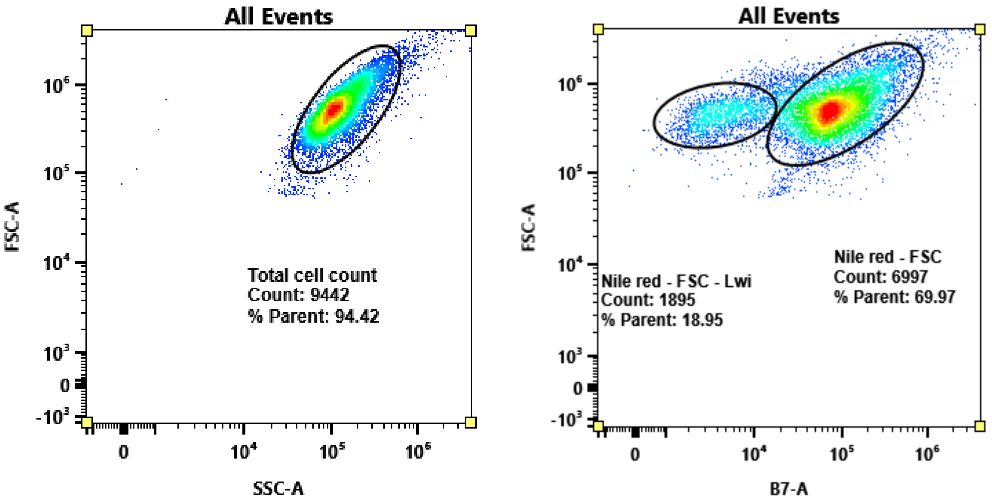


Figure 51: Biexponential scattergrams for Nile red – stained C. macerans at the 24th hour of cultivation. The left scattergram shows the dependence of size of the cells (FSC – Forward Scatter Channel) on internal cell complexity (SCS – Side Scatter Channel). The Right scattergram shows dependence of size of the cells (FSC – Forward Scatter Channel) on fluorescence intensity of Nile red (B7 – Fluorescence emission detection channel 652 – 669 nm for blue excitation laser – 488 nm).

6 CONCLUSIONS

This study addresses the cultivation of carotenogenic yeasts on waste substrates from the food industry (e.g., coffee grounds, spent grain, coffee oil, waste frying oil, animal fat, poultry fat, feathers, and spent brewery yeast) as well as waste from biofuel production (waste glycerol). Carotenogenic yeasts, as confirmed in this study, have the ability to utilize industrial waste for their growth while enriching themselves with various bioactive compounds, including carotenoids, ergosterol, and ubiquinone. These compounds possess antioxidant activity, and carotenoids and ergosterol also have provitamin activity. Another valuable product are fatty acids (lipids), which these yeasts can accumulate in high concentrations, classifying them as oleaginous yeasts. In some cases, accumulation of antioxidants or other lipidic substances (such as fatty acids) absorbed from the medium is observed within the yeast biomass, specifically vitamin E (tocopherol), which is present in high concentrations in extracted coffee oil.

Many waste materials are unsuitable for direct use in cultivation, requiring essential pretreatment to convert them into usable nutrient sources, primarily carbon and nitrogen. Pretreatments included lipid extraction (for coffee grounds), acid and enzymatic hydrolysis (for coffee grounds and spent grain, both lignocellulosic waste), and filtration to remove solid components. Feather waste underwent alkaline hydrolysis, while spent yeast was subjected to autolysis at elevated temperatures. Waste frying oil was pretreated by removing water and filtration. The obtained lipidic materials and hydrolysates were characterized for their carbon and nitrogen content and used as substrates for cultivation.

For cultivation in this study, the following carotenogenic yeast strains were used: *Rhodotorula (Rhodosporidium) toruloides* (CCY 62-2-4), *Rhodotorula kratochvilovae* (CCY 20-2-26), *Rhodotorula mucilaginosa* (CCY 20-9-7), *Rhodotorula mucilaginosa* (CCY 19-4-6), *Cystofilobasidium macerans* (CCY 10-1-2), *Cystofilobasidium infirmominiatum* (CCY 17-18-4), *Sporobolomyces (Sporidiobolus) pararoseus* (CCY 19-9-6), *Sporidiobolus metaroseus* (CCY 19-6-20), *Sporidiobolus salmonicolor* (CCY 19-6-4), *Phaffia rhodozyma* (CCY 77-1-1).

For flask cultivation, two C/N ratios (25 and 50) were selected, and various conditions such as the type of nitrogen source and combinations of carbon substrates were tested to determine their impact on the final yield. Among the measured parameters in the resulting biomass, only the top 2 to 3 results for each C/N ratio in the following text.

For a C/N ratio of 25, the highest carotenoid production was observed in *S. pararoseus* grown on a medium containing a mixture of coffee oil and animal fat as carbon sources, with lipase induction in the inoculum, achieving 11.554 mg/g biomass. The second-highest carotenoid production was recorded in *R. toruloides* on a mixture of frying oil, animal fat, and 25 % glycerol as the carbon source, yielding 11.454 mg/g biomass. Both cultivations used ammonium sulphate as the nitrogen source. The highest ubiquinone production was achieved by *P. rhodozyma* on waste frying oil with feather hydrolysate (25.416 mg/g) and by *S. pararoseus* on coffee oil with ammonium sulphate and inoculum induction (21.141 mg/g). Ergosterol production peaked in *S. pararoseus* on purified glycerol with ammonium sulphate (10.946 mg/g) and *R. toruloides* on frying oil with urea (10.429 mg/g). The highest tocopherol accumulation was observed in *R. mucilaginosa* (CCY 19-4-6) grown on pure coffee oil (22.971 mg/g) and on coffee oil with 10 % coffee hydrolysate (19.080 mg/g), both with potassium nitrate as the nitrogen source. The highest biomass growth was recorded in *R. mucilaginosa* (CCY 19-4-6) on poultry fat with urea (29.50 g/l) and *R. toruloides* on glucose with autolyzed yeast extract from commercial granulated yeast (27.50 g/l). The highest lipid accumulation was observed in *R. toruloides* on glycerol medium (pure glycerol) with ammonium nitrate (19.77 %) and in *S. metaroseus* (19.03 %). The strains that showed the highest results for the C/N ratio of 25 were *S. pararoseus*, *R. toruloides*, and *R. mucilaginosa* (CCY 19-4-6).

For a C/N ratio of 50, the most significant results are highlighted in this section. The highest carotenoid content was recorded in *R. mucilaginosa* (CCY 19-4-6) on glycerol medium (pure glycerol) with urea (6.102 mg/g) and on a medium containing poultry fat with 25 % glycerol and feather hydrolysate (5.641 mg/g). Ubiquinone production peaked in *R. kratochvilovae* on glycerol medium with feather hydrolysate (15.505 mg/g) and on its equivalent with urea (13.149 mg/g). Ergosterol production was the highest in *S. pararoseus* on glycerol medium with urea (14.365 mg/g) and in *R. kratochvilovae* on a medium with poultry fat, 10 % glycerol, and feather hydrolysate (11.584 mg/g). Tocopherol significantly enriched the biomass of *R. mucilaginosa* (CCY 19-4-6) (62.273 mg/g) and *S. pararoseus* (45.791 mg/g) on coffee oil medium with 20 % coffee hydrolysate and urea as the nitrogen source. The highest biomass yields were observed in *R. toruloides* on a medium with a triple amount of inorganic nitrogen supplemented with yeast autolysate from granulated yeast and glucose as the carbon source (33.28 g/l), as well as on a medium with yeast autolysate from spent brewery yeast completely replacing the inorganic nitrogen source with glucose as the carbon source (33.23 g/l). *S. pararoseus* achieved 31.12 g/l on a medium containing coffee oil and 10 % coffee hydrolysate. High lipid accumulation, confirming the oleaginous nature of these strains, was observed in *R. mucilaginosa* (CCY 20-9-7) on glycerol medium (pure glycerol) with urea (79.00 %) and in *S. pararoseus* on a medium with coffee oil and 30 % coffee hydrolysate with urea as the nitrogen source (65.00 %). The strains that yielded the highest results for a C/N ratio of 50 were *S. pararoseus*, *R. kratochvilovae*, and *R. mucilaginosa* (CCY 19-4-6).

A portion of the experiments was conducted in bioreactor cultivations, testing various waste substrates on several different strains. The most significant biomass growth and lipid accumulation were observed in *S. pararoseus*, cultivated on coffee oil with 10 % coffee hydrolysate, with 40.56 % lipids at 70 hours and a biomass yield of over 40 g/l at 57 hours. High lipid accumulation was also observed in the bioreactor using glycerol and feather hydrolysate with *R. toruloides* (40.81 %). The highest carotenoid and ubiquinone production was achieved by *R. toruloides* on a medium with coffee oil, ammonium sulphate and potassium nitrate, at 30 hours for ubiquinone (14.993 mg/g) and at 119 hours for carotenoids (11.984 mg/g). The highest ergosterol production was recorded in the same strain on coffee oil medium with 10 % coffee hydrolysate and urea at 144 hours (9.783 mg/g), with the highest tocopherol accumulation at 40 hours (19.706 mg/g). In the bioreactor using waste glycerol and *C. macerans*, the biomass yield coefficient was 0.415, a relatively high value, although biomass growth was low, making it less suitable for industrial use in terms of time efficiency. The high biomass and lipid accumulation in *S. pararoseus* and the significant accumulation of specific metabolites in *R. toruloides* make these strains potential industrial producers of these compounds and biomass on waste substrates.

Another significant experiment in this study involved testing and visualizing the initial adaptation phases (lag phase) of cells subjected to a medium shift by subculturing from a complex medium to a mineral medium. Visualization was performed using fluorescence techniques (cytometry and microscopy), and a comprehensive overview of yeast culture development is provided in chapter 5.2. It was found that most strains remained in the lag phase during the first 4 hours, as the cell count did not increase substantially. However, a gradual increase in biomass weight was observed in some strains, which can be attributed to the gradual synthesis of enzymes and accumulation of metabolites necessary for the transition into the exponential phase. A visible transition to accelerated growth or the exponential phase was observed in all strains except *R. kratochvilovae* after 12 hours of cultivation, evidenced by increases in both cell count and biomass weight, indicating intensive cell division.

Under fluorescence microscopy, the autofluorescence of carotenoids and FAD was visible, excited by blue excitation light and emitting green to yellow fluorescence. This fluorescence likely originates from clusters in organelles, probably mitochondria, where the Krebs cycle and beta-oxidation of fatty acids occur, processes in which FAD is involved. Additionally, weak reddish autofluorescence was observed under green light excitation, which was diffuse throughout the cell, likely caused by carotenoids with a broad absorption spectrum extending into longer wavelengths. When stained with Nile red, different phenomena were observed based on its binding to phospholipids and triglycerides. Nile red bound to phospholipids exhibits a fluorescence absorption maximum in the green excitation filter range and emits red light, corresponding to the emission spectrum. This causes the entire cell surface to emit red light under green light excitation, as the cytoplasmic membrane is composed of phospholipids. In contrast, Nile red bound to triglycerides has absorption maxima in the blue range and emits yellow-orange fluorescence, highlighting lipid droplets within the cells.

The study confirms the original hypothesis that carotenogenic yeast strains can be used to utilize waste materials and produce valuable biomass enriched with carotenoids, ubiquinone, ergosterol, and lipids. These strains also show potential for industrial applications in waste utilization (see bioreactor cultivations). Growth-phases experiments demonstrate that under a C/N ratio of 50, the transition from the lag phase to accelerated growth occurred in nearly all studied strains between 4-12 hours. For future experiments, it would be beneficial to make more frequent samples during this time interval to better investigate this phenomenon. The selected strains, representing different genera, were chosen to serve as model microorganisms for potential industrial applications. Since short lag phases are crucial for industrial processes, these experiments should also be performed in future using media with waste substrates.

7 LIST OF ABBREVIATIONS

1N	Standard nitrogen amount
3N	Triple nitrogen amount
ACP	Acyl carrier protein
Add	Addition of autolysate
ATP	Adenosine triphosphate
BCRC	Bioresource Collection and Research Center (Taiwan)
C/N	Carbon to Nitrogen ratio
CA	Commercial autolysate
CCY	Culture Collection of Yeasts
CM	*Cystofilobasidium macerans*
Co	Coffee oil
CoA	Coenzyme A
Cont	Control experiment
DMAPP	Dimethylallyl pyrophosphate
DSM	German Collection of Microorganisms and Cell Cultures GmbH
ELSD	Evaporative light scattering detector
F.H.	Feather Hydrolysate
FAA	Fatty acid
FACS	Fluorescence activated cell sorting
FAD	Flavin adenine dinucleotide
FAME	Fatty acid methyl ester
FC	Fluorescence cytometry
FID	Flame ionization detector
Fo	Frying oil
FPP	Farnesyl pyrophosphate
FSC	Forward scatter channel
GC	Gas chromatography
GGPP	Geranylgeranyl pyrophosphate
Glu	Glucose
Gly	Glycerol
GPP	Geranyl pyrophosphate
GR	Granulated yeasts
HI	High intensity
HPLC	High performance liquid chromatography
IPA	Isopropyl alcohol
IPP	Isopentenyl pyrophosphate
LSCM	Laser scanning confocal microscopy
LI(Lwi)	Low intensity
MUFA	Monounsaturated fatty acids
MVA	Mevalonic acid
MVP	Mevalonate phosphate
MVPP	Mevalonate pyrophosphate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCIM	National Collection of Industrial Microorganisms (India)
NR	Nile red
PDA	Poly diode array detector
Pf	Poultry fat
PHA	Polyhydroxyalkanoates
PR	*Phaffia rhodozyma*
PTT	Poly-tri-methylene terephthalate
PUFA	Polyunsaturated fatty acids
RCMB	The Regional Center for Mycology and Biotechnology (Egypt)

RI	Refractive index
RK	*Rhodotorula kratochvilovae*
RM (19-4-6)	*Rhodotorula mucilaginosa* (CCY 19-4-6)
RM (20-9-7)	*Rhodotorula mucilaginosa* (CCY 20-9-7)
Rpm	Rounds per minute
RT	*Rhodotorula (Rhodosporidium) toruloides*
SM	*Sporidiobolus metaroseus*
SP	*Sporobolomyces (Sporidiobolus) pararoseus*
SS	*Sporidiobolus salmonicolor*
SSC	Side scatter channel
SY	Spent brewery yeasts
TAG	Triacylglycerol
TISTR	Thailand Institute of Scientific and Technological Research
TSE	Transmissible spongiform encephalopathy
Ur	Urea
UV	Ultraviolet light
VIS	Visible light
WG	Waste glycerol
WH	Whey

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9 LIST OF ATTACHMENTS

Attachments are included separately as original full texts of articles and conference abstracts.

Attachment 1:

SZOTKOWSKI, Martin; PLHALOVÁ, Žaneta; SNIEGOŇOVÁ, Pavlína; HOLUB, Jiří; CHUJANOV, Oleg et al., 2023. Conversion of Mixed Waste Food Substrates by Carotenogenic Yeasts of Rhodotorula sp. Genus. Online. *Microorganisms*. Roč. 11, č. 4. ISSN 2076-2607. Available at: https://doi.org/10.3390/microorganisms11041013.

Attachment 2:

HOLUB, Jiří; SZOTKOWSKI, Martin; CHUJANOV, Oleg; ŠPAČKOVÁ, Dominika; SNIEGOŇOVÁ, Pavlína et al., 2023. Production of Enriched Biomass by Carotenogenic Yeasts Cultivated on by-Products of Poultry Processing—A Screening Study. Online. *Microorganisms*. Roč. 11, č. 2. ISSN 2076-2607. Available at: https://doi.org/10.3390/microorganisms11020321.

Attachment 3:

HOLUB, J.; CHUJANOV, O.; SZOTKOWSKI, M.; ŠIMANSKÝ, S.; DZURICKÁ, L.; MÁROVÁ, I.; SCHILDOVÁ, V. Optimization of cultivations of carotenogenic yeasts on poultry waste substrates. Proceedings of the 9 th International Conference on Chemical Technology. 1. Prague: 2022. p. 356 ISBN: 978-80-88307-11-2

Attachment 4:

HOLUB, J.; CHUJANOV, O.; ŠPAČKOVÁ, D.; ŠIMANSKÝ, S.; OBRAČAJ, J.; MÁROVÁ, I. CULTIVATION OF CAROTENOGENIC YEAST ON VARIOUS FOOD INDUSTRIAL WASTE SUBSTRATES. Vyšehradská 320/49 128 00 Prague 2 Czech Republic: AMCA, spol. s r.o., 2024. p. 201-201.

Attachment 5:

HOLUB, J.; SZOTKOWSKI, M.; ŠIMANSKÝ, S.; HUBAČOVÁ, K.; PLHALOVÁ, Ž.; MÁROVÁ, I. Production of carotenoid and lipid enriched red yeasts biomass on a combination of whey and waste animal fatmal fat. Poland: Sciendo, 2021. p. 90-90.

Attachment 1

SZOTKOWSKI, Martin; PLHALOVÁ, Žaneta; SNIEGOŇOVÁ, Pavlína; HOLUB, Jiří; CHUJANOV, Oleg et al., 2023. Conversion of Mixed Waste Food Substrates by Carotenogenic Yeasts of Rhodotorula sp. Genus. Online. *Microorganisms*. Roč. 11, č. 4. ISSN 2076-2607. Available at: https://doi.org/10.3390/microorganisms11041013.

microorganisms

Article

Conversion of Mixed Waste Food Substrates by Carotenogenic Yeasts of *Rhodotorula* sp. Genus

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Abstract: The consequence of the massive increase in population in recent years is the enormous production of mainly industrial waste. The effort to minimize these waste products is, therefore, no longer sufficient. Biotechnologists, therefore, started looking for ways to not only reuse these waste products, but also to valorise them. This work focuses on the biotechnological use and processing of waste oils/fats and waste glycerol by carotenogenic yeasts of the genus *Rhodotorula* and *Sporidiobolus*. The results of this work show that the selected yeast strains are able to process waste glycerol as well as some oils and fats in a circular economy model and, moreover, are resistant to potential antimicrobial compounds present in the medium. The best-growing strains, *Rhodotorula toruloides* CCY 062-002-004 and *Rhodotorula kratochvilovae* CCY 020-002-026, were selected for fed-batch cultivation in a laboratory bioreactor in a medium containing a mixture of coffee oil and waste glycerol. The results show that both strains were able to produce more than 18 g of biomass per litre of media with a high content of carotenoids (10.757 ± 1.007 mg/g of CDW in *R. kratochvilovae* and 10.514 ± 1.520 mg/g of CDW in *R. toruloides*, respectively). The overall results prove that combining different waste substrates is a promising option for producing yeast biomass enriched with carotenoids, lipids, and beta-glucans.

Keywords: carotenogenic yeasts; β-glucans; lipids; carotenoids; waste glycerol; waste animal fat; waste frying oil; waste coffee oil

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1. Introduction

Nowadays, more than ever, it is necessary to start using sustainable sources of raw materials and thus try to minimise the waste products of the industry. Thus, the transition from a linear model of the economy to a circular model is one of the most discussed topics in the field of biotechnology as well. The circular economy is a discipline whose main goal is to maximise the use of raw materials and, if possible, eliminate waste production [1]. Human society in the agricultural and food industry produces a huge amount of waste, which increases yearly with the growing population and resulting demands for food [2–5]. An example is the coffee industry, which produces millions of tons of coffee grounds annually [6–13]. Another example is the biofuel production industry boom in the form of fatty acids methyl esters (FAME), the waste of which is a large amount of waste glycerol [10]. Conventional waste processing methods, e.g., combustion or composting, massively release CO_2 into the atmosphere, which greatly increases the burden on the environment [11,12]. On the other hand, biotechnological processing of waste materials provides many possibilities for producing substances as an alternative to the expensive and synthetic compounds used in many fields, and could decrease CO_2 emissions into the atmosphere [13].

Many waste by-products are processed into products with low added value by conventional processing methods. Therefore, the potential of the waste by-product is not fully exploited. However, biotechnological processing of food and agricultural waste by particular microorganisms allows us to produce many substances with a higher added value and, thus, better use the potential of these substances [14,15]. This work focuses on the processing of some glycerol- and oil-based waste substrates by carotenogenic yeasts, and aims to find suitable media for carotenogenic yeasts to maximize biomass and bioactive compound production in order to develop a method for low-cost and large-scale cultivations in the future.

Carotenogenic yeasts are a disparate group of microscopic fungi capable of producing large amounts of carotenoid pigments (up to 35.0 mg/g dry weight [14]), resulting in their characteristic red colour. Furthermore, they produce ergosterol, squalene, ubiquinone (which are products of the active mevalonate pathway) [16], and a large amounts of proteins and beta-glucans which are present within their cell walls [17,18]. Red yeasts are also able to utilize a large number of different carbon sources (oils, fats, glycerol, etc.) and accumulate a large number of lipids in the biomass (up to 80%) [19,20]. Thus, red yeasts offer the possibility of producing biomass enriched by all substances mentioned above [16–18,21].

In this work, some lipid waste substrates were tested as a C-source for red yeast cultivation. This article is devoted not only to coffee oil, which is a lipidic material rich in carbon compounds and is originated from spent coffee grounds (SCG), which is waste material that mainly consist of polysaccharides, lignin, and lipids (10–20 wt.%) [13]. Due to the high lipid content, coffee oil rich in saturated and unsaturated fatty acids (mainly PUFA) and tocopherol can be obtained from SCG by extraction processes [22,23]. As demonstrated in this study, coffee oil can be used as a rich carbon source for cultivation of carotenogenic yeasts and other microorganisms, with tocopherol accumulation induced in yeast cells [13,21,24].

A cheaper and more readily available waste lipid substrate is waste frying oil (WFO), which is a very rich C-source with a high content of unsaturated fatty acids (MUFA and PUFA). On the other hand, it also contains products of lipid degradation and peroxidation formed by its overheating during frying [25–28]. As already demonstrated in a recent study, carotenogenic yeasts show a significant production of valuable metabolites in frying oil [21].

Another lipid waste substrate is animal fat. Due to the intensive production of edible animal fat, significant amounts of waste lipid by-product (waste fat) are continuously generated [28]. Waste fat can be used for re-incorporation into the human organism via supplementation of the food chain through microbial biotechnological products [17].

In the production of biogas or biofuels, as a result of the processing of lipid substrates, waste glycerol is also produced. It is a trivalent alcohol that is very easily used by microorganisms. The possibility of using carotenogenic yeasts to valorise waste and technical glycerol was confirmed as well [29]. Due to the fact that other waste lipidic substrates are non-polar and their transport into the cells could be a limiting factor, contrary glycerol solubility in water allows its faster transport into the cells and, owing to this fact, it is a more suitable substrate for early stages of cultivations [30].

Utilization of waste substrates for cultivation of microorganisms often requires their pretreatment and/or enzymatic sugar release to improve their availability to metabolic processing. During harsh pretreatment, toxic compounds can be generated, which can affect culture viability. Moreover, some microbial growth inhibitors can be found as a part of some waste materials, and a detoxification process is needed. The costs of waste processing and detoxification could be balanced by the fact that media based on waste substrates are rich in macro- and micro-nutrients essential for microbial growth. Therefore, any further addition of expensive supplements is not required [21,31].

In this work, the usage of combined food and agricultural waste substrates as a possible source of nutrients necessary for growth of microorganisms is further examined. Our experimental strategy is based on gradual selection and valorisation of waste by-products, first using simple, rapidly metabolizable processed waste material that provides the microorganisms with energy nutrients in the first hours of cultivation to produce biomass and necessary enzymes for the later stages. The second, more complex material will serve as the microorganism's main carbon source. The complex material induces the production of secondary and stress-induced metabolites (carotenoids, lipids, ergosterol, ubiquinone), which are economically advantageous and increase the profitability of the whole biotechnological process. This approach reduces the use of expensive simple C-sources, but also significantly extends the production time of microorganisms and exerts higher stress levels on carotenogenic yeasts. On the other hand, using a complex source lowers biomass production, and, thus, some compromise conditions must be found.

2. Materials and Methods

2.1. Yeast Strains

Based on our results on yeast biotechnology [17,21,32] regarding the ability to process waste materials and crude glycerol, several yeast strains of genus *Rhodotorula* were enrolled in this study as follows: *Rhodotorula toruloides* CCY 062-002-004, *Rhodotorula kratochvilovae* CCY 020-002-026, *Rhodotorula mucilaginosa* CCY 019-004-006, and *Sporidiobolus pararoseus* CCY 019-009-006 as a comparison. All strains were purchased from Culture Collection of Yeasts (CCY; Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic) and preserved in cryovials (YPD media with 50% glycerol solution) at −82 °C.

2.2. Microorganisms Cultivation, Hydrolysate, and Media Preparation

2.2.1. Yeast Inoculation

YPD media was used for all inoculation steps. For proper propagation, cultures from cryovials were inoculated on Petri dishes with YPD agar and kept under laboratory temperature for 96 h. After sufficient proliferation, using an inoculation loop, the yeasts were inoculated into 50 mL of liquid YPD medium in a 250 mL Erlenmeyer flask with the ratio of 1 loop per 10 mL of media. After 24 h, 25 mL of culture from Inoculum I was transferred to a 500 mL Erlenmeyer flask with 125 mL of YPD media (Inoculum II). After 24 h, the culture was inoculated into production media. All cultivations were performed at room temperature 22 °C under constant illumination of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of photons on a reciprocal shaker with 110 rpm. The composition of YPD media was as follows: 20 g glycerol, 20 g peptone, 10 g yeast extract, and 1000 mL of tap water. Two-stage cultivation in nutrient-rich liquid inoculation media (Inoculum I and II) was used to produce a sufficient amount of yeast cells for the next step, cultivation in production media [17,21,32].

2.2.2. Yeast Production Media

The experimental scheme was divided into several parts and continued the results of our previous works [16,21,33]. The first part focused on extensive screening cultivation of various yeasts on different combinations of waste glycerol and different waste oils and two ratios: simple:complex carbon source. The second part followed by studying lipase induction's effect on whole biomass and metabolite production during the inoculation step. The media contained only lipid waste substrates for this part, except for control glycerol media. The induction was performed by adding 0.5 g of waste frying oil into Inoculum II.

Studied yeast strains were cultivated on basic mineral medium with composition as follows: 4 g KH_2PO_4, 0.696 g $MgSO_4\cdot7H_2O$, 5.12 g $NaNO_3$, 4 g $(NH_4)_2SO_4$, 46.44 g Glycerol, and 1000 mL of tap water. Composition of the media corresponds to a C/N ratio of 25, and Glycerol-based media were used as a control. All salts, glycerol, glucose, acids, and bases used in this work were purchased from Penta company (Prague, Czech Republic). Yeast extract and peptone were purchased from Himedia company, Thane, India. The studied parameters in this work were combinations of waste glycerol and waste fats/oils as carbon sources in the media, different ratios between simple and complex carbon sources, and the effect of yeast lipase production induced during the inoculation step. Yeasts were cultivated in 250 mL Erlenmeyer flasks with 55 mL of media (50 mL production media + 5 mL Inoculum II). Cultivations were performed on reciprocal shakers with a shaking amplitude of 110 rpm, under constant illumination, and at a laboratory temperature of 22 °C for 96 h. All laboratory cultivations were performed in triplicate. The Table 1 below lists the abbreviations describing the media that were used. Figure 1 shows a simplified experimental scheme.

Table 1. List of abbreviations and explanations.

Media Type	Explanation
Control	Control glycerol media
CO	Media with a waste coffee oil as sole carbon source
FO	Media with a waste frying oil as sole carbon source
Fat	Media with a mixed waste animal fat as sole carbon source
CO+Fat	Media with a combination coffee oil:animal fat 50:50 as carbon source
FO+Fat	Media with a combination frying oil:animal fat 50:50 as carbon source
FO+Gly10	Media with a combination frying oil:glycerol 90:10 as a carbon source
CO+Gly10	Media with a combination coffee oil:glycerol 90:10 as a carbon source
Fat+Gly10	Media with a combination animal fat:glycerol 90:10 as a carbon source
FO+Gly25	Media with a combination frying oil:glycerol 75:25 as a carbon source
CO+Gly25	Media with a combination coffee oil:glycerol 75:25 as a carbon source
Fat+Gly25	Media with a combination animal fat:glycerol 75:25 as a carbon source
(I)	Lipase induction media—yeast inoculum culture was cultivated with the addition of oil to induce the lipase production

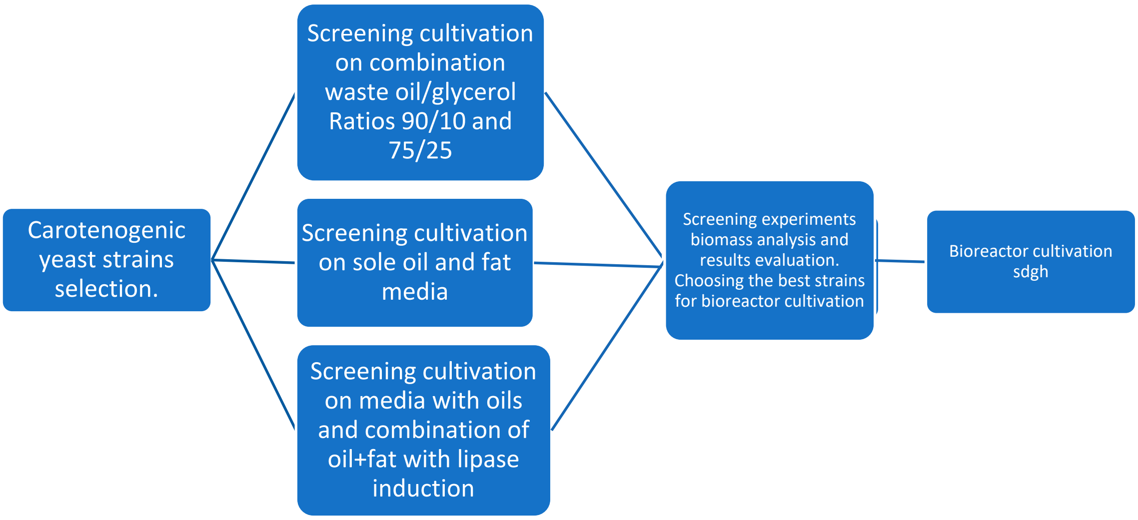

Figure 1. Experimental scheme diagram.

2.2.3. Large-Scale Bioreactor Co-Cultivation

The last part of the experiment was performed in a laboratory 7.0 L bioreactor (RALF model, Bioengineering AG, Wald, Switzerland) under conditions based on previous data [21] and the results obtained from this work. The yeasts *Rhodotorula kratochvilovae* CCY 020-002-026 and *Rhodotorula mucilaginosa* CCY 019-004-006 were chosen as the best strains for bioreactor cultivation. For large-scale cultivation, higher glycerol:oil ratios were chosen with the fed-batch cultivation style. Waste glycerol served as a fast and easy-to-process carbon source in combination with a second complex source, which was an untreated waste lipid source. The ratio between carbon sources was 25:75% SCG glycerol:waste oil.

Before cultivation, the glycerol solution was diluted with tap water to match the carbon source concentration, then poured into the reactor vessel together with the salts and 10% of the calculated waste lipid source amount. After that, the bioreactor was equipped with a pre-calibrated pH probe, dissolved oxygen probe, aeration ring, stirrer, and a 0.2 µm pore size filter for aeration and temperature sensor. Subsequently, the fermenter and the equipment were sterilised in an autoclave and then cooled to room temperature. In the separate bottle, the rest of the calculated waste lipid source (90%) was autoclaved in a separate bottle and, after cooling down, connected to the bioreactor via a pump controlled by the bioreactor programme.

The bioreactor was also equipped with two bottles containing 5% KOH and 5% H_2SO_4 and was sterilised in an autoclave at 121 °C for 15 min. After cooling, the medium's pH was adjusted to 6.5 and the temperature to 25 °C with gentle stirring at 60 rpm. Under these conditions, the calibration of the oxygen probe was conducted. Zero pO_2 value was set immediately after pH and temperature setting, and 100% pO_2 value was set after vigorous stirring and aeration. Yeasts were inoculated into a fermenter with a ratio of 1:20. Before inoculation, yeast culture was supplemented with an antifoam agent. Cultivations lasted seven days, i.e., 168 h. This time was chosen based on the first cultivation until all the oil was pumped into the system and consumed by the yeast with a time delay. Comparative cultivation of the second strain was carried out under the same conditions. The addition of an additional lipid waste source was controlled by oxygen consumption. Process parameters used during cultivation are listed in the following Table 2. All bioreactor cultivations were performed in duplicate.

Table 2. Bioreactor process values during co-cultivation.

Parameters	Values
Media volume	5.25 L
Stirring	300–800 rpm—regulated by oxygen consumption
pH	6.5
pO_2	30%
Temperature	25 °C
Aeration	4 L per minute
Illumination	200 $\mu mol \cdot m^2 \cdot s^{-1}$ of photons
Inoculation ratio	1:10
Lipid waste feeding	regulated by oxygen consumption

2.3. Waste Materials

2.3.1. Waste Glycerol

A waste glycerol solution was obtained as a waste by-product of acid high-temperature transesterification of vegetable oil and animal fat from a concurrent commercial project in another research group. The waste glycerol solution was sterilised immediately in an autoclave at 121 °C for 15 min upon receipt. It was subsequently stored at temperatures of 4–8 °C in the dark. Before use, waste glycerol was analysed for glycerol, free fatty acid, and lipid content using methods described in the Section 2.4.5.

2.3.2. Waste Oils and Fats

Waste coffee grounds (SCG) were obtained from a commercial coffee shop using coffee-type Robusta. To prepare SCG hydrolysate, collected SCG were dried for 24 h at 80 °C. Dried SCG were then milled in a laboratory grinder to obtain particles with size 100–500 µm. The next step was the oil extraction from dried SCG using the Soxhlet apparatus. The procedure went as follows: 80 g of milled SCG was extracted with 500 mL of hexane:IPA 60:40 in the Soxhlet apparatus for approximately 90 min. The extraction was kept until a colourless liquid extract was produced. Coffee oil was obtained during the SCG hydrolysis as a by-product during Soxhlet extraction of the spent coffee grounds. Coffee oil extract in IPA:Hexane 40:60 solution was evaporated under a vacuum. Defatted SCGs were stored and used to prepare SCG hydrolysate for ongoing research on processing carbohydrate substrates by heterotrophic microorganisms.

Waste frying oil (sunflower frying oil) was obtained from a household kitchen and was filtered before use under a vacuum through filtration paper to remove any leftover food particles. Waste animal fat was obtained from the company Norillia, Norway, and its composition is described in our previous work [17]. All waste lipid materials were used in natural form and were not subjected to any kind of hydrolysis. Lipid wastes were stored at 4–8 °C for further use. Its composition was analysed using gas chromatography, described in Section 2.4.5.

2.4. Analytical Methods

2.4.1. Waste Glycerol Analysis

Obtained waste glycerol was analysed for total fermentable glycerol, free fatty acids, and lipid content. For total fermentable glycerol analysis, a sample of waste glycerol solution was diluted with MiliQ water and filtered through a 0.45 µm Nylon filter into the vial. The prepared sample was analysed using Dionex UltiMate 3000 series HPLC with RI detector (Thermo Fischer Scientific, Waltham, MA, USA) on Luna Omega Sugar column 250 mm × 4.6 mm × 2.6 µm (Phenomenex, Washington, DC, USA) using isocratic elution with mobile phase acetonitrile (ACN):H_2O 75:25 at flowrate 1.0 mL/min and temperature 35 °C. Total glycerol content was identified and evaluated using commercial Glycerol standards (Merck, Burlington, MA, USA). Free fatty acids and lipids were analysed using gas chromatography, described in Section 2.4.5 [17].

2.4.2. Phenolics in Coffee Oil

For total phenolic content, a sample of extracted coffee oil was diluted with hexane and filtered through a 0.45 µm PTFE filter into a vial. Samples were measured on Dionex Ultimate series HPLC with Vanquish DAD detector (Thermo Fischer Scientific, Waltham, MA, USA) on Kinetex F5 column 150 mm × 4.6 mm × 2.6 µm (Phenomenex, Washington, DC, USA) with flowrate 0.4 mL/min using a gradient elution described in Table S1. Separation was performed at 35 °C. Phenolic compounds were identified using commercial standards (Merck, Burlington, MA, USA). Chromatographic data were evaluated using Chromeleon 7.2. software. Total phenolic content was calculated as a sum of all of them [21].

2.4.3. Cell Dry Weight

Samples from cultivation media (40 mL) were centrifuged at 7000 rpm for 3 min. The supernatant was collected for further analyses and stored at −30 °C. The yeast cells were then washed twice with the mixture of distilled water and hexane 1:1 (*v*/*v*) and suspended in 1 mL of distilled water. Then, purified biomass was quantitatively transferred into Eppendorf tubes, frozen at −82 °C and then freeze-dried. After determining their weight, to calculate CDW, dried cells were used to analyse lipid-soluble metabolites, carotenoids, ergosterol, ubiquinone, glucans and lipids.

2.4.4. Lipid Metabolite Analysis

Total sterols, coenzyme Q, chlorophylls, tocopherol, and carotenoid content were determined using the HPLC/PDA method. Samples of freeze-dried yeast biomass were properly mixed, weighed (approx. 20–25 mg), and rehydrated with 1 mL of MiliQ water for 30 min. Excess water was removed by centrifugation at 10,000 rpm, and 1 mL of methanol and about 0.5 mL of glass beads (0.2–0.5 mm diameter) were added to the sample. An Eppendorf tube with the sample was then placed into a homogeniser for two cycles (30 s at 4000 rpm). The sample was then transferred to a 15 mL tube and washed with 2 mL of chloroform. The mixture was further vortexed for 15 min. Then, 1 mL of demi-water was added, and the tube was allowed to stabilise for two phases after shaking. The lower chloroform phase was quantitatively transferred to a clean tube and dried under an inert nitrogen atmosphere. The dried sample was dissolved in 2 mL of mixture ethyacetate (EtAc):ACN (2:1) and filtered through a 0.45 µm PTFE filter into the vial. Samples were measured on Dionex Ultimate series HPLC with Vanquish DAD detector (Thermo Fischer Scientific, USA) on Kinetex C18-EVO column 150 mm × 4.6 mm × 5 µm (Phenomenex, USA) using gradient separation with mobile phase A (ACN: MeOH: Tris HCl pH = 8; 84:2:14) and mobile phase B (MeOH: EtAc; 60:40) at flowrate 1.2 mL/min and 25 °C. The gradient program is listed in [21]. The productions are displayed in mg/g dry cell weight (CDW).

Carotenoid pigments were detected at 445 nm. Sterols, ubiquinone, and tocopherol, were detected at 285 nm. Chromatographic data were evaluated using Chromeleon 7.2. software. Total carotenoid, tocopherol, sterol, and ubiquinone production were identified and evaluated using commercial standards (Merck, Burlington, MA, USA) and external calibration as in [21]. Those standards were concretely ergosterol, ubiquinone, tocopherol, and of the carotenoids betacarotene, lycopene, torularhodin, and torulene. Carotenoids which were not identifiable by spectra and retention time were calculated by betacarotene standard.

2.4.5. Lipids and Fatty Acids

Total lipids and individual fatty acids were determined by optimised GC/FID analysis. Approx. 10–15 mg of freeze-dried yeast biomass was put into a 2.0 mL crimp neck vial together with 1.8 mL 15 % (v/v) H_2SO_4 in methanol, capped with an aluminium cap and heated at 85 °C for 2 h. After the transesterification process, the mixture was transferred quantitatively into a 5 mL vial and neutralized with 0.5 mL of 0.005 M NaOH. The FAMEs were converted to the non-polar phase by adding 1 mL of n-hexane and shaking vigorously for 10 min using a vortex. The total lipids and fatty acids profile were determined by gas chromatography/flame ionisation detection (GC/FID) analysis. G.C. analysis of FAMEs was carried out on a TRACETM 1300 Gas Chromatograph (Thermo Fischer Scientific, USA) equipped with a flame ionisation detector, an Al 1310 autosampler, and a Lion FAME column (30 m, 0.25 mm, 0.20 µm) (Chromservis, Praha-Petrovice, Czech Republic). The temperature program is listed in Chyba! Nenalezen zdroj odkazů. Individual FAMEs were identified using commercial standard Supelco 37 Component FAME Mix (Merck, Darmstadt, Germany, SRN). The internal standard method was used for quantification by adding 0.5 mg/mL of heptadecanoic acid (Sigma Aldrich, SRN) into the transesterification mixture. Chromatography data were evaluated using Chromeleon software 7.2 [21,32].

2.4.6. β-Glucan Determination

To determine the amount of β-glucans from yeast, a kit from Megazyme corporation (Megazyme, Wicklow, Ireland) was used, which is based on the enzymatic breakdown of polysaccharides into monomers of D-glucose. The enzymatic method is divided into two parts: total glucan assay and α-glucan assay. The amount of β-glucans is then calculated as the difference between the total amount of glucans and alpha glucans. The concentration of β-glucans was then calculated using the Excel program with a pre-prepared calculation sheet provided by the manufacturer. The method was an optimised procedure from our previous work [33].

Total Glucan Assay

Lyophilised biomass and a control sample from the kit were weighted in amounts of 25 mg into glass tubes with screw caps. Samples in tubes were hydrolysed with 1 mL of 12 M sulfuric acid solution for 2 h in an ice bath. Samples were vortexed several times during hydrolysis to ensure complete hydrolysis. Subsequently, 5 mL of distilled water was added to each sample, and the mixture was intensively mixed. Then the samples were capped and placed on a block heater and incubated there for 2 h at 100 °C. After cooling the samples to room temperature, the tube contents were neutralized by adding 1.5 mL of 10 M KOH. The mixtures were subsequently mixed and quantitatively transferred into a 50 mL centrifuge tubes each containing 17.5 mL of 200 mM acetate buffer (pH 5), and the mixtures were again mixed. Then the samples were centrifuged for 5 min at 10,000 rpm. Supernatant was subsequently collected in amount of 0.1 mL to the bottom of each glass screw tube as an aliquot sample. Then, 0.1 mL of Exo-1,3-β-glucanase + β-glucosidase mixture in 200 mM acetate buffer was added to the bottom of the tubes. The contents of the tubes were subsequently mixed and incubated at 40 °C for 60 min. Then, 3 mL of GOPOD reagent was added to each tube. A blank was prepared by pipetting 0.2 mL of 200 mM acetate buffer into 3 mL of GOPOD reagent. A standard sample of D-glucose was prepared by mixing 0.1 mL of the standard solution D-glucose, 0.1 mL of 200 mM acetate buffer, and 3 mL of GOPOD reagent. All prepared samples were subsequently incubated for 20 min at 40 °C. Then, absorbance was measured at a wavelength of 510 nm against a blank.

α-Glucan Assay

Lyophilised biomass and a control sample of α-glucan (from the glucan kit) were weighted in amounts of 25 mg into screw-cap glass tubes. Then, 1 mL of 1.7 M NaOH was added, and the samples were cooled in an ice bath for 20 min. The samples were continuously vortexed. Then, 4 mL of 1.2 M acetate buffer (pH 3.8) was added to each tube, and 0.1 mL of a mixture of amyloglucosidase with invertase from bottle two was added immediately. The contents of the tubes were subsequently mixed on a vortex, and the tubes were placed on a block heater, where they were incubated at a temperature of 40 °C for 30 min. Then the contents of the tubes were homogenised on a vortex, poured into 50 mL centrifuge tubes, and centrifuged at 10,000 rpm for 10 min. Centrifuged supernatant was taken in amounts of 0.1 mL as an aliquot sample into screw-cap glass tubes. Then 0.1 mL of 200 mM acetate buffer (pH 5) and 3 mL of GOPOD reagent were added to each sample. Samples were incubated on a block heater for 20 min at 40 °C. The absorbance was measured at a wavelength of 510 nm against a blank.

2.5. Statistical Analysis

The growth screening experiments on yeasts in Erlenmeyer flasks were carried out in triplicate. Bioreactor cultivations were carried out in duplicate. The presented results are the mean of the replicates, and the standard deviations are shown as error bars in the figures. Data handling and statistics were performed using the Excel software package (Microsoft Excel 2013, Microsoft Corp., Redmond, WA, USA). Experimental data obtained through screening cultivations were subjected to analysis using the statistical program Statistica (Stanford, CA, USA). The analysis was focused on the correlation of the amount of lipidic waste substrate and the production of biomass and β-glucans by yeasts. The Shapiro-Wilks test was used for determination of the normality of the data. Subsequently, one-way ANOVA or its non-parametric alternative (the Kruskal-Wallis test) was performed on data to determine whether there were any statistical differences in means or medians of the analysed data (p values were set to 0.05). Furthermore, corelation analysis was performed, which describes (via corelation coefficient) whether there are any corelations between analysed groups.

3. Results

3.1. Phase I and Phase II Screening Cultivation Results

Our experiment results proved that all studied strains were able to process and utilize used waste substrates. In all our strains, the lipase induction led to higher overall productivity. Both small-volume screening phases for a given strain are always discussed together in the results below. The results of lipid, beta-glucan, biomass production, and fatty acid profiles are shown in graphs. HPLC analysis of secondary metabolites carotenoids, ergosterol, ubiquinone, and tocopherol (for bioreactor cultures only) are presented in the tables.

3.1.1. *Rhodotorula kratochvilovae* CCY 020-002-026 Cultivation

For the strain *Rhodotorula kratochvilovae*, control glycerol proved to be very suitable for biomass production (Figures 2 and 3), and of the waste substrates, frying oil, or its mixture with waste animal fat, was the most suitable. Due to its solid state, animal fat was more difficult to utilize. The highest value (14.30 ± 1.37 g/L) was achieved using a medium with a mixture of frying oil and waste animal fat without adding glycerol and without lipase induction. Due to its solid state and small active surface, animal fat alone is the most difficult to process, but after creating a liquid mixture with frying oil, it is much more usable. For the production of carotenoids, media containing waste animal fat were the most suitable. The highest production of carotenoids was achieved during cultivation on a medium with waste animal fat with the induction of lipase activity (4.930 ± 0.530 mg/g). Additionally, on this media, one of the highest productions of torularhodin (2.557 ± 0.221 mg/g) made up 52% of the total carotenoids (Table 3). The yeast *Rhodotorula kratochvilovae* preferred torularhodin as the major carotenoid pigment in all cultivations.

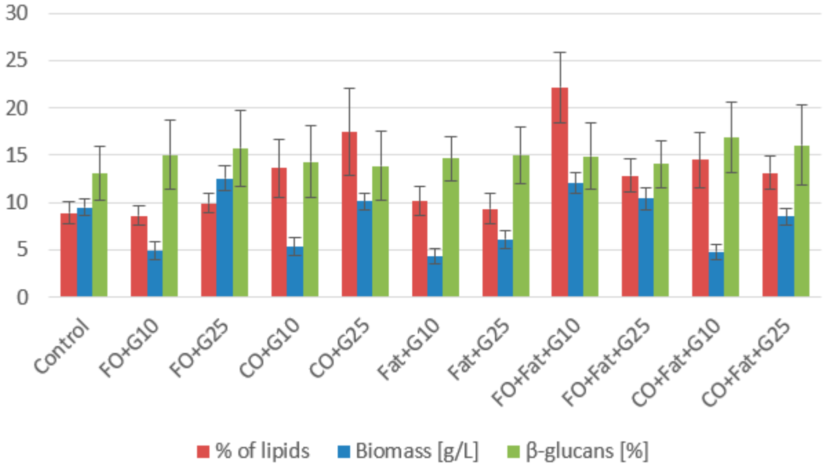

Figure 2. Phase I: Biomass, lipid, and β-glucan production of *Rhodotorula kratochvilovae* cultivated on a combination of waste lipids and glycerol.

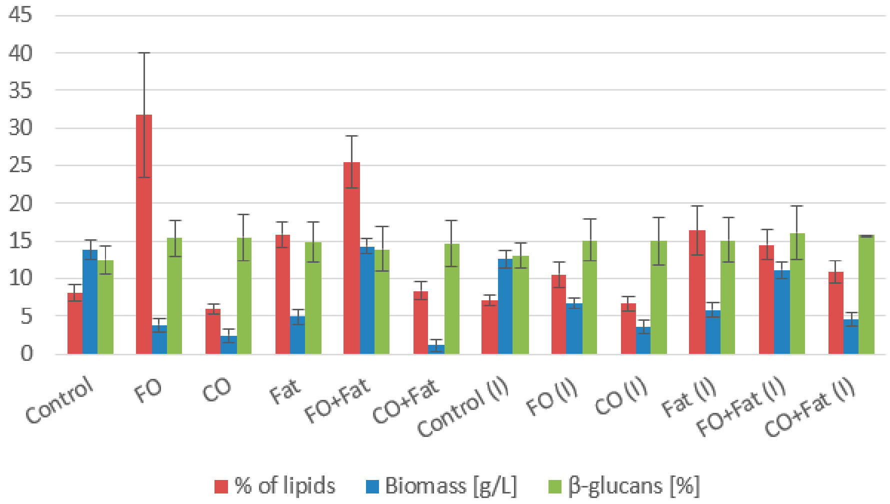

Figure 3. Phase II: Biomass, lipid, and β-glucan production of *Rhodotorula kratochvilovae* cultivated on waste lipid media with and without lipase induction.

Table 3. HPLC analysis of Phase II screening cultivations *Rhodotorula kratochvilovae* cultivated on waste lipid media with and without lipase induction. Productions are listed in mg/g of cell dry weight.

Sample Name	Betacarotene	Torularhodin	Torulene	Total Carotenoids	Ubiquinone	Ergosterol
Control	0.269 ± 0.019	1.352 ± 0.098	0 ± 0	1.705 ± 0.121	4.940 ± 0.531	3.239 ± 0.294
F.O.	0.013 ± 0.001	0.077 ± 0.005	0 ± 0	0.081 ± 0.006	7.427 ± 0.672	2.987 ± 0.291
C.O.	0 ± 0	0 ± 0	0 ± 0	0.035 ± 0.002	3.555 ± 0.274	4.531 ± 0.385
Fat	0.170 ± 0.012	2.354 ± 0.208	0.113 ± 0.008	2.744 ± 0.216	5.426 ± 0.392	3.621 ± 0.312
FO+Fat	0.124 ± 0.009	1.496 ± 0.107	0.118 ± 0.008	1.897 ± 0.143	5.797 ± 0.675	3.607 ± 0.378
CO+Fat	0.132 ± 0.009	2.916 ± 0.215	0.114 ± 0.008	3.212 ± 0.288	4.438 ± 0.371	3.985 ± 0.432
Control (I)	0.229 ± 0.016	1.479 ± 0.122	0.126 ± 0.009	1.922 ± 0.170	5.940 ± 0.716	3.457 ± 0.351
F.O. (I)	0 ± 0	0 ± 0	0 ± 0	0.209 ± 0.015	2.401 ± 0.175	2.846 ± 0.255
C.O. (I)	0.110 ± 0.008	2.813 ± 0.199	0.116 ± 0.008	3.058 ± 0.215	3.481 ± 0.257	4.462 ± 0.478
Fat (I)	0.098 ± 0.007	2.557 ± 0.197	0.139 ± 0.010	4.930 ± 0.560	5.260 ± 0.547	3.728 ± 0.322
FO+Fat (I)	0.141 ± 0.010	1.986 ± 0.142	0.150 ± 0.011	2.471 ± 0.232	7.127 ± 0.714	3.980 ± 0.359
CO+Fat (I)	0.120 ± 0.008	2.997 ± 0.282	0.120 ± 0.009	3.334 ± 0.273	5.504 ± 0.570	3.991 ± 0.437

High productions of ergosterol were obtained in the cultivation on media with the coffee oil or its mixture with waste fat. The highest value was achieved on media with coffee oil without induction of lipase activity 4.531 ± 0.380 mg/g, and 1.5% less (4.462 ± 0.442 mg/g) with induction (Tables 3 and 4). This is an increase of more than 50% compared to the control media. Media with the addition of glycerol were the most suitable for the production of ubiquinone. A production of 9.262 ± 0.987 mg/g was achieved by culturing with a mixture of frying oil and animal fat with the addition of 10% glycerol, which is more than 80% better than the control glycerol medium.

Table 4. HPLC analysis of Phase I screening cultivations *Rhodotorula kratochvilovae* cultivated on a combination of waste lipids and glycerol. Productions are listed in mg/g of cell dry weight.

Sample Name	Betacarotene	Torularhodin	Torulene	Total Carotenoids	Ubiquinone	Ergosterol
Control	0.216 ± 0.016	1.357 ± 0.097	0.110 ± 0.008	4.420 ± 0.396	1.719 ± 0.139	2.787 ± 0.243
FO+G10	0 ± 0	0 ± 0	0 ± 0	0.202 ± 0.014	4.754 ± 0.360	3.594 ± 0.323
FO+G25	0 ± 0	0 ± 0	0 ± 0	0.014 ± 0.001	6.808 ± 0.858	3.336 ± 0.240
CO+G10	0.090 ± 0.006	1.191 ± 0.088	0 ± 0	1.933 ± 0.171	0.72 ± 0.0530	3.771 ± 0.393
CO+G25	0.205 ± 0.015	1.880 ± 0.165	0.114 ± 0.008	2.352 ± 0.171	8.486 ± 0.908	3.303 ± 0.319
Fat+G10	0.133 ± 0.009	2.275 ± 0.210	0.125 ± 0.009	2.567 ± 0.216	4.462 ± 0.390	3.381 ± 0.241
Fat+G25	0.220 ± 0.016	1.480 ± 0.115	0.109 ± 0.008	1.912 ± 0.167	4.738 ± 0.443	3.804 ± 0.380
FO+Fat+G10	0.238 ± 0.017	2.186 ± 0.190	0.122 ± 0.009	2.808 ± 0.233	9.262 ± 1.427	4.318 ± 0.321
FO+Fat+G25	0.173 ± 0.012	1.180 ± 0.084	0 ± 0	1.468 ± 0.118	8.021 ± 1.090	3.417 ± 0.306
CO+Fat+G10	0.217 ± 0.015	2.241 ± 0.179	0.123 ± 0.009	2.681 ± 0.205	6.547 ± 0.684	3.801 ± 0.402
CO+Fat+G25	0.296 ± 0.021	2.539 ± 0.210	0.109 ± 0.008	3.058 ± 0.256	7.381 ± 0.590	3.894 ± 0.360

Waste lipid substrates are suitable for increasing lipid accumulation in the *Rhodotorula kratochvilovae* biomass. Media with frying oil, waste animal fat, and their mixture were the best. The highest production was achieved when yeast was cultivated on a frying oil medium without induction (31.74 ± 5.77% of lipids), approximately four times the value of the control production media. When induction was used, the lipid representation decreased almost to the value obtained in control media cultures. Using media with coffee oil without the addition of glycerol led to a decrease in the lipid content of the biomass. From the fatty acid profile shown in the graphs below (Figures 4 and 5), we see the most suitable substrate for accumulating polyunsaturated fatty acids (PUFA) in the strain *Rhodotorula kratochvilovae* was coffee oil or frying oil. In the same type of media, without the addition of glycerol and without the use of lipase induction, their representation reached 54.0%. By using lipase induction, the representation of PUFA in the biomass decreased by less than 2%.

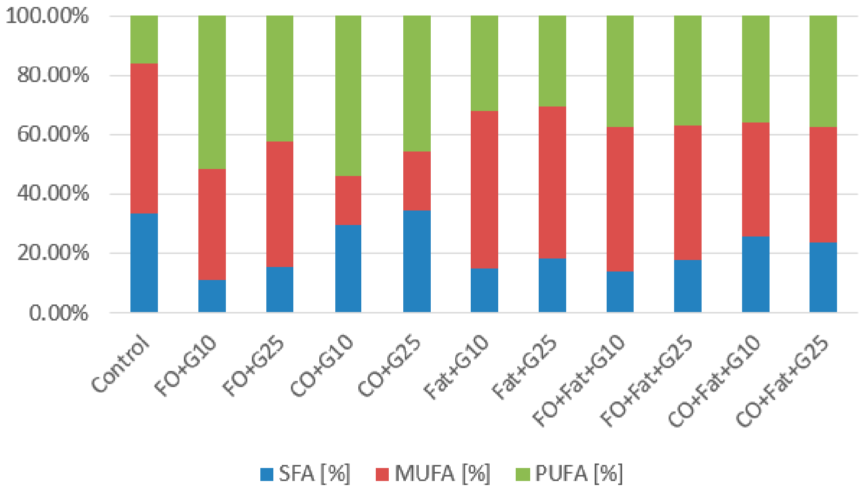

Figure 4. Phase I: Fatty acid production of *Rhodotorula kratochvilovae* cultivated on a combination of waste lipids and glycerol.

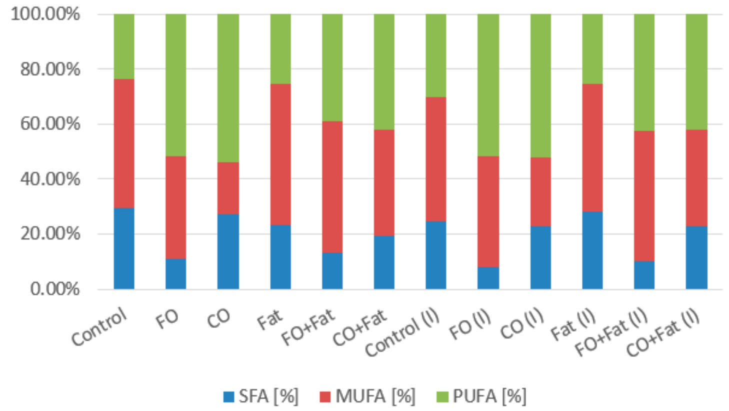

Figure 5. Phase II: Fatty acid production of *Rhodotorula kratochvilovae* cultivated on waste lipid media with and without lipase induction.

The highest production of monounsaturated fatty acids (MUFA) was obtained from cultivations on control glycerol and all media supplemented with glycerol. With this strain, only waste animal fat positively affected MUFA production. The highest production was achieved when yeast was cultured on a medium with animal fat with a smaller addition of glycerol (53.5%). With a higher addition, the representation decreased by 2.5%, and a similar result was also achieved when using fat alone. The total UFA representation was highest overall in control media cultivations and on media containing coffee oil. Saturated fatty acids (SFA) are best accumulated from coffee oil on media with a higher addition of glycerol. A value of 34.61% was reached, and with a lower addition of glycerol, the representation of SFA decreased by 5%.

Rhodotorula kratochvilovae CCY 020-002-026 Cultivation—Statistical Analysis

Due to the non-normal distribution of the analysed data (Shapiro-Wilk test) in the case of the *R. kratochvilovae* strain, the Kruskal-Wallis test was applied to determine variability of data, where can be proven (on the basis of $p = 0$) that the medians of individual groups (production of biomass and glucans depending on the concentration of the lipid substrate in the medium) varies. Furthermore, there is an observable negative correlation of the dependence of the increase in biomass on the increasing representation of lipids in the medium; this trend is expressed by a correlation coefficient of -0.585. The low degree of positive correlation of the dependence of the increasing production of glucans with the increasing representation of lipids in the medium is expressed by a correlation coefficient with a value of 0.232 (Figure S9).

3.1.2. *Rhodotorula toruloides* CCY 062-002-004 Cultivation

The *Rhodotorula toruloides* strain showed an excellent ability to utilize waste lipid substrates with a high production of carotenoids and lipid substances (Tables 5 and 6). The highest biomass production was shown by cultures using control glycerol media (more than 12 g/L on average). Cultivations of waste substrates led to a decrease in biomass production. The medium with frying oil with glycerol addition of 25% was the best for biomass production, with a value of 8.76 ± 1.11 g/L, which is almost 30% less than the average of the control glycerol media (Figures 6 and 7). For the production of carotenoids by the *Rhodotorula toruloides* strain, the most suitable medium was a mixture of frying oil with animal fat and the addition of 25% glycerol. The value of total carotenoids rose to 11.256 ± 1.394 mg/g thanks to the highest content of torularhodin (10.232 ± 0.844 mg/g), which made up 91% of all carotenoids (Table 5). A suitable medium was also a mixture of coffee oil and animal fat with the addition of 25% glycerol, where the second highest value of carotenoid production was reached with a value of 10.912 ± 1.050 mg/g (decrease of 3%). Animal fat, despite its solid state, is an excellent substrate for this strain, as it can be seen from the data (Table 6) that without the addition of glycerol, the highest production is precisely on the medium with waste animal fat with the induction of lipase activity (9.323 ± 0.767 mg/g). Waste animal fat is also very suitable for the production of ergosterol. All media using it in any combination achieved values of at least 7 mg/g. The highest production of ergosterol was achieved by *Rhodotorula toruloides* cultivated with waste animal fat using lipase induction (8.644 ± 1.169 mg/g). With the same type of cultivation (with induction), the difference in ergosterol production was 9.3% in the mixture of animal fat with frying oil and only 3% when using the mixture with coffee oil. The production of ubiquinone was highest in media using a mixture of coffee oil and animal fat with the addition of glycerol. With the addition of 10% glycerol, ubiquinone reached the highest value of 10.224 ± 1.380 mg/g, and with a higher addition of glycerol, it was only 7% smaller (9.532 ± 1.072 mg/g) (Table 6).

Table 5. HPLC analysis of Phase I screening cultivations *Rhodotorula toruloides* cultivated on a combination of waste lipids and glycerol. Productions are listed in mg/g of cell dry weight.

Sample Name	Betacarotene	Torularhodin	Torulene	Total Carotenoids	Ubiquinone	Ergosterol
Control	0.262 ± 0.019	2.745 ± 0.209	0.210 ± 0.015	4.961 ± 0.436	3.371 ± 0.308	1.881 ± 0.144
FO+G10	0.138 ± 0.010	2.692 ± 0.223	0.136 ± 0.010	2.948 ± 0.233	2.024 ± 0.155	6.059 ± 0.602
FO+G25	0.315 ± 0.022	5.604 ± 0.686	0.147 ± 0.010	6.091 ± 0.720	2.705 ± 0.224	4.667 ± 0.503
CO+G10	0.200 ± 0.014	4.182 ± 0.320	0.152 ± 0.011	4.544 ± 0.522	3.741 ± 0.323	4.464 ± 0.401
CO+G25	0.365 ± 0.026	5.395 ± 0.591	0.158 ± 0.011	5.957 ± 0.755	2.56 ± 0.206	5.528 ± 0.599
Fat+G10	0.409 ± 0.030	7.213 ± 1.001	0 ± 0	7.904 ± 1.016	4.785 ± 0.336	7.758 ± 0.850
Fat+G25	0.351 ± 0.025	5.380 ± 0.431	0 ± 0	6.109 ± 0.577	5.142 ± 0.515	6.195 ± 0.588
FO+Fat+G10	0.481 ± 0.035	7.806 ± 0.806	0.119 ± 0.008	8.611 ± 0.896	4.971 ± 0.376	7.134 ± 0.941
FO+Fat+G25	0.746 ± 0.058	10.232 ± 1.369	0.139 ± 0.010	11.256 ± 0.997	3.964 ± 0.394	6.854 ± 0.751
CO+Fat+G10	0.385 ± 0.028	6.455 ± 0.754	0.106 ± 0.007	7.084 ± 0.933	10.224 ± 1.665	7.114 ± 0.647
CO+Fat+G25	0.637 ± 0.046	8.480 ± 0.802	0.129 ± 0.009	10.912 ± 1.164	9.532 ± 1.289	7.739 ± 1.076

Table 6. HPLC analysis of Phase II screening cultivations *Rhodotorula toruloides* cultivated on waste lipid media with and without lipase induction. Productions are listed in mg/g of cell dry weight.

Sample Name	Betacarotene	Torularhodin	Torulene	Total Carotenoids	Ubiquinone	Ergosterol
Control	0.467 ± 0.035	2.650 ± 0.234	0.118 ± 0.008	6.484 ± 0.651	5.479 ± 0.595	2.318 ± 0.213
F.O.	0.087 ± 0.006	0.745 ± 0.054	0 ± 0	0.843 ± 0.064	8.091 ± 0.802	4.703 ± 0.530
C.O.	0.446 ± 0.032	6.091 ± 0.617	0.144 ± 0.010	6.735 ± 0.549	2.175 ± 0.192	7.080 ± 0.776
Fat	0.225 ± 0.016	4.017 ± 0.293	0.132 ± 0.009	4.423 ± 0.382	2.409 ± 0.186	7.097 ± 0.766
FO+Fat	0.198 ± 0.014	3.745 ± 0.382	0.096 ± 0.007	4.101 ± 0.349	3.051 ± 0.282	7.488 ± 0.880
CO+Fat	0.356 ± 0.026	6.526 ± 0.534	0.131 ± 0.009	7.597 ± 0.987	2.881 ± 0.266	7.720 ± 0.566
Control (I)	0.602 ± 0.044	3.339 ± 0.333	0.142 ± 0.010	4.382 ± 0.336	4.391 ± 0.388	2.987 ± 0.273
F.O. (I)	0.001 ± 0	0.903 ± 0.069	0.099 ± 0.007	1.367 ± 0.097	5.653 ± 0.640	5.066 ± 0.539
C.O. (I)	0.408 ± 0.029	6.377 ± 0.656	0.161 ± 0.011	6.966 ± 0.940	4.606 ± 0.477	6.558 ± 0.861
Fat (I)	0.424 ± 0.030	8.688 ± 1.332	0.120 ± 0.008	9.323 ± 0.663	4.917 ± 0.350	8.644 ± 0.744
FO+Fat (I)	0.357 ± 0.026	6.144 ± 0.587	0.136 ± 0.010	7.026 ± 0.888	3.661 ± 0.272	8.023 ± 0.933
CO+Fat (I)	0.415 ± 0.030	7.526 ± 0.577	0.138 ± 0.012	8.105 ± 1.011	2.940 ± 0.282	8.393 ± 1.197

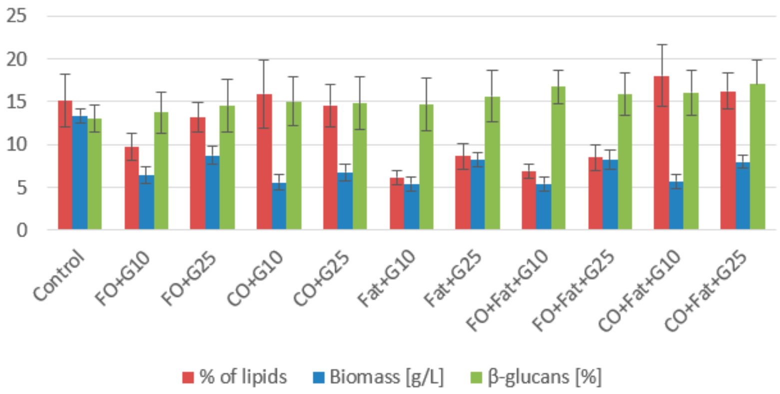

Figure 6. Phase I: Biomass, lipid, and β-glucan production of *Rhodotorula toruloides* cultivated on a combination of waste lipids and glycerol.

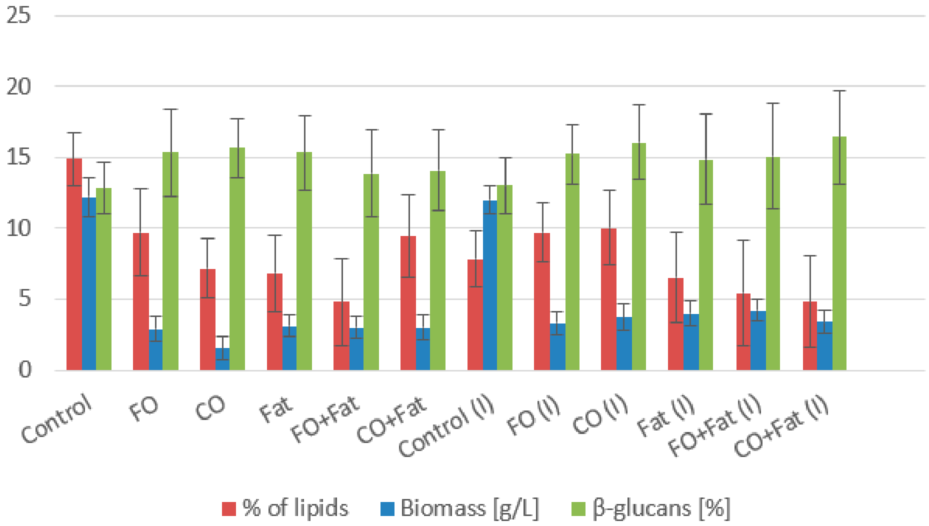

Figure 7. Phase II: Biomass, lipid, and β-glucan production of *Rhodotorula toruloides* cultivated on waste lipid media with and without lipase induction.

Fatty acid profiles in *Rhodotorula toruloides* cultures are shown in the graphs below (Figures 8 and 9). The type of substrate used strongly influences the fatty acid profile. It is noticeable that when cultivating on the control media, the representation of polyunsaturated fatty acids (PUFA) is the lowest. To increase PUFA, coffee oil or frying oil is the most suitable. The highest value of PUFA representation was achieved by the strain using coffee oil without fat induction of lipases (54.86%) and then using frying oil with induction of lipase activity (50.88%). For the accumulation of monounsaturated fatty acids, media with frying oil or waste animal fat or their mixtures are the most suitable. Values of 66.04% MUFA were achieved on media with frying oil and a higher addition of glycerol and with a lower addition of glycerol (64.37%) (Figure 8). With the same type of cultivation (with the addition of glycerol) using waste animal fat and its mixture with frying oil, values of MUFA production in the range of 52–60% were achieved. We observe a significant reduction of MUFA to 16.7–22.5% with coffee oil. The choice of coffee oil is suitable if we want to achieve a high representation of saturated fatty acids (SFA). When coffee oil was used, similar values were achieved as with the control media. The highest value when using a waste substrate was achieved during cultivation on a medium with coffee oil and a lower addition of glycerol (40.32%) (Figure 8).

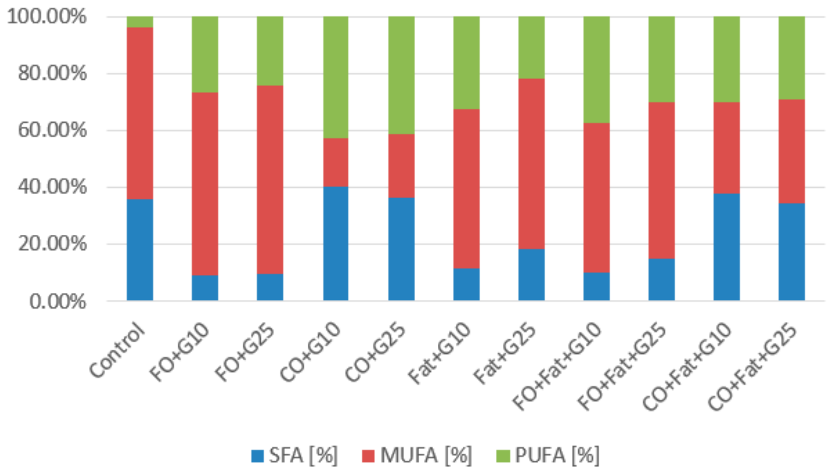

Figure 8. Phase I: Fatty acid production of *Rhodotorula toruloides* cultivated on a combination of waste lipids and glycerol.

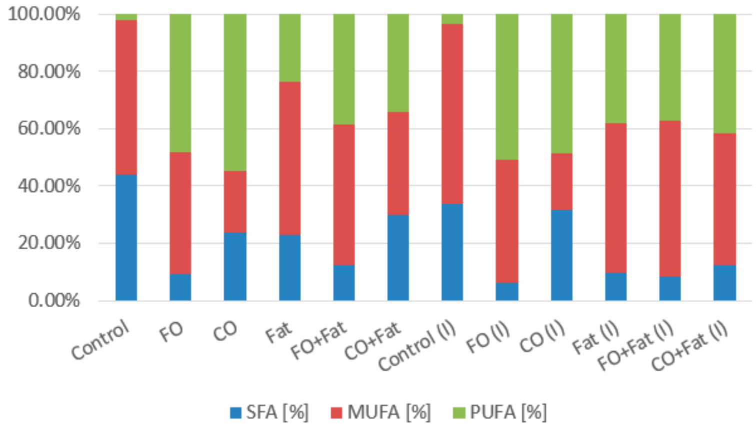

Figure 9. Phase II: Fatty acid production of *Rhodotorula toruloides* cultivated on waste lipid media with and without lipase induction.

Rhodotorula toruloides CCY 062-002-004 Cultivation—Statistical Analysis

The Shapiro-Wilk test for normality determination was applied to analyse the data of the *R. toruloides* strain. A normal data distribution was determined for the strain; therefore, the ANOVA method was chosen with the result (p = 0). The mean values differed statistically significantly for the individual investigated groups. At the same time, it is possible to observe a negative correlation of biomass production with the increasing representation of lipids in the medium (correlation coefficient reached a value of −0.893). The production of beta-glucans shows a lower degree of positive correlation (correlation coefficient with a value of 0.301) with increasing representation of lipids (Figure S10).

3.1.3. *Rhodotorula mucilaginosa* CCY 019-004-006 Cultivation

As can be seen from the tables (Tables S4 and S10), waste lipid sources are not a suitable substrate for the strain *Rhodotorula mucilaginosa* high carotenoids production. On the other hand, they are suitable for the production of lipid substances by this strain. The best medium for producing carotenoids was the medium with waste animal fat with the addition of 25% glycerol. Total carotenoids reached a value of 6.450 ± 0.801 mg/g, of which more than 65% was lycopene (with the highest value of 4.369 ± 0.494 mg/g), and almost 30% was torularhodin with its highest value of 1.918 ± 0.149 mg/g. Very similar results were achieved using a medium with a mixture of coffee oil and animal fat with 10% glycerol (a decrease in total carotenoids by almost 5%, lycopene by 0.6%, and torularhodin by 9% compared to the previous medium). Waste lipid substrates had the highest effect on ubiquinone production. Several times higher production than on the control medium, 12.710 ± 0.959 mg/g was achieved when using frying oil without adding glycerol. Using the lipase induction with the same substrate, the production decreased by almost 25% (9.774 ± 1.372 mg/g), but the production of ergosterol, on the contrary, rose by 26%. To produce ergosterol, media with waste animal fat or its mixtures worked the best. The most ergosterol was produced by this strain on waste animal fat media supplemented with 25% glycerol (7.587 ± 1.055 mg/g) (Table S7). The highest biomass production values were found for cultivations on the control glycerol medium (8.50–11.10 g/L) (Figures S1 and S5). In cultures with a higher addition of glycerol, the values range from 6.50 to 8.60 g/L, approximately 75%, compared to the control media. In purely lipidic substrate media, biomass production values were reduced to only 30% compared to the control media.

Rhodotorula mucilaginosa strain biomass generally contained few lipids. For control glycerol media, the value was around 6–7%. Frying oil was the most suitable waste lipid substrate for producing the highest amounts of lipids in the biomass. The highest value (20.17 ± 2.15%) was achieved when cultured on a medium with frying oil without adding glycerol and without lipase induction. With the same type of cultivation, only with a mixture of frying oil and animal fat, the lipid content decreased by 6%. When lipase activity induction was used, the values were halved. From the graphs with profiles of the fatty acid content (Figures S2 and S6), the highest values of PUFA content were achieved by cultivations using frying oil (47–50.5% PUFA). The highest content of MUFA, more than 70%, was obtained on all control media. Of the waste substrates used, waste animal fat is the most suitable for increasing MUFA values in biomass.

Rhodotorula mucilaginosa CCY 019-004-006 Cultivation—Statistical Analysis

In the case of the *R. mucilaginosa* strain, statistical analysis revealed the same results as in the case of the *R. toruloides* strain (normal data distribution). The mean values of the obtained data differ significantly. As it was mentioned in a previous case, a negative correlation was observed in the production of biomass by yeast depending on the increasing content of the lipid substrate in the medium (correlation coefficient reached the value of -0.712). The production of beta-glucans shows a low degree of positive correlation (correlation coefficient with a value of 0.243) with the increasing representation of lipids (Figure S11).

3.1.4. *Sporidiobolus pararoseus* CCY 019-009-006 Cultivation

The results of the cultivation of the *Sporidiobolus pararoseus* strain on the selected media show that the yeast did not utilize the fat substrate well under the given conditions, and from an overall point of view, the production of studied metabolites and biomass is low. The highest production of total carotenoids was achieved in the control glycerol medium of 3.329 ± 0.256 mg/g of biomass (Table S9), where the highest representation was achieved by torularhodin with a production of 2.583 ± 0.225 mg/g (almost 80% of total carotenoids), followed by beta-carotene (0.464 ± 0.033 mg/g) and torulene (0.203 ± 0.014 mg/g). The best results were obtained on a medium containing frying oil with induction of lipase production. Here the highest biomass production of 14.40 ± 1.30 g/L was found (Figure S3) and with the second highest total carotenoid content of 1.332 ± 0.095 mg/g (40% compared to the highest value produced on the control glycerol medium). In this media, the highest torulene production (0.270 ± 0.019 mg/g) and the second highest production of torularhodin (0.639 ± 0.049 mg/g) were found. The highest production of 5.241 ± 0.414 mg/g of ergosterol was achieved using a medium with frying oil, without glycerol and without lipase induction, which is less than half of the highest production value when using the control glycerol medium (10.946 ± 1.612 mg/g). Waste lipids appear to be a good substrate for ubiquinone production, which reached the highest value on the coffee oil medium with 10% glycerol, almost double that of the control medium (12.810 ± 1.453 mg/g). A similar production of ubiquinone was also achieved during cultivation on a medium with waste animal fat and the addition of 10% glycerol (12.601 ± 1.967 mg/g).

The chromatographic analysis of lipid production by the yeast *Sporidiobolus pararoseus* shows a different ability of the yeast to utilize the selected waste substrates. Higher biomass and lipid production occurs in the case of media containing a combination of lipid substrate and glycerol. Overall, the highest lipid content of 60.22 ± 16.81% was determined on media containing coffee oil with a lower addition of glycerol (Table S8). The excellent ability of the yeast *Sporidiobolus pararoseus* to utilize coffee oil was already confirmed in our previous work [21,32]. As can be seen from the results in the graphs (Tables S8 and S14), yeast on mixed fat media, especially the coffee oil mixture, produced more biomass compared to pure fat media and control glycerol media. The application of induction of lipase activity in this type of medium subsequently led to a further increase in biomass production.

On media with lipase induction, the highest lipid production of 62.63 ± 13.25% was achieved again on media with coffee oil. From an overall perspective, we can see that the application of individual combinations of fats and glycerol additions positively affected the production of biomass and lipids. From the point of view of the fatty acid profile (Figures S4 and S8), we can see that the application of different types of lipid waste significantly affects the fatty acid profile. Compared to the control glycerol medium, where the content of unsaturated fatty acids (UFA) prevails (>85%), there is an increased accumulation of saturated fatty acids, which make up more than 30–40%, on media with waste lipids. This phenomenon can be attributed to a faster metabolism and greater biomass production. It can be assumed that with prolonged cultivation, there would be a slowdown in growth and the accumulation of a higher UFA content. Increased UFA content is observed only on media containing frying oil, where the UFA content is comparable to the glycerol control media. From the point of view of the representation of individual groups, we see that the application of frying oil leads to an increase in polyunsaturated fatty acids (PUFA).

Sporidiobolus pararoseus CCY 019-009-006 Cultivation—Statistical Analysis

The Shapiro-Wilk test which was applied to determine normality showed a normal distribution of the data. A subsequent ANOVA analysis with a p = 0 result confirmed significant statistical variability between individual groups of data. For this strain, there is an observable positive correlation of biomass production with increasing lipid content in the medium (correlation coefficient reached a value of 0.618). The production of beta-glucans shows almost no correlation (correlation coefficient with a value of 0.089) with increasing representation of the lipidic carbon source in the medium (Figure S12).

3.2. Bioreactor Cultivation of the Yeasts

3.2.1. *Rhodotorula kratochvilovae* CCY 020-002-026 Bioreactor Cultivation

As a follow-up to previous screening cultivations, large-volume bioreactor cultivation was carried out with the *Rhodotorula kratochvilovae* strain using coffee oil with waste glycerol as a simple substrate and coffee oil as a complex substrate, which were fed by a fed-batch system. Cultivation took place for seven days in a 7 L bioreactor.

The graph (Figure 10) shows a steadily growing culture. Even with a high inoculation ratio, the culture is coping very well with the conditions. The maximum value of 18.82 ± 1.06 g/L was reached at the 168th h. With the steadily growing biomass production curve, we can assume that the culture would continue growing if the cultivation were prolonged and a carbon source was added. At the 8th h, total carotenoid content reaches the second highest maximum (10.714 ± 1.802 mg/g) (Table 7). Then as the culture grows, carotenoid content drops to the lowest concentration of 5.648 ± 0.578 mg/g at 40 h. After that, the carotenoid content slowly grows to its maximum 10.757 ± 0.943 mg/g at the 144th h, with the major carotenoid pigment produced by this strain being torularhodin, with a maximum production of 10.349 ± 0.987 mg/g. In the 8th h, ergosterol reached a value of 9.064 ± 1.020 mg/g. Then its content decreased to almost half until the end of the second day of cultivation. After that, it started to grow again. It reached its maximum at the 144th h with a value of (9.783 ± 0.934 mg/g). Ubiquinone reached its maximum at the 48th h (14.225 ± 2.217 mg/g). Its production during cultivation was unstable and shifted between 5 and 14 mg/g. A measurement error could also cause this. During the whole cultivation, the yeast steadily accumulated tocopherol from the coffee oil, reaching its maximum of 9.203 ± 0.489 mg/g at the end of cultivation.

In the graph (Figure 10), we can see that lipid content in the biomass was slowly declining as the lipids were used as an energy source on the first days of cultivation. Then, at the 56th h, we observe a maximum of 25.38 ± 8.91%, which corresponds to a decrease in yeast metabolic rate and a transition to lipid utilization. After the successful metabolism shift, the lipid content stabilizes at 15–18%. The graph below (Figure 11) shows the distribution of the individual groups of fatty acids. The mixture of coffee oil and waste glycerol resulted in a high accumulation of polyunsaturated and saturated fatty acids. The representation of SFA was around 30–35%. From the 40th to 56th h, there was an increase to 37.5–42.5%. Monounsaturated fatty acids were in the range of 10–18% and reached their maximum at the last sampling at the 168th h with a value of 18.3%. Polyunsaturated fatty acids reached the highest content and were present in the 47–54% range. They reached their maximum (53.92%) at the 72nd h, followed by decreases, and in the last sample, their content was 48.63%.

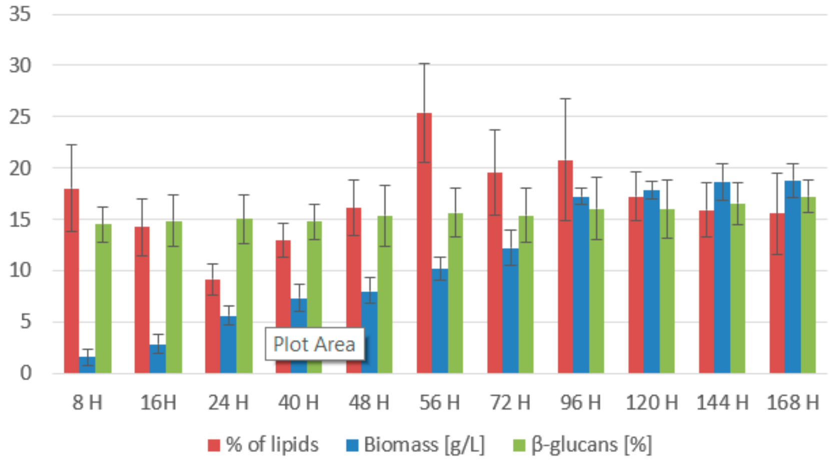

Figure 10. Biomass, lipid, and β-glucan production of *Rhodotorula kratochvilovae* in a bioreactor cultivation on coffee oil and glycerol with lipase induction.

Table 7. HPLC analysis of bioreactor cultivation of *Rhodotorula kratochvilovae* cultivated on coffee oil and glycerol with lipase induction. Productions are listed in mg/g of cell dry weight.

Sample Name	Betacarotene	Torularhodin	Torulene	Total Carotenoids	Ubiquinone	Ergosterol	Tocopherol
8 H	0.245 ± 0.018	10.313 ± 1.144	0.115 ± 0.008	10.714 ± 1.63	5.135 ± 0.443	9.064 ± 0.919	0.105 ± 0.085
16H	0.186 ± 0.013	4.340 ± 0.460	0.141 ± 0.010	4.754 ± 0.375	3.122 ± 0.222	5.503 ± 0.598	0.142 ± 0.103
24 H	0.204 ± 0.014	5.918 ± 0.641	0.155 ± 0.011	6.330 ± 0.577	12.137 ± 1.255	6.430 ± 0.589	0.245 ± 0.153
40 H	0.176 ± 0.013	5.355 ± 0.547	0.102 ± 0.007	5.648 ± 0.540	4.747 ± 0.419	6.520 ± 0.521	2.416 ± 0.421
48 H	0.232 ± 0.016	7.238 ± 0.948	0.105 ± 0.007	7.614 ± 0.719	14.225 ± 2.380	7.432 ± 0.937	3.042 ± 0.503
56 H	0.242 ± 0.017	7.648 ± 0.943	0.121 ± 0.009	8.046 ± 0.836	13.306 ± 1.788	9.165 ± 1.085	3.412 ± 0.548
72 H	0.176 ± 0.013	6.804 ± 0.882	0.108 ± 0.008	7.107 ± 0.518	4.572 ± 0.513	7.705 ± 0.687	5.487 ± 0.603
96 H	0.143 ± 0.010	1.592 ± 0.119	0.129 ± 0.009	8.597 ± 0.191	6.071 ± 0.749	6.573 ± 0.761	8.412 ± 0.640
120 H	0.210 ± 0.015	9.386 ± 0.786	0.124 ± 0.009	9.752 ± 0.774	11.762 ± 1.647	9.359 ± 0.963	9.031 ± 0.584
144 H	0.258 ± 0.018	10.349 ± 1.647	0.111 ± 0.008	10.757 ± 1.104	2.835 ± 0.269	9.783 ± 1.198	8.716 ± 0.706
168 H	0.212 ± 0.015	8.646 ± 1.293	0.123 ± 0.009	9.007 ± 0.846	12.806 ± 1.276	8.522 ± 1.011	9.203 ± 0.489

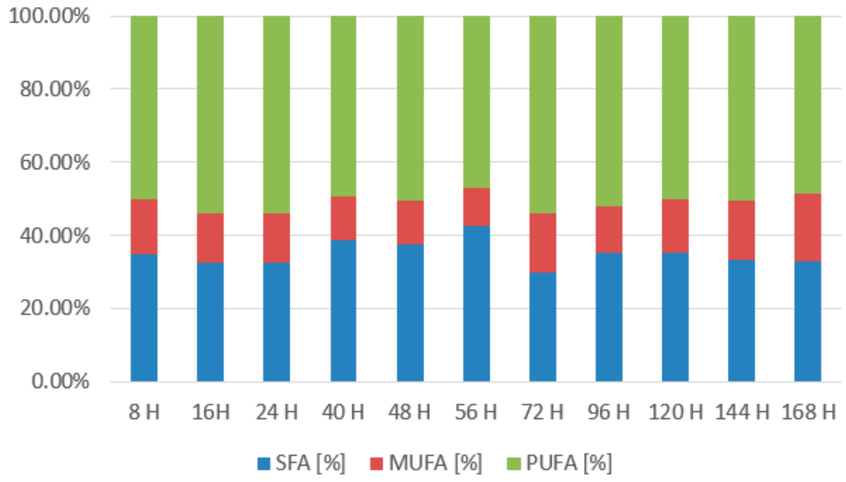

Figure 11. Fatty acid production of *Rhodotorula kratochvilovae* in a bioreactor cultivation on coffee oil and glycerol with lipase induction.

3.2.2. *Rhodotorula toruloides* CCY 062-002-004 Bioreactor Cultivation

Rhodotorula toruloides was chosen as a second tested strain for comparison due to its high production of lipid metabolites. The strain was cultivated under the same conditions. The results of bioreactor cultivation show (Figures 12 and 13) that the chosen media composition suits the yeast very well. The culture shows steady biomass production, reaching a maximum on the final day of cultivation of 18.82 ± 2.439 g/L. As with the results of the previous strain *Rhodotorula kratochvilovae*, we can again assume that by prolonging the cultivation, the biomass production of the strain *Rhodotorula toruloides* would be higher. Throughout the cultivation, the yeast displays an increasing production of carotenoids, reaching its maximum (10.302 ± 1.657 mg/g) at the 96th h of cultivation (Table 8). Then, for the rest of the cultivation, the carotenoid production stabilises between 9.7 and 10.5 mg/g. For this strain, torulene serves as the main carotenoid pigment, which reaches its maximum production at the end of cultivation at the 168th h (9.146 ± 1.140 mg/g).

At that time, ergosterol production also reached its production peak (10.952 ± 1.697 mg/g). During the initial stages of cultivation, the yeast displays quite an unstable production of ergosterol and ubiquinone, which ranges between 6.8 and 9.0 mg for ergosterol and 5.6–7.5 mg/g of ubiquinone. Production of both metabolites stabilizes in the latter phase of the cultivation and starts to increase. Ubiquinone reaches its peak at 120th h of 10.520 ± 1.124 mg/g per CDW. Tocopherol accumulation shows similarities with strain *R. kratochvilovae* and reaches a maximum (9.203 ± 0.489 mg/g) at the end of the cultivation. Results of lipid production (Figure 12) show a fast decline of lipids on the first day of cultivation, reaching a minimum of 8.46 ± 1.21% by the 24th h. Then, the lipid content increases steadily and reaches its peak of 28.46 ± 7.02% at the 96th h. Then, the lipid content drops again to 23.12 ± 3.76%. The fatty acid profile is stable (Figure 13). The results show that with an increased amount of metabolised coffee oil, the content of PUFA increases at the expense of SFA and MUFA. At the end of cultivation, the UFA content was 89.54% (64.19% PUFA and 25.34% MUFA).

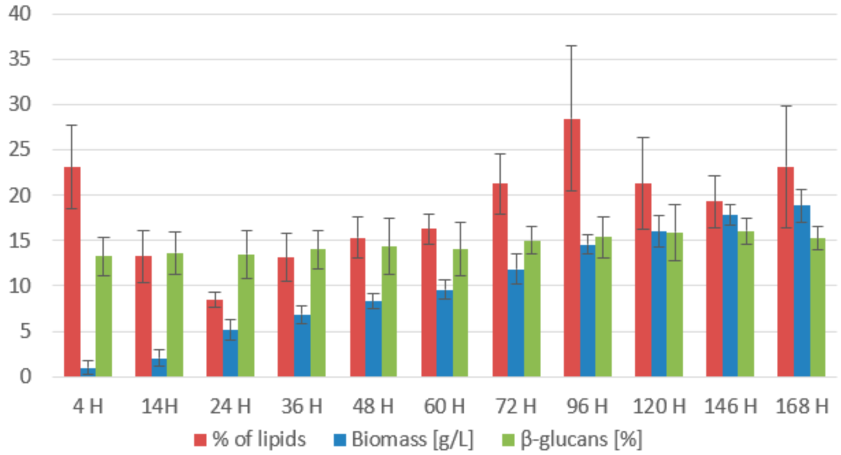

Figure 12. Biomass, lipid, and β-glucan production of *Rhodotorula toruloides* in a bioreactor cultivation on coffee oil and glycerol with lipase induction.

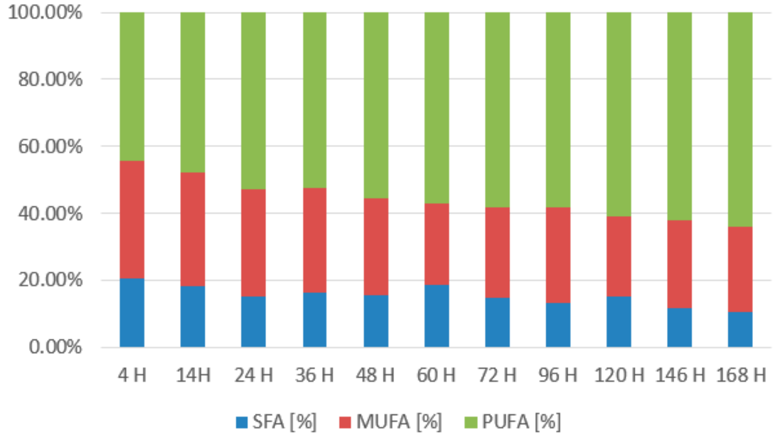

Figure 13. Fatty acid production of *Rhodotorula toruloides* in a bioreactor cultivation on coffee oil and glycerol with lipase induction.

Table 8. HPLC analysis of bioreactor cultivation of *Rhodotorula toruloides* cultivated on coffee oil and glycerol with lipase induction. Productions are listed in mg/g of cell dry weight.

Sample Name	Betacarotene	Torularhodin	Torulene	Total Carotenoids	Ubiquinone	Ergosterol	Tocopherol
4 H	0.345 ± 0.024	0.426 ± 0.031	5.125 ± 0.587	6.512 ± 0.812	7.592 ± 0.873	8.042 ± 1.181	0.105 ± 0.085
14H	0.286 ± 0.020	0.314 ± 0.022	5.122 ± 0.567	6.125 ± 0.436	8.195 ± 0.846	7.159 ± 0.650	0.142 ± 0.103
24 H	0.302 ± 0.021	0.928 ± 0.071	6.152 ± 0.591	7.412 ± 0.799	5.599 ± 0.545	6.865 ± 0.880	0.245 ± 0.153
36 H	0.284 ± 0.020	0.403 ± 0.030	6.243 ± 0.805	7.648 ± 0.960	6.952 ± 0.934	7.592 ± 0.711	2.416 ± 0.421
48 H	0.262 ± 0.018	0.215 ± 0.015	7.614 ± 0.566	8.612 ± 1.196	7.020 ± 0.823	8.456 ± 1.145	3.042 ± 0.503
60 H	0.413 ± 0.030	0.476 ± 0.033	7.046 ± 0.733	8.621 ± 0.719	6.562 ± 0.529	9.295 ± 1.063	3.412 ± 0.548
72 H	0.125 ± 0.009	0.701 ± 0.052	7.107 ± 0.797	8.462 ± 1.284	7.520 ± 0.628	8.952 ± 0.941	5.487 ± 0.603
96 H	0.416 ± 0.029	0.562 ± 0.041	8.513 ± 1.029	10.302 ± 1.699	8.195 ± 0.791	9.620 ± 0.920	8.412 ± 0.640
120 H	0.713 ± 0.054	0.345 ± 0.024	8.925 ± 0.969	10.513 ± 1.602	10.52 ± 1.587	9.880 ± 0.901	9.031 ± 0.584
146 H	0.842 ± 0.060	0.379 ± 0.028	8.012 ± 0.581	9.715 ± 1.062	9.520 ± 1.320	10.295 ± 1.66	8.716 ± 0.706
168 H	0.453 ± 0.032	0.418 ± 0.031	9.146 ± 1.077	10.514 ± 1.425	10.195 ± 0.871	10.952 ± 1.38	9.203 ± 0.489

3.2.3. β-Glucan Production Results

The results of all cultivation and all strains share the same characteristics. The yeast reacts to exposure to waste lipids and oils by slightly increasing the β-glucan content. The overall β-glucan production on standard artificial media is between 12.5% and 14.0% of dry biomass matter. Our experiments show that β-glucan production is induced up to 17% in different oil-based media. However, it is not possible to state unequivocally which of the oils, or which of their combinations, has a greater influence.

4. Discussion

Waste substrates from the agricultural and food industry are suitable sources of nutrients for the biotechnological processing of microorganisms in the circular economy model [34]. We can mention easily processed waste substrates: glycerol [35,36], alcohols, animal fats [17], vegetable oils [21]. Furthermore, with the use of pretreatments, a number of lignocellulosic substrates can be processed with yeast: coffee grounds [21], straw [37] and grape pomace [38]. Furthermore, carotenogenic yeasts demonstrated high productivity and the ability to biotransform poultry industry waste (feathers and fat) into products with higher added value [39]. In the biotechnology of carotenogenic yeasts, however, we encounter a problem. Complex waste substrates used for yeast cultivation must be hydrolysed to a certain extent. It is caused by the low activity of hydrolytic enzymes produced by carotenogenic yeasts or their absence [16,17,21]. In the first case of low enzyme activity, it is sufficient to hydrolyse the substrate partially, and this partially hydrolysed substrate becomes more accessible to the yeast. This procedure then leads to higher biomass yields. However, it may not always lead to a higher production of individual metabolites. In the second case, an example is cellulose substrates, which must be completely hydrolysed [21].

An example can be the preparation of SCG hydrolysate [21]. The increased production of metabolites is primarily induced in carotenogenic yeasts by the action of the native untreated substrate. The waste material in its natural form exerts a higher stress on the yeast leading to increased production of desired metabolites [17]. The goal is to find a compromise that will ensure sufficient production of yeast biomass and simultaneously induce the production of valuable metabolites. In the case of animal fats and vegetable oils, which represent complex substrates, there are two routes. The first is simple hydrolysis to release glycerol. The disadvantage is the simultaneous release of a high concentration of fatty acids, which strongly inhibits yeast growth. In our previous work, we confirmed that the best compromise in this situation is partial hydrolysis releasing enough simple glycerol for the initial exponential phase of yeast growth [17,21]. At the same time, this method increases the availability of fats, especially animal fats, which are solid at the cultivation temperatures used. At the same time, non-hydrolysed fat acts as a stress factor and an inducer of increased production of metabolites. The second way consists in using two substrates as simultaneous sources of carbon. In previous work, we studied this combination of representatives of the genus *Sporidiobolus* with the focus on maximal possible valorisation of spent coffee grounds by carotenogenic yeasts. Beneficial nutrients in coffee grounds for yeasts were hydrolysed lignocellulose as a carbohydrate source and coffee oil acting as a complex substrate [21]. The main idea of this work is similar: to supply the yeast with a sufficient amount of an easily metabolizable carbon source leading to a sufficient biomass production in the exponential growth phase. In this work, the effect of the combination of waste oils and fats with waste glycerol from biofuel production on yeasts of the genus *Rhodotorula* was studied. Waste glycerol is studied here as an alternative to SCG hydrolysate [21]. This work was started in the first phase by screening cultivation of selected yeast strains on media containing a combination of waste glycerol and waste oil. Furthermore, the influence of the ratio of simple and complex carbon sources on carotenogenic yeasts' growth and overall productivity was studied. The following ratios were chosen for these experiments: 10:90 and 25:75 (glycerol:waste lipid). Cultivation results confirmed the ability of yeast to grow on all studied combinations of oils and glycerol. At the same time, the yeast demonstrated a high level of resistance to antimicrobial substances (e.g., phenolics) [21] present in the medium. In terms of biomass production for all strains, it was found that media with a higher glycerol content (higher glycerol:fat ratio) were significantly better for biomass production than identical media with a lower glycerol content. The results of the *Rhodotorula kratochvilovae* CCY 020-002-026 strain, in comparison with other studied yeast strains, was the only one to produce more biomass on lipid-containing media than on the control glycerol medium.

Sporidiobolus pararoseus CCY 019-009-006, which was chosen as a comparison strain based on the results of our previous works [17,21], produced very low amounts of biomass on control glycerol media. On the other hand, in media with combined substrates, it produced more biomass and was characterized primarily by a high accumulation of lipids, which was the highest compared to the other strains (50–60% of lipids). It can therefore be stated that on purely glycerol media, the strain growth is inhibited, as can be also seen in our previous work [17]. The application of lipid substrates in all studied strains increased beta-glucan production. The production of carotenoids was also higher on media with waste substrates. The maximum yield was produced by the *Rhodotorula toruloides* CCY 062-002-004 strain. The results show the same trends for the production of ubiquinone and ergosterol; again, the yeast produced metabolites better in media with a higher glycerol content. The results in this work and the results in our previous research show that representatives of the genus *Sporidiobolus* are not able to utilize glycerol, especially if pure glycerol is the majority carbon source [17]. This interesting phenomenon was also observed in other representatives of the *Fungi* kingdom [30]. Additionally, we would like to study this more deeply in the future.

The second part followed by studying the effect of the induction of lipase production during the inoculation step of the production of whole biomass and metabolites. For these experiments, yeasts were cultured only on lipid substrates and their combinations. The biomass production results show that lipase induction had a generally positive effect in all monitored parameters. However, biomass production in the genus *Rhodotorula* was limited by the insufficient supply of nutrients with an exception for the strain *Rhodotorula kratochvilovae* CCY 020-002-026, which grew very well on a combination of frying oil and animal fat. The same results and trends as in the case of the first phase of the experiments were observed in the second experimental phase for the strain *Sporidiobolus pararoseus* CCY 019-009-006. The strain was characterised by the highest biomass production under these conditions.

Based on the results of the first and the second phase of the screening experiments, strains of *Rhodotorula kratochvilovae* CCY 020-002-026 and *Rhodotorula toruloides* CCY 062-002-004 were selected for controlled fed-batch cultivation in a laboratory bioreactor 7L. Although the strain *Sporidiobolus pararoseus* CCY 019-009-006 was characterised by high biomass production its carotenoid production was significantly lower in comparison with *Rhodotorula* strains and therefore it was not included in the last part of the experiment. Yeasts were cultivated in a bioreactor on a combination of two substrates (waste glycerol and coffee oil), which were chosen due to the high content of phenolic substances and tocopherol, which could be incorporated by red yeasts into their cells. Cultivation was carried out using the fed-batch method. At the beginning, all the glycerol and 10% of the total oil were present in the fermenter, and the rest was gradually dosed through the pump. The higher content of the untreated substrate corresponded with our goal to reduce the financial costs of the entire process and, at the same time, induce a high production of secondary metabolites. The results show that the chosen combination of substrates induced biomass production and, simultaneously, the production of target yeast metabolites, which confirmed our assumption [17,21].

In conclusion, the present study showed that the proposed procedure for cultivating carotenogenic yeasts using a combination of two successively utilized waste C-sources is an effective method for obtaining a high yield of enriched red yeast biomass. The ratio of 25:75 for simple:complex sources appears optimal for a sufficient increase of yeast biomass in the initial stages of growth. The used strategy allows us to replace expensive artificial raw materials. Moreover, the combining of these substrates without the need for pre-treatment offers wide possibilities for modulating the biotechnological process and optimising the production of individual metabolites. Furthermore, it was confirmed that the choice of waste oil can modulate the ratio of individual groups of fatty acids in the biomass. Significant assimilation of the unsaturated fatty acids present in the coffee oil was measured when the yeast was cultivated on the coffee oil-based media. After replacing the lipid substrate with frying oil, the fatty acid profile changed towards higher MUFA. This phenomenon was observed also in our previous work, where yeasts were cultivated on combination SCG hydrolysate:oil [21]. Unsaturated fatty acids generally have a beneficial effect on human and animal health. Carotenogenic yeast biomass produced in this way, containing a high content of unsaturated fatty acids and other valuable substances, can serve as a dietary supplement for humans or as feed for animals [40].

Cultivation on animal fat encounters the problem of its solid state, which reduces the effective surface area for the action of yeast lipases and leads to low production. Our proposed mixture of 50:50 animal fat and vegetable oil resulted in a change in physical properties, and this mixture was more fluid under cultivation conditions, resulting in better results on heavy combinations than pure fat [17]. The overall results show that even higher production of biomass and metabolites can be achieved by further optimising the process, consisting of the ratio of both substrates, optimising the mineral composition of the medium, the length of cultivation, and other parameters. In addition, the results of this study confirm that the tested carotenogenic yeasts are suitable for the industrial production of yeast biomass and metabolites with high added value within the concept of the circular economy.

5. Conclusions

In this work, three strains of the genus *Rhodotorula* (*Rhodotorula toruloides* CCY 062-002-004, *Rhodotorula kratochvilovae* CCY 020-002-026, and *Rhodotorula mucilaginosa* CCY 019-004-006) were cultivated on combined waste glycerol and oil/fat-based media. For comparison, one strain of the genus *Sporidiobolus* (*Sporidiobolus pararoseus* CCY 019-009-006) was studied as well. The preliminary tests confirmed that all tested yeasts were able to utilize all tested oils, either alone or in combination with waste glycerol. The highest biomass production was achieved by red yeasts growing on media with a higher glycerol:oil ratio (25:75). At the same time, for most of the strains studied, the amount of complex lipid substrate used was a sufficient stress factor to induce the production of lipid-soluble metabolites (carotenoids, ergosterol, ubiquinone) and lipid accumulation. A common effect of the growth of red yeasts on a medium containing some waste lipids was the increased content of beta-glucans in the yeast cell walls. The increased activity of lipases induced in the inoculation step of the cultivation had additional positive effects on the overall productivity of the yeast. *Rhodotorula kratochvilovae* CCY 020-002-026 and *Rhodotorula toruloides* CCY 062-002-004 were selected as the best-producing strains for fed-batch cultivation in a laboratory bioreactor on medium with combined waste substrates glycerol:coffee oil. The results confirmed our assumption that using a combination of waste substrates can stimulate a higher production of the studied metabolites and, with the use of a sufficient simple carbon source, also the production of yeast biomass. Yeast of the genus *Rhodotorula* sp. can be recommended as highly productive strains suitable for industrial use and processing of these wastes.

The application of oils to the medium had an interesting effect on the yeast's fatty acid profile. At the same time, the incorporation of substances contained in oils into the yeast biomass was observed. An example is a highly valued tocopherol. Overall, the combination of glycerol and fat/oil waste materials is an effective method of producing carotenoids and lipid-enriched biomass within the concept of a circular economy. Adding untreated oleic substrate can further reduce the cost of the entire biotechnological process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11041013/s1. Figure S1. Phase I: Biomass, lipid and β-glucan production of *Rhodotorula mucilaginosa* cultivated on combination of waste lipids and glycerol. Figure S2. Phase I: Fatty acid production of *Rhodotorula mucilaginosa* cultivated on combination of waste lipids and glycerol. Figure S3. Phase I: Biomass, lipid and β-glucan production of *Sporidiobolus pararoseus* cultivated on combination of waste lipids and glycerol. Figure S4. Phase I: Fatty acid production of *Sporidiobolus pararoseus* cultivated on combination of waste lipids and glycerol. Figure S5. Phase II: Biomass, lipid and β-glucan production of *Rhodotorula mucilaginosa* cultivated on waste lipid media with and without lipase induction. Figure S6. Phase II: Fatty acid production of *Rhodotorula mucilaginosa* cultivated on waste lipid media with and without lipase induction. Figure S7. Phase II: Biomass, lipid and β-glucan production of *Sporidiobolus pararoseus* cultivated on waste lipid media with and without lipase induction. Figure S8. Phase II: Fatty acid production of *Sporidiobolus pararoseus* cultivated on waste lipid media with and without lipase induction. Figure S9. Statistical analysis results of *Rhodotorula kratochvilovae* cultivated in Erlenmeyer flasks on media with different content of waste carbon lipid source. Figure S10. Statistical analysis results of *Rhodotorula toruloides* cultivated in Erlenmeyer flasks on media with different content of waste carbon lipid source. Figure S11. Statistical analysis results of *Rhodotorula mucilaginosa* cultivated in Erlenmeyer flasks on media with different content of waste carbon lipid source. Figure S12. Statistical analysis results of *Sporidiobolus pararoseus* cultivated in Erlenmeyer flasks on media with different content of waste carbon lipid source. Table S1. Phenolic content analysis: gradient elution used during HPLC/DAD analysis. Table S2. HPLC/DAD analysis of yeast, microalgae lipid metabolites: changes in mobile phase composition during gradient elution. Table S3. Temperature programme of GC/FID analysis of FAMEs. Table S4. Phase I: Biomass, lipid production, fatty acid profile and β-glucan production of *Rhodotorula kratochvilovae* cultivated on combination of waste lipids and glycerol. Table S5. Phase I: Biomass, lipid production, fatty acid profile and β-glucan production of *Rhodotorula toruloides* cultivated on combination of waste lipids and glycerol. Table S6. Phase I: Biomass, lipid production, fatty acid profile and β-glucan production of *Rhodotorula mucilaginosa* cultivated on combination of waste lipids and glycerol. Table S7. HPLC analysis of Phase I screening cultivations of *Rhodotorula mucilaginosa* cultivated on combination of waste lipids and glycerol. Productions are listed in mg/g of cell dry weight. Table S8. 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Attachment 2

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Production of Enriched Biomass by Carotenogenic Yeasts Cultivated on by-Products of Poultry Processing—A Screening Study

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Abstract: Carotenogenic yeasts are a group of microorganisms producing valuable metabolites such as carotenoids, ergosterol, ubiquinone or fatty acids. Their exceptional adaptability allows them to grow in diverse conditions. Owing to their extracellular lipase activity, they are capable of processing many lipid-type waste substrates. This study discusses the processing of poultry waste, specifically fat and feathers by using carotenogenic yeasts. Poultry fat does not require any pre-treatment to be utilized by yeast, but hydrolytic pre-treatment is required for the utilization of the nitrogen contained in feathers. Glycerol was used as a supplementary substrate to support the culture in the early stages of growth. Seven yeast strains were used for the experiments, of which the strain *Rhodotorula mucilaginosa* CCY19-4-25 achieved exceptional results of biomass production: 29.5 g/L on poultry fat + 10% glycerol at C/N ratio 25 and 28.3 g/L on media containing poultry fat + 25% glycerol at C/N 50. The bioreactor cultivation of the *Rhodosporidium toruloides* strain in media containing glycerol and feather hydrolysate as a nitrogen substrate achieved a biomass yield of 34.92 g/L after 144 h of cultivation. The produced enriched yeast biomass can be used as a component for poultry feeding; thus, the study is performed under the biorefinery concept.

Keywords: carotenogenic yeasts; poultry waste fat; poultry feather; enriched biomass; carotenoids; lipids; biorefinery

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1. Introduction

Red yeasts are a group of diverse yeast species (*Rhodotorula* sp., *Sporidiobalus* sp., *Cystofilobasidium* sp.) capable of carotenoid biosynthesis via the mevalonate pathway [1,2]. These yeasts also include oleogenous species that are capable of accumulating lipids within their cells in a total content of more than 20% of dry biomass [3,4]. In many cases, carotenogenic yeasts possess extracellular lipase activity, which results in their ability to decompose triglycerides into glycerol and fatty acids and use them as a carbon source for the propagation and production of metabolites [5,6].

Carotenogenic yeasts are considered to be a species of interest due to their ability to biotransform waste substrates of a lipid, sugar or polyol (glycerol) nature (which has been observed in many studies [7–12]) to valuable metabolites, which include coenzyme Q10, ergosterol, carotenoids, fatty acids (lipids) and β-glucans [13].

The growing world population increases the demands on all sectors of industrial and agricultural production, the burden on the environment is being increased, and as a result it is necessary to intensify the transition from a linear economic model to a circular economic model [14,15]. Carotenogenic yeasts fit into the circular economic model with their metabolic activity, which enables the production of biomass and metabolites from waste organic substrates under the biorefinery concept. Furthermore, it is a significant advantage that the biotechnological production of biomass can be carried out in any place equipped with a suitable technology base, independently of the agricultural ground or the climate conditions [12]. The produced biomass enriched with beneficial substances (carotenoids, ergosterol, ubiquinone, lipids) has great potential as a feed additive, and at the same time, it is a cheaper alternative to complex carotenoid mixtures used as a feed additive [16,17]. According to a recent study, animals supplemented with enriched carotenogenic yeast biomass achieved better body growth and condition than animals whose diet was not supplemented with yeast [18]. Biomass or its components have the potential to be used in supplements for human nutrition, cosmetics, or pharmacy [19]. Feeding of poultry with red yeast biomass cultivated on waste poultry fat as the only carbon source and poultry feather hydrolysate as the only nitrogen source fully accepts the conception of the circular economy.

According to [20], the annual production of meat is 337 million tons, and more than 1/3 belongs to the production of chicken meat (118.02 million tons). Such mass production produces several usable by-products, including poultry feathers (40 million tons per year [21]) and fat (11% of total chicken body fat—not all waste by-products [22]). Poultry fat is rich in fatty acids (30.7% SFA, 30.2% MUFA and 30.9% PUFA) and can be considered a valuable carbon source [23]. As a source of nitrogen, it is possible to consider using feathers, the composition of which is 91% protein (keratin), 1% lipids and 8% water [24]. Due to the rigid structure of keratin, in which protein chains are tightly packed and stabilized through hydrophobic interactions and disulfide bonds, degradation by common proteolytic enzymes such as pepsin, papain or trypsin is unattainable [25].

By processing poultry material, a series of waste by-products can be obtained, which, after appropriate treatment, could be considered a carbonaceous or even complex substrate for biotechnological processing. The keratin contained in feathers is classified as a scleroprotein and due to its insolubility in water, it is difficult to utilize it in its native form by microorganisms without any specific enzymatic apparatus. For this reason, its basic or basic-enzymatic hydrolysis pre-treatment is necessary for its use in the cultivation of carotenogenic yeasts [26]. Under normal conditions, chicken fat is in a liquid form and there is no need for its pre-treatment as carotenogenic yeasts contain enzyme equipment (extracellular lipases) for splitting triglycerides.

2. Materials and Methods

2.1. Strains

The following types of red (carotenogenic) yeasts from the Bratislava collection of CCY (Culture Collection of Yeasts) strains were used for cultivation: *Cystofilobasidium macerans* CCY 10-1-2, *Rhodotorula kratochvilovae* CCY 20-2-26, *Rhodosporidium toruloides* CCY 062-002-001, *Rhodotorula mucilaginosa* CCY 20-9-7, *Rhodotorula mucilaginosa* CCY 19-4-25, *Sporidiobolus metaroseus* CCY 19-6-20, and *Sporidiobolus pararoseus* CCY 19-9-6.

2.2. Waste Materials and Their Pre-Treatment

Animal waste materials such as chicken fat and poultry feathers were used for the cultivations in combination with purified glycerol (which also served as a model for cultivations on waste glycerol from biofuel production; further GLY). While chicken fat was used for cultivation purposes in its pure form without any hydrolyzing treatments, degreased feathers were subjected to basic hydrolysis. The process consisted of hydrolyzing 80 g of degreased feathers in 800 mL of 1 M NaOH solution in sterilizing bottles at 100 °C for 30 min. The determination of nitrogen in the final hydrolysate was carried out using the biuret method. According to the results of nitrogen determination, 80 mL of hydrolysate per liter of medium should be used for cultivation purposes.

2.3. Description of Cultivation Experiments

Cultivations were carried out in 250 mL Erlenmeyer flasks with a medium content of 50 mL. The media contained a common mineral base (Table 1) and differed in the types and amounts of carbonaceous (chicken fat and glycerol; further F and GLY) and nitrogenous substrates (urea and feather hydrolysate—further as F.H.) used. Cultivation experiments were performed at C/N ratios of 25 and 50 and divided into series: the 1st series without the addition of hydrolysate and the 2nd series with the addition of hydrolysate. Both series included a control media containing glycerol as a carbon source, one containing urea as a nitrogen source and the second with addition of feather hydrolysate as a nitrogen source (Tables 2 and 3).

Table 1. Mineral basis of production media.

Component	**Amount (g)**
KH_2PO_4 (g)	4
$MgSO_4 \cdot 7H_2O$ (g)	0.7
Tap water	1000

Table 2. Composition of production media at C/N ratio of 25 for volume of 1000 mL of media.

1st series					
Media name	GLY	FAT	F + 10% GLY	F + 25% GLY	GLY + F.H.
Poultry fat (g)	0	17.66	15.89	13.24	0
Glycerol (g)	46.26	0	4.63	11.56	46.26
Urea (g)	1.81	1.81	1.81	1.81	0
F.H. (mL)	0	0	0	0	80
2nd series					
Media name	GLY (−F.H.)	FAT + F.H.	F + 10% GLY + F.H.	F + 25% GLY + F.H.	GLY + F.H. (F.H)
Poultry fat (g)	0	17.66	15.89	13.24	0
Glycerol (g)	46.26	0	4.63	11.56	46.26
Urea (g)	1.81	0	0	0	0
F.H. (mL)	0	80	80	80	80

Table 3. Composition of production media at C/N ratio of 50 for volume of 1000 mL of media.

1st series					
Media name	GLY	FAT	F + 10% GLY	F + 25% GLY	GLY + F.H.
Poultry fat (g)	0	35.31	31.78	26.49	0
Glycerol (g)	92.51	0	9.25	23.13	92.51
Urea (g)	1.81	1.81	1.81	1.81	0
F.H. (mL)	0	0	0	0	80
2nd series					
Media name	GLY (F.H.)	FAT + F.H.	F + 10% GLY + F.H.	F + 25% GLY + F.H.	GLY + F.H. (F.H)
Poultry fat (g)	0	35.31	31.78	26.49	0
Glycerol (g)	92.51	0	9.25	23.13	92.51
Urea (g)	1.81	0	0	0	0
F.H. (mL)	0	80	80	80	80

2.4. Cultivation in Flasks

Yeast cultivation was carried out via double inoculation in Erlenmeyer flasks in YPD media (Table 4), according to an inoculation ratio of 1:5, on reciprocal shakers ensuring constant shaking. The double inoculation process was used based on experience and previously published results [12]. This cultivation scheme is sufficient for production of exponential culture with high cell density. Each inoculum was re-inoculated 24 h after first inoculation. Inoculation into the production media from the 2nd inoculum was performed at an inoculation ratio of 1:5 to 50 mL of the production media in 250 mL Erlenmeyer flasks. Production cultivation lasted 96 h at constant shaking on reciprocating shakers. Cultivation was terminated by separating the biomass from the medium by repeated centrifugation and subsequent freezing of the biomass at −80 °C to prepare it for the lyophilization procedure.

Table 4. YPD media composition.

Component	Amount
Yeast autolysate (g)	10
Bacteriological peptone (g)	20
Glycerol (g)	20
Tap water (mL)	1000

2.5. Cultivation in a Bioreactor

Cultivation was carried out in a 3.5 L bioreactor with a working volume of 2.2 L (2 L of production medium and 200 mL of inoculum). The bioreactor was filled with the production medium (Table 5). The sterility of the process was achieved by subjecting the bioreactor to sterilizing conditions in an autoclave (121 °C for 15 min). After cooling and connecting the bioreactor to the control unit, the pH was adjusted by using 10% (m/m) KOH and 10% (*v*/*v*) H_2SO_4 solutions to a working pH of 5.5. The temperature was set to 25 °C and constant stirring and aeration was ensured.

Table 5. Composition of the bioreactor medium for volume of 2000 mL (the remaining volume was supplemented with tap water).

Component	Amount
KH_2PO_4 (g)	8
$MgSO_4·7H_2O$ (g)	1.392
Glycerol (g)	92.51
F.H. (mL)	160

2.6. Gravimetry and Pigment Extraction

The frozen biomass was lyophilized and subjected to the gravimetric determination of biomass production (g/L). The hydrated biomass in microtubes filled with glass beads and methanol was subsequently subjected to a disintegration process using a laboratory disintegrator for the purpose of extracting pigments, ergosterol and ubiquinone according to Folch´s extraction [27]. The final extract was stripped of the extraction solvent (chloroform) and was dissolved in a 2:1 HPLC grade solvent mixture of ethyl acetate:acetonitrile in a volume suitable for HPLC analysis with PDA detection [11,12].

2.7. Transesterification and FAME Extraction

The biomass (approx. 10 mg) was weighed into crimping vials filled with 1.8 mL of the transesterification mixture, which consisted of 15% (*v*/*v*) H_2SO_4 dissolved in methanol with the addition of heptadecenoic acid as an internal standard at a concentration of 0.5 mg/mL. The vials were crimped and tempered in a thermo-block at 85 °C for 120 min. The contents of the cooled vials were transferred to screw-top vials containing 1 mL of HPLC-grade hexane as an extractant and 0.5 mL of a neutralizing solution of 0.05 M NaOH. The mixture was intensively vortexed, and a proportional part (depending on the weight of the biomass) was taken from the hexane phase for the quantitative and qualitative analysis of fatty acids on a GC-FID apparatus.

2.8. HPLC Analysis

The qualitative and quantitative analysis of pigments, ergosterol and ubiquinone was performed on a Thermo Fischer Scientific HPLC apparatus on a Kinetex EVO C18 column (particle size 2.6 µm, length 150 mm, Phenomenex) with PDA detection and gradient elution. The flow rate was set at 1.2 mL/min with a total sample analysis duration of 25 min. The scanned wavelength channels were set to 450 nm for the detection of carotenoids and 280 nm for the detection of sterols and ubiquinone. The composition and gradient dosing of the mobile phases are introduced in Tables 6 and 7.

Table 6. Composition of mobile phases in HPLC analysis.

Mobile Phase	Component	Volume Parts
A	Acetonitril	84
	100 mM trisHCl buffer pH 8	14
	Methanol	2
B	Methanol	60
	Ethylacetate	40

Table 7. Elution gradient.

Retention Time [min]	MF A (%)	MF B (%)
0	100	0
13	0	100
19	0	100
20	100	0
25	100	0

2.9. GC Analysis

The analysis of fatty acids was carried out on the following apparatus: a Thermo Scientific TRACE 1300 TM Gas Chromatograph, with a Thermo Scientific Al 1310 autosampler and an automatic dispenser equipped with a splitter. FAME (fatty acid methyl ester) separation was performed on a LION GC-FAME column with dimensions of 30 m × 0.25 mm × 0.25 µm and detection was performed on a flame ionization detector (FID). The analysis conditions were as follows:

The temperature of the Injector was set to 240 °C, the split was set to 10, the flow rate of the mobile phases was 1 mL/min, and the injection volume was 1 µL. The temperature of the detector was set to 240 °C with the following set flow rates: air 350 mL/min, hydrogen 35 mL/min and nitrogen (makeup gas) 30 mL/min. The temperature gradient during separation was as follows: 80 °C from injection to t_R = 1 min, temperature increase to 140 °C with a gradient of 15 °C/min to t_R = 5 min, temperature increase to 190 °C with a gradient of 3 °C/min up to t_R = 21.7 min, increase with a temperature gradient of 25 °C/min to 260 °C with maintenance of this temperature for 1 min until the end of separation at t_R = 25.5 min [3,4].

3. Results

As a model red yeast with previously verified high production properties, the strain *Rhodotosporidium toruloides* (CCY 062-002-001) was tested first. The cultivation was performed in Erlenmeyer flasks at C/N 25 and C/N 50 and in a laboratory fermenter as well. The results are expressed in the form of graphs. The other strains were tested in

Erlenmeyer flasks at C/N 25 and C/N 50 only. These results are documented in Tables S3–S5 (Supplementary Material). The strain R. mucilaginosa CCY 19-4-25 was found to be the best producer of biomass from the poultry wastes; thus, there results are also presented in some graphs. Experimental data are introduced in Table S4 (Supplementary).

3.1. Flask Cultivations of Strain Rhodotosporidium toruloides (CCY 062-002-001)

3.1.1. Results for C/N 25

The cultivations on media of the first series showed the highest biomass growths in GLY (19.0 g/L) and FAT (20.4 g/L) media. Furthermore, two inverse trends can be observed in media containing a mixed carbonaceous substrates. In the first series, as the concentration of fat in the medium increases, the biomass production increases; in the second series, the trend is inverse. Significant carotenoid productions were achieved on GLY (4.891 mg/g) and F + 10% GLY (5.667 mg/g) media. The highest ergosterol productions achieved were observed on GLY (F.H.) (5.544 mg/g) and FAT + F.H. (6.911 mg/g) media. The highest accumulations of ubiquinone were achieved on F + 25% GLY (7.748 mg/g) and F + 10% GLY + F.H. (9.711 mg/g) media. A higher percentage content of lipids in the biomass was recorded in the mixed media of the second series, in which the highest accumulation of lipids in the dry biomass was recorded in F + 10% GLY + F.H. (25.04%). The highest productions of SFA (1.57 g/L medium) and MUFA (2.01 g/L medium) were observed in the FAT medium. The highest PUFA production (0.85 g/L medium) was recorded in the F + 25% GLY + F.H. medium. Results of cultivations are presented in Figures 1–3.

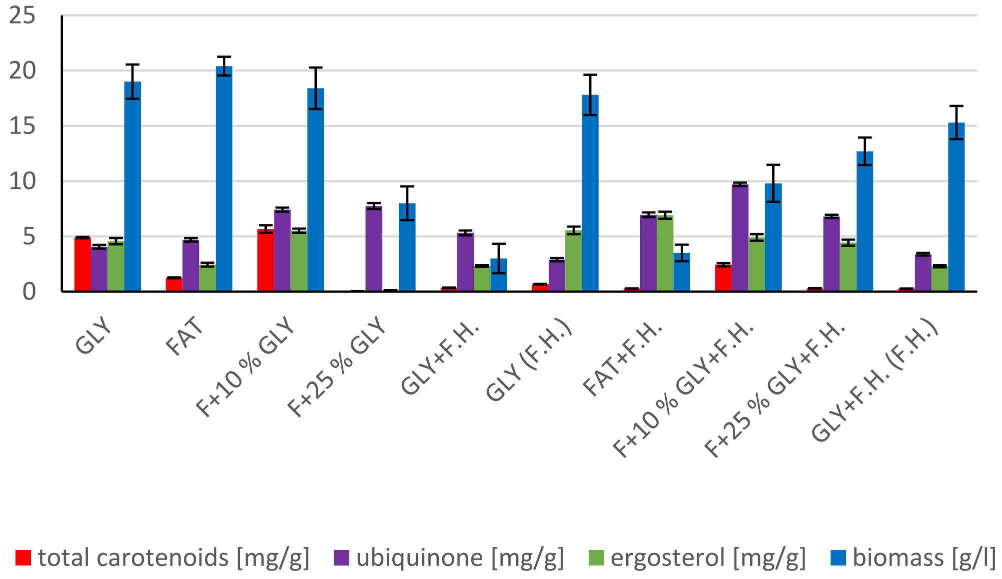

Figure 1. Metabolite production by biomass of yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 25.

3.1.2. Results for C/N 50

The most significant biomass productions were achieved on the glycerol media GLY + F.H. (21 g/L) and GLY (24 g/L). In media with mixed carbonaceous substrates, there were observable trends in both series, in which the biomass production increased as the concentration of chicken fat in the medium decreased. The highest values of produced carotenoids were achieved in GLY (2.533 mg/g) and F + 25% GLY (4.582 mg/g) media. Significant ergosterol productions were achieved on F + 25% GLY + F.H. (8.724 mg/g) and F + 10% GLY + F.H. (8.944 mg/g) media. Significant values of ubiquinone production were achieved in F + 25% GLY + F.H. (9.706 mg/g) and GLY + F.H. (F.H) (11.611 mg/g) media. The highest percentage content of lipids in the biomass was achieved during cultivation on the F + 10% GLY (31.14%) medium. The highest production of individual types of fatty acids was observed in cultivations in different media: for SFA, it was on GLY + F.H.

(2.09 g/L medium); for MUFA, it was on GLY (2.29 g/L medium); and for PUFA, it was on F + 25% GLY (0.81 g/L medium). In the second series, in media with a mixed carbon substrate, a trend of decreasing lipid accumulation can be observed depending on the decreasing concentration of fat in the media. Results of cultivations are presented in Figures 4–6.

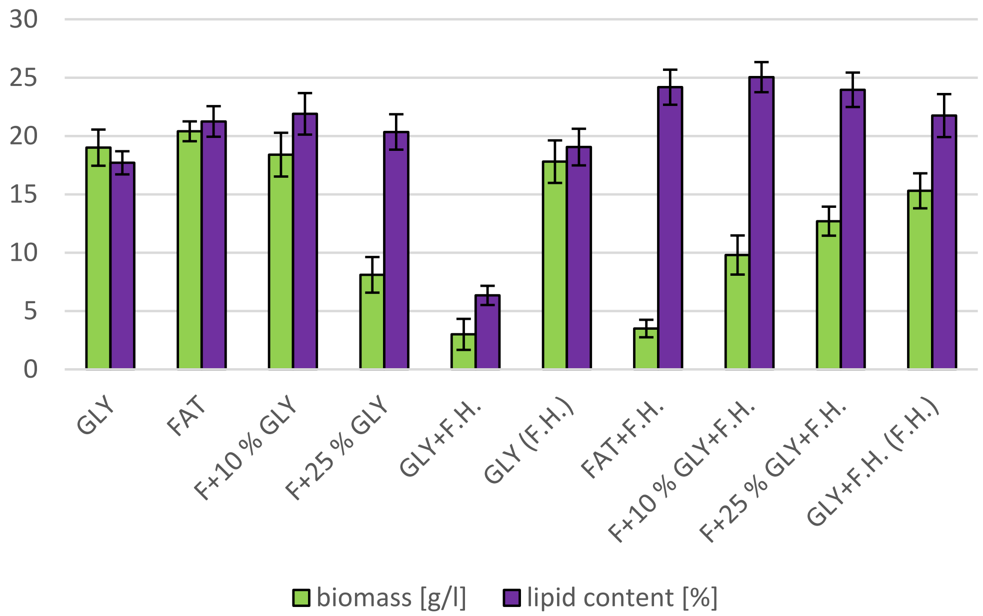

Figure 2. Lipid and biomass content in yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 25.

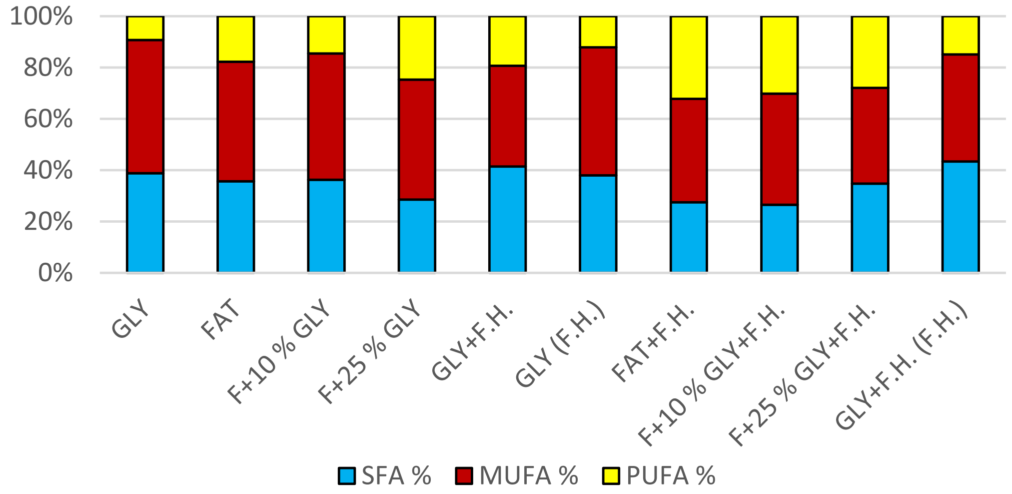

Figure 3. Fatty acid distribution in lipids of yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 25.

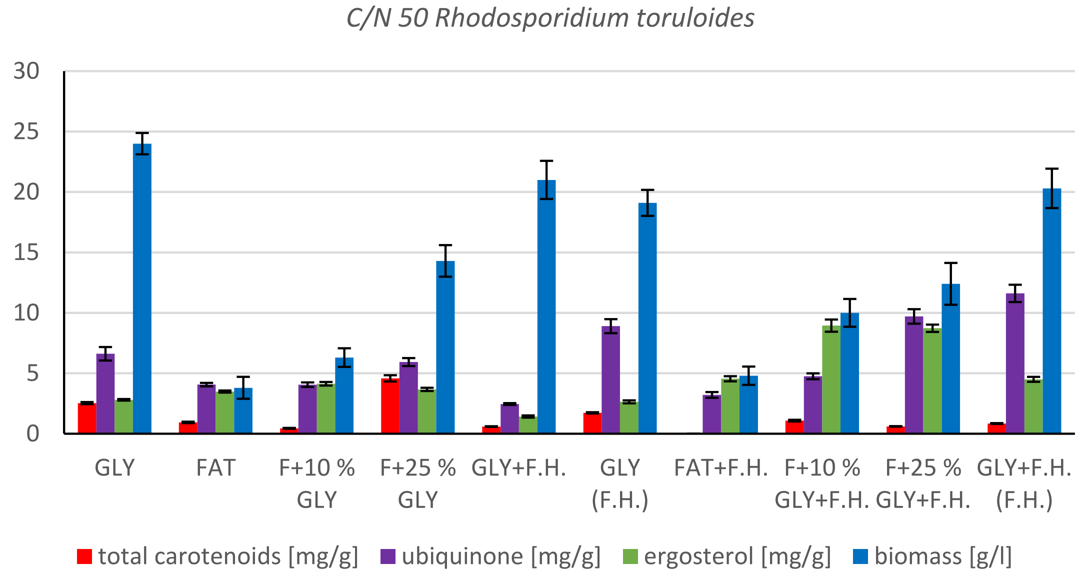

Figure 4. Metabolite production by biomass of yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 50.

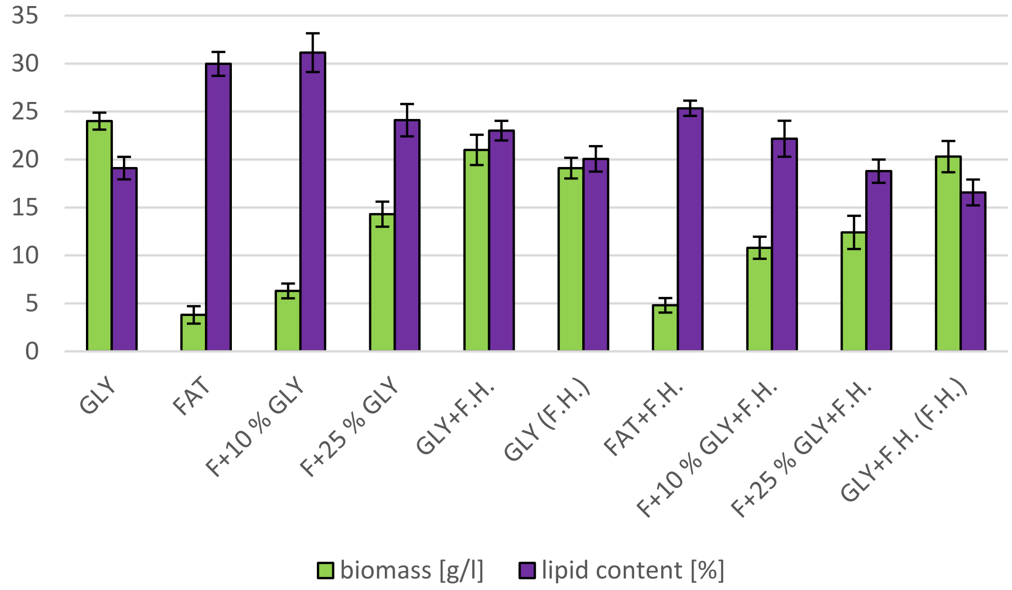

Figure 5. Lipid and biomass content in yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 50.

3.1.3. Bioreactor Cultivation of Strain *Rhodotosporidium toruloides* (CCY 62-2-4)

The cultivation in a laboratory bioreactor showed that under controlled conditions, a significant growth in biomass can be achieved, as can be observed in Figure 7 in the 144th hour of cultivation (34.92 g/L). The production of carotenoids did not report significant increments; its maximum was reached at the 22nd hour of cultivation (0.831 mg/g). The concentration of ergosterol in the biomass reached its maximum at the 28th hour of cultivation (8.280 mg/g). The highest observed production of ubiquinone was at the beginning of cultivation (20.942 mg/g), but significant production was also achieved in the culture at the 144th hour of cultivation (12.059 mg/g). The highest percentage content of lipids in the biomass was observed at the 72nd hour of cultivation (40.81%), but the most significant production of all types of fatty acids was achieved at the 144th hour of cultivation

(SFA 4.50 g/L, MUFA 5.15 g/L, PUFA 2.08 g/L). Results of cultivation are presented in Figures 7–9.

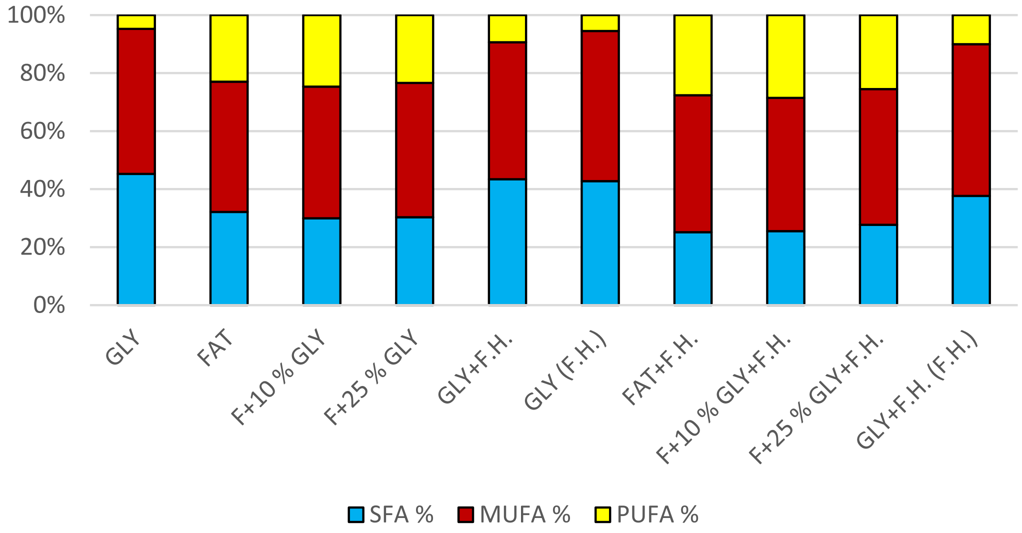

Figure 6. Fatty acid distribution in lipids of yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 50.

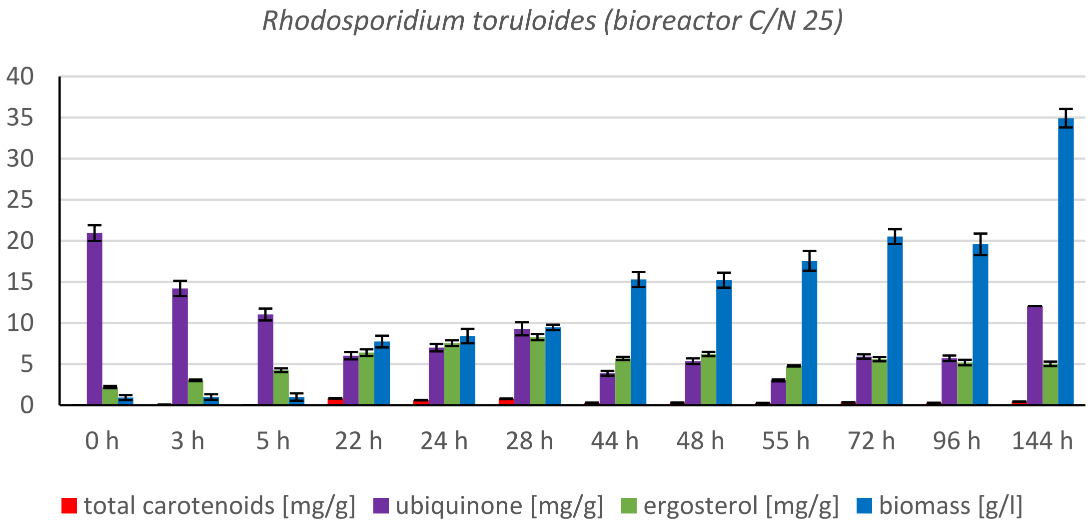

Figure 7. Metabolite production by biomass of yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 25 cultivated in bioreactor.

3.2. Flask Cultivations of Strain Rhodotorula kratochvilovae (CCY 20-2-26)

3.2.1. Results for C/N 25

The highest biomass production in the first series was recorded in mixed carbon substrate media, F + 10% GLY (17.7 g/L) and F + 25% GLY (19.2 g/L). Significant productions of carotenoids and ergosterol were achieved in the control media of the first series; in the GLY medium, the production of carotenoids reached 1.602 mg/g and ergosterol reached 3.443 mg/g of biomass, while in GLY + F.H., the production of carotenoids was 1.439 mg/g and ergosterol achieved a biomass of 3.778 mg/g. The highest ubiquinone productions were observed in the GLY medium (10.495 mg/g) and in the F + 25% GLY + F.H. medium (6.238 mg/g). A significant lipid production was recorded in the FAT + F.H. medium (40.82% of dry biomass), which generally showed the highest production of all types of fatty acids (SFA 2.26 g/L, MUFA 2.18 g/L and PUFA 1.31 g/L medium). There is also an observable trend in the second series of samples with F.H., in which with the increasing concentrations of fat in the media, the percentage accumulation of lipids in the biomass also increases (Table S3, Supplementary).

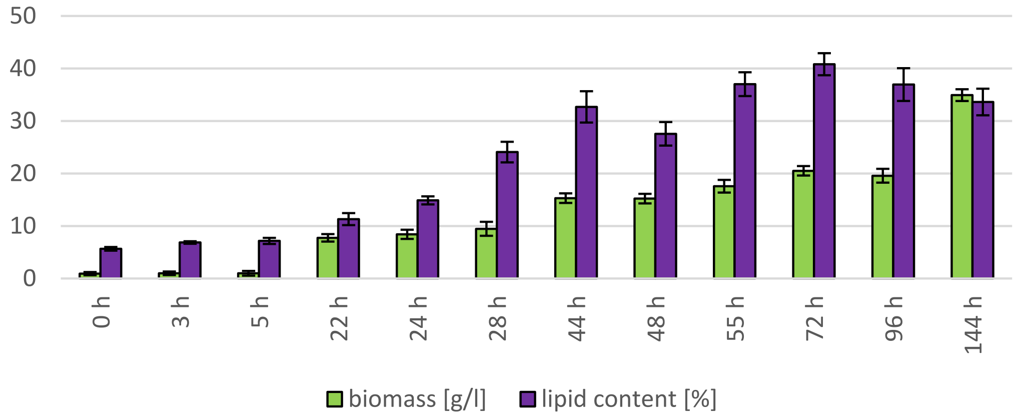

Figure 8. Lipid and biomass content in yeast strain *R. toruloides* in conditions of C/N ratio 25 cultivated in bioreactor.

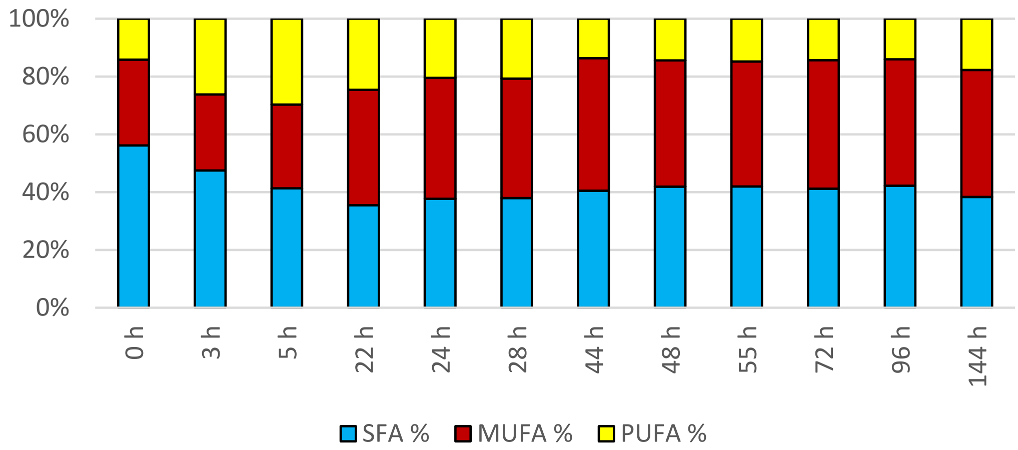

Figure 9. Fatty acid distribution in lipids of yeast strain *Rhodotosporidium toruloides* in conditions of C/N ratio 25 cultivated in bioreactor.

3.2.2. Results for C/N 50

In the first series with chicken fat, a significant biomass growth was observed, the highest biomass yield was achieved on F + 25% GLY (21.2 g/L) media. In the second series, with addition of fat, there is a trend of increasing growth of biomass with a decreasing content of fat in the media. The highest overall biomass concentration of both series was achieved in the media consisting of F + 25% GLY + F.H. (21.5 g/L). The production of carotenoids on the mixed media did not exceed the increments in the control glycerol media, GLY (1.196 mg/g) and GLY (F.H.) (1.324 mg/g). The increased productions of ergosterol were observed in F + 10% GLY + F.H. (11.583 mg/g) media. The highest ubiquinone productions were recorded on the control media of the second series, GLY (F.H.) (13.149 mg/g) and GLY + F.H. (F.H) (15.505 mg/g). Lipid accumulation achieved the greatest values in the cultivation of *Rhodotorula kratochvilovae* in F + 25% GLY + F.H. (37.87% dry biomass) together with the highest productions of all types of fatty acids (SFA 2.09 g/L, MUFA 3.90 g/L and PUFA 2.15 g/L medium).

3.3. Flask Cultivations of Strain Cystofilobasidium macerans (CCY 10-1-2)

3.3.1. Results of C/N Ratio 25

In cultivations in media with a mixed carbon source, there is an observable trend of increasing biomass production with a decreasing fat concentration in the medium.

The highest yields of products were achieved in F + 25% GLY + F.H. (15.1 g/L) and GLY + F.H. (F.H.) (16.6 g/L) media. The notable carotenoid productions were observed in GLY + F.H. (1.325 mg/g) and GLY (1.963 mg/g) media. The highest reported results of ergosterol production were achieved on F + 25% GLY + F.H. (6.587 mg/g) and FAT + F.H. (9.932 mg/g) media. Noteworthy values of production of ubiquinone were achieved in GLY (10.939 mg/g) and F + 25% GLY + F.H. (7.951 mg/g) media. The highest values of accumulated lipids by biomass (31.88%) and produced SFA (2.31 g/L) were observed after cultivation in FAT + F.H. media. The highest accumulation of MUFA by the strain was achieved in GLY + F.H. (F.H.) (1.41 g/L) and PUFA reached its highest value in F + 25% GLY + F.H. (1.02 g/L). The percentage content of lipids in biomass reported two reverse trends in media with a mixed carbon source. In the first series, with an increasing level of fat, the amount of accumulated lipids decreased. This trend is inverse in the second series (Table S3, Supplementary).

3.3.2. Results for C/N 50

The most significant biomass growth of the yeast *Cystofilobasidium macerans* at a C/N ratio of 50 was achieved in GLY + F.H. (F.H.) (17.5 g/L) and GLY (F.H.) (21.3 g/L) media. In media with mixed carbon substrates of the first series, an increasing trend of biomass production can be observed depending on the decrease in fat concentration in the media composition. Significant carotenoid productions were observed in cultivation in FAT (4.916 mg/g) and F + 10% GLY (4.056 mg/g) media. The highest ergosterol products were achieved in F + 25% GLY + F.H. (6.456 mg/g) and F + 10% GLY + F.H. (8.72 mg/g) media. The highest amount of ubiquinone was achieved in GLY (F.H.) (8.228 mg/g) and GLY + F.H. (F.H) (9.864 mg/g) media. The notable percentage content of lipids in dry biomass was observed after cultivation in the FAT + F.H. (26.46%) medium. The most significant production of SFA (1.73 g/L) and MUFA (2.80 g/L) were recorded in GLY (F.H.) media. PUFA were produced to the greatest extent in F + 25% GLY + F.H. (0.83 g/L) media.

3.4. Flask Cultivation of Strain Rhodotorula mucilaginosa (CCY 19-4-25)

3.4.1. Results of C/N Ratio 25

The trend of decreasing biomass production depending on the decreasing concentration of fat in the media can be observed in media with a mixed carbon substrate of the first series. The highest biomass yields were achieved in F + 10% GLY (24.8 g/L) and FAT (29.5 g/L) media. Significant carotenoid productions were achieved in F + 25% GLY (7.430 mg/g) and GLY (10,469 mg/g) media, together with highest ergosterol productions of 5.121 mg/g in F + 25% GLY and 6.832 mg/g in GLY media. Ubiquinone was produced notably in media GLY (5.726 mg/g) and FAT + F.H. (8.932 mg/g). The highest lipid accumulation (25% dry biomass) and production of SFA (3.0 g/L), MUFA (3.12 g/L) and PUFA (1.39 g/L) were observed on the FAT medium. The lipid content percentage of the mixed carbon source media in the second series reported an increasing tendency with the decreasing fat content of the media (Table S4 Supplementary). Results of cultivations are also presented in Figures 10–12.

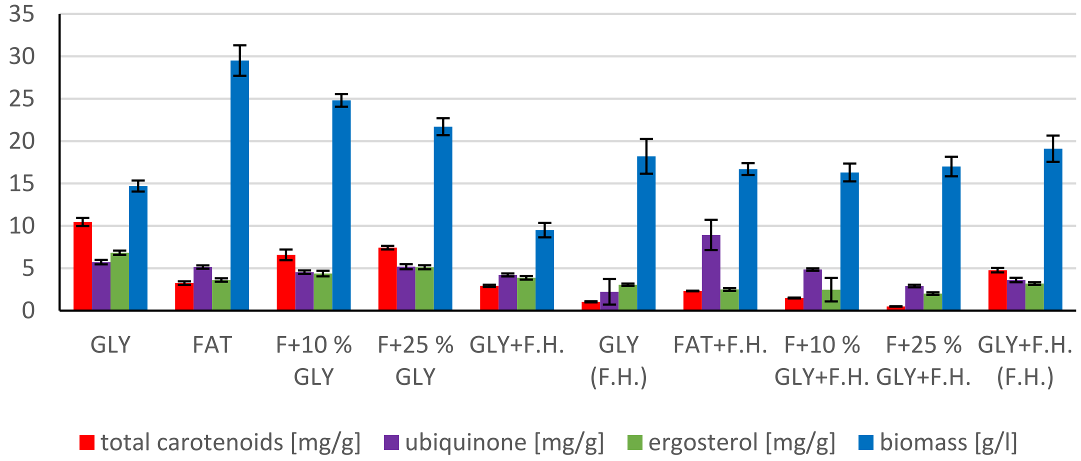

Figure 10. Metabolite production by biomass of yeast strain *Rhodotorula mucilaginosa* CCY19-4-25 in conditions of C/N ratio 25.

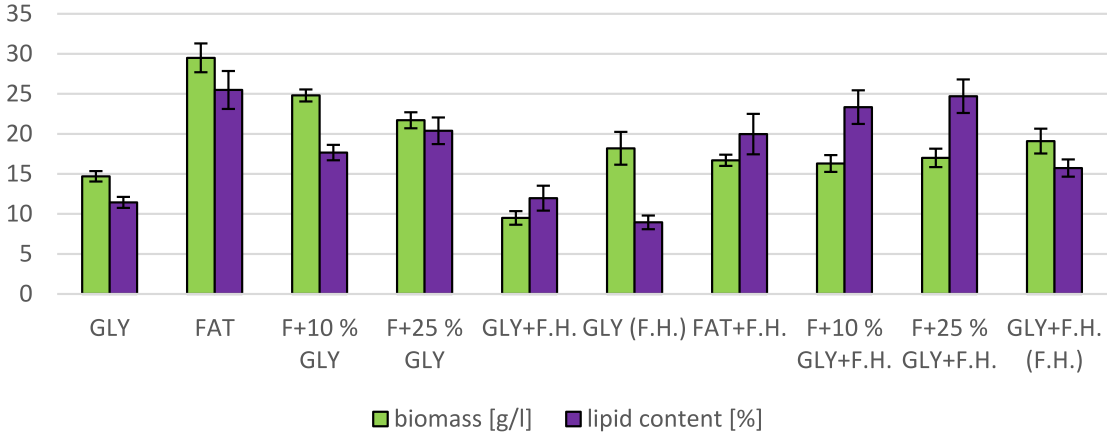

Figure 11. Lipid and biomass content in yeast strain *R. mucilaginosa* at C/N ratio 25.

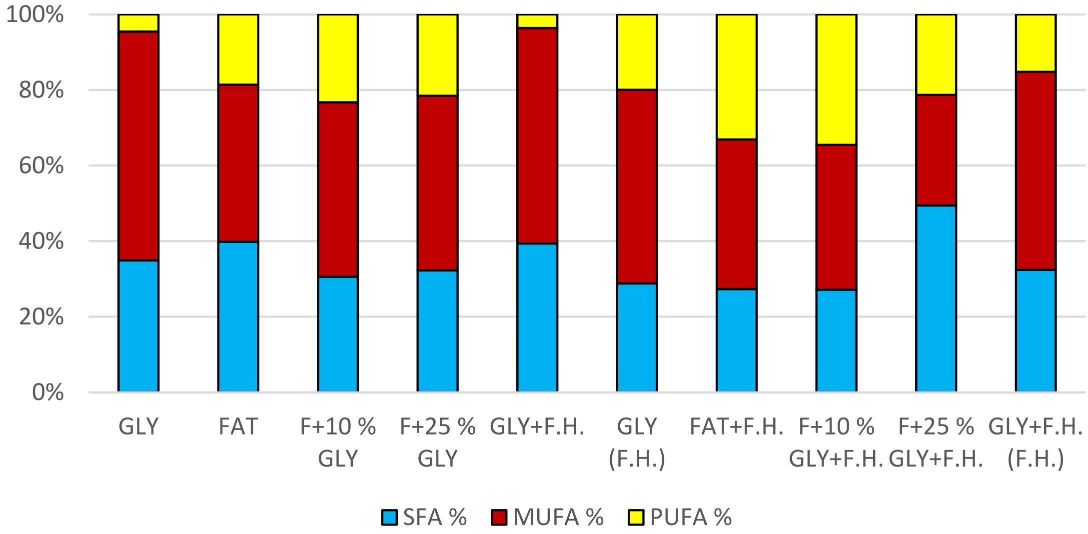

Figure 12. Fatty acid distribution in lipids of yeast strain *Rhodotorula mucilaginosa* in conditions of C/N ratio 25.

3.4.2. Results of C/N Ratio 50

The most significant biomass productions were achieved in FAT + F.H. (28.3 g/L) and F + 25% GLY + F.H. (28.3 g/L) media. Significant carotenoid productions were observed in cultures in F + 25% GLY + F.H. (5.612 mg/g) and GLY (F.H.) (6.044 mg/g) media. The highest concentrations of ergosterol were achieved in GLY (F.H.) (6.118 mg/g) and GLY + F.H. (F.H.) (4.621 mg/g) media. Ubiquinone was produced in the highest concentrations in F + 25% GLY + F.H. (7.573 mg/g) and FAT + F.H. (7.838 mg/g) media. The percentage of lipid content (38.33%) and SFA production (5.76 g/L) was observed in the culture in the F + 10% GLY + F.H. medium. The highest production of MUFA (3.43 g/L) and PUFA (2.43 g/L) was observed in the FAT + F.H. medium. Results of cultivations are presented in Figures 13–15.

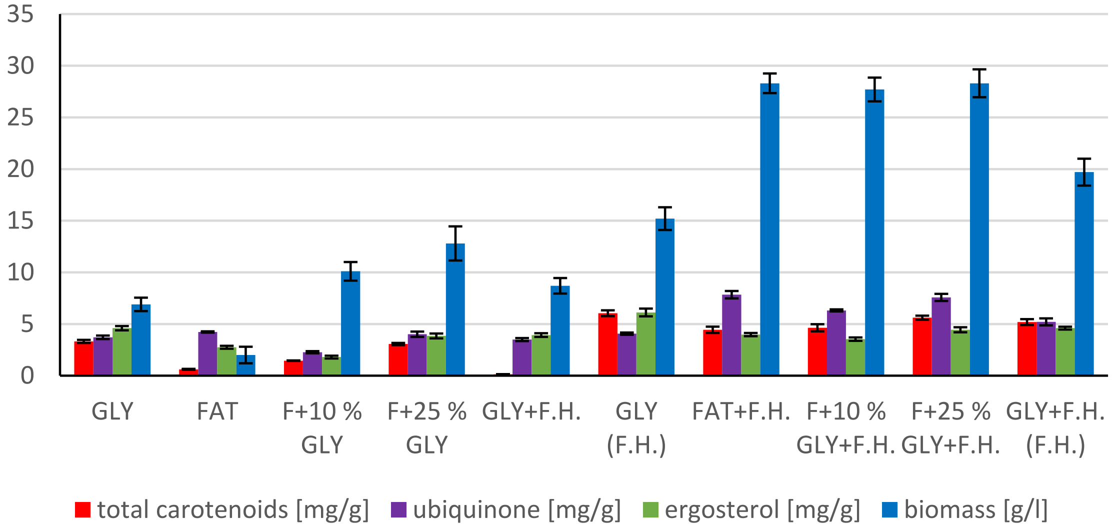

Figure 13. Metabolite production by biomass of yeast strain *Rhodotorula mucilaginosa* CCY19-4-25 in conditions of C/N ratio 50.

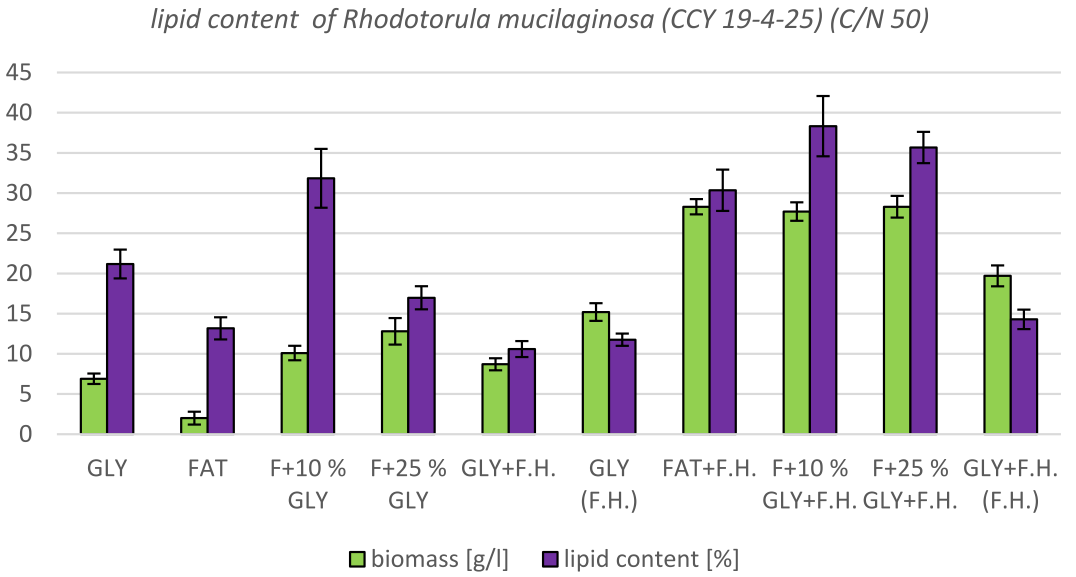

Figure 14. Biomass and lipid production by biomass of yeast strain *Rhodotorula mucilaginosa* (CCY 19-4-25) in conditions of C/N ratio 50.

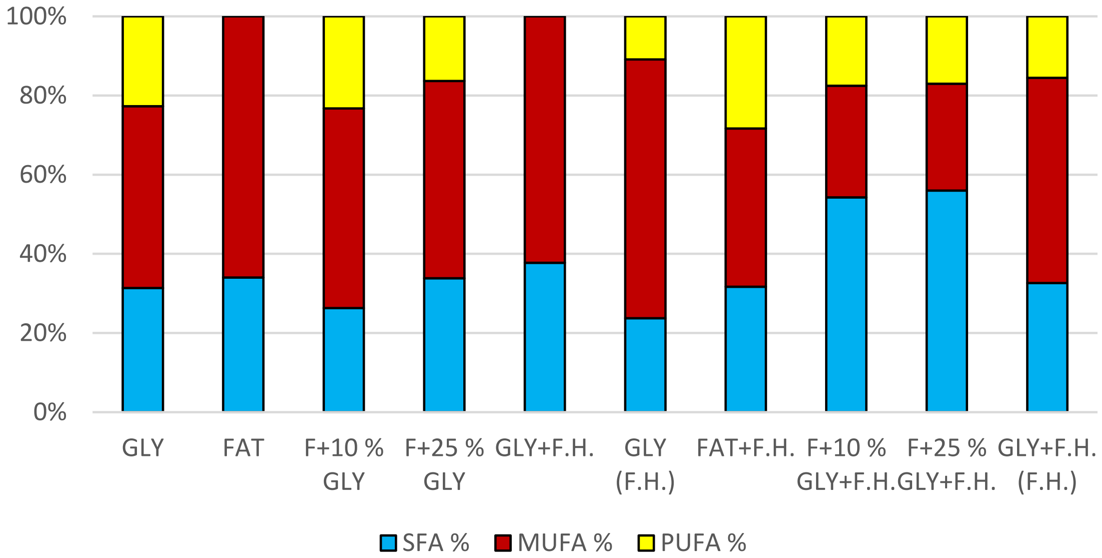

Figure 15. Fatty acid distribution in lipids of yeast strain *Rhodotorula mucilaginosa* in conditions of C/N ratio 50.

3.5. Flask Cultivations of Strain Rhodotorula mucilaginosa (CCY 20-9-7)

3.5.1. Results of C/N Ratio 25

Biomass production reached its two highest values in F + 25% GLY + F.H. (17.2 g/L) and GLY (F.H.) (17.8 g/L) media. The highest concentrations of carotenoids in the biomass were observed in cultivations in F + 10% GLY (1.324 mg/g) and GLY (2.241 mg/g) media. Ergosterol was observed in high concentrations in the biomass cultured in GLY (F.H.) (4.945 mg/g) and GLY + F.H. (F.H.) (4.969 mg/g) media. The highest ubiquinone productions were achieved in FAT + F.H. (5.418 mg/g) and GLY + F.H. (F.H.) (5.988 mg/g) media. The most significant lipid accumulation was achieved in the FAT medium (46.79%), but the highest production of SFA (2.97 g/L) was observed in the GLY (F.H.) medium and the highest production of MUFA (1.24 g/L) and PUFA (1.22 g/L) was recorded in cultivation in the F + 10% GLY + F.H. medium (Table S4, Supplementary).

3.5.2. Results of C/N Ratio 50

Significant biomass production values were achieved in F + 25% GLY + F.H. (24.8 g/L) and FAT + F.H. (26.1 g/L) media. The highest values of accumulated carotenoid concentrations were achieved in cultivation in GLY (1.187 mg/g) and F + 10% GLY (1.221 mg/g) media. Ergosterol was produced in greater quantities in FAT + F.H. (3.895 mg/g) and F + 10% GLY + F.H. (3.704 mg/g) media. Ubiquinone was produced on a greater scale in F + 10% GLY (6.084 mg/g) and FAT + F.H. (7.894 mg/g) media. A slightly increasing trend of lipid accumulation, depending on the decreasing concentration of fat in the media, is observable in the second series in media with mixed carbonaceous substrates. The highest accumulation of lipids (34.59%) and the highest production of SFA (4.66 g/L) were observed in the F + 25% GLY + F.H. medium. The highest production of MUFA (2.88 g/L) was recorded in the FAT + F.H. medium and the highest PUFA production (1.91 g/L) was observed in cultivation in the F + 10% GLY + F.H. medium.

3.6. Flask Cultivation of Strain Sporidiobolus metaroseus (CCY 19-6-20)

3.6.1. Results for C/N Ratio 25

The highest biomass productions were observed in the second series of cultivations in F + 10% GLY + F.H. (14 g/L) and F + 25% GLY + F.H. (15.2 g/L) media. Carotenoids were produced by biomass to the greatest extent in GLY (F.H.) (1.247 mg/g) and GLY + F.H. (F.H.) (1.675 mg/g) media. Ergosterol reached the highest productions in GLY + F.H. (3.757 mg/g) and GLY (4.295 mg/g) media. The most significant ubiquinone productions were observed in GLY (F.H.) (5.490 mg/g) and F + 25% GLY + F.H. (5.610 mg/g) media. In media with mixed carbon substrates of the second series, a trend of increasing biomass production and lipid accumulation can be observed with decreasing fat concentration in the media. The highest percentage content of lipids in the biomass (36.41%) and the highest production of SFA (2.81 g/L) and MUFA (1.70 g/L) were observed in the F + 25% GLY + F.H. medium. The highest production of PUFA (1.17 g/L) was observed in the F + 10% GLY + F.H. medium (Table S5, Supplementary).

3.6.2. Results for C/N Ratio 50

The highest biomass productions were achieved in F + 10% GLY + F.H. (11 g/L) and F + 25% GLY + F.H. (15.7 g/L) media. The most notable carotenoid productions were observed in GLY (F.H.) (0.931 mg/g) and GLY + F.H. (F.H.) (1.255 g/L) media. The highest productions of ergosterol were measured in the same media, 2.810 mg/g in GLY (F.H.) and 3.754 mg/g in GLY + F.H. (F.H.). Ubiquinone productions were the highest in F + 25% GLY + F.H. (7.482 mg/g) and GLY + F.H. (F.H.) (7.845 mg/g) media. There is a noticeable trend of an increasing biomass growth and at the same time a decreasing percentage of lipids in the biomass, depending on the decreasing concentration of fat in the media. This phenomenon is observable in media with a mixed carbon substrate in the second series. The highest percentage of lipids was observed on the FAT + F.H. (44.23%) medium. The highest production of all types of fatty acids was observed in the F + 25% GLY + F.H. (SFA 1.18 g/L, MUFA 2.18 g/L, PUFA 1.66 g/L) medium.

3.7. Flask Cultivation of Strain Sporidiobolus pararoseus (CCY 19-9-6)

3.7.1. Results of C/N Ratio 25

The most significant biomass growths were achieved in F + 25% GLY (15.9 g/L) and FAT + F.H. (16.1 g/L) media. The notable carotenoid productions were observed after cultivation in F + 10% GLY (1.845 mg/g) and GLY (2.690 mg/g) media. The concentrations of ergosterol in the biomass reached the highest values in GLY (F.H.) (8.231 mg/g) and GLY (9.247 mg/g) media. Ubiquinone was produced by biomass in the greatest values in FAT + F.H. (14.712 mg/g) and F + 10% GLY + F.H. (15.251 mg/g) media. In the second series of media with mixed carbonaceous substrates, a trend of decreasing lipid accumulation can be observed according to the decreasing fat content in the media. The highest accumulation of lipids (32.30%) and the production of SFA (2.48 g/L) and MUFA (1.85 g/L) were observed in cultivation in FAT + F.H. The highest production of PUFA (0.92 g/L) was observed in the F + 25% GLY + F.H. medium (Table S5, Supplementary).

3.7.2. Results of C/N 50

The most significant biomass productions were achieved in media of the second series, in F + 25% GLY + F.H. (28.2 g/L) and F + 10% GLY + F.H. (30.1 g/L). The culture achieved the highest production of carotenoids in GLY (F.H.) (1.496 mg/g) and F + 10% GLY + F.H. (2.425 mg/g) media. The highest values of ergosterol were achieved on GLY (7.181 mg/g) and GLY (F.H.) (14,365 mg/g) media. Significant ubiquinone productions were recorded in F + 25% GLY + F.H. (9.008 mg/g) and GLY (F.H.) (9.460 mg/g) media. In the first series, it can be observed that in media with a mixed carbon substrate, as the concentration of fat in the medium decreases, the production of biomass and accumulated lipids decrease slightly. The highest achieved percentage content of lipids in the biomass was observed in the FAT medium (35.13%). The highest production of all types of fatty acids was observed on the F + 10% GLY + F.H. (SFA 4.29 g/L, MUFA 4.04 g/L, PUFA 1.99 g/L) medium.

4. Discussion

Carotenogenic yeasts are ubiquitous microorganisms capable of utilizing various types of substrates and able to grow under various conditions. Their ability to accumulate lipids in higher amounts than 20% of the cell content places them in the group of oleaginous microorganisms. Many studies have confirmed their ability to utilize lipid substrates such as waste coffee oil, waste animal fat or waste frying oil [11,12]. According to these studies, this work focuses on the usability of poultry processing by-products, namely poultry fat and feathers, in media for the cultivation of carotenogenic yeasts. Carbon substrate was enriched with the addition of glycerol as a substrate that was easier to process in case of diauxia in order to provide to the yeast culture sufficient time to adapt to a more complex fatty substrate.

The data show that the significant producer of biomass at C/N 25 is the yeast *Rhodotorula mucilaginosa* (CCY 19-4-25), which showed the highest biomass growths in the FAT (29.5 g/L) and F + 10% GLY medium (24.8 g/L). At the same time the highest accumulations of carotenoids were recorded in GLY (10,469 mg/g) and F + 25% GLY (7.430 mg/g) media. The most significant accumulations of ergosterol were recorded in *Cystofilobasidium macerans* strains in the FAT + F.H. medium (9.932 mg/g) and *Sporidiobolus pararoseus* in the GLY medium (9.247 mg/g). The *Sporidiobolus pararoseus* strain showed the highest ubiquinone accumulation in F + 10% GLY + F.H. (15.251 mg/g) and FAT + F.H. (14.712 mg/g) media. The most accumulated lipids were recorded in the yeast culture *Rhodotorula mucilaginosa* (CCY 20-9-7) in the FAT medium (46.79%) and *Rhodotorula kratochvilovae* in the FAT + F.H. medium (40.82% with lipid production of 5.75 g/L). The highest lipid production was recorded in the yeast *Rhodotorula mucilaginosa* (CCY 19-4-25) in the FAT medium (7.52 g/L)

During cultivations at C/N 50, significant increases in biomass were observed in the yeast strain *Sporidiobolus pararoseus* in the F + 10% GLY + F.H. (30.1 g/L) medium and *Rhodotorula mucilaginosa* (CCY 19-4-25) in FAT + F.H. and F + 25% GLY + F.H. media (28.3 g/L). On the same strain, significant carotenoid production was also found in the GLY (F.H.) medium (6.044 mg/g) and F + 25% GLY + F.H. medium (5.613 mg/g). The most significant ergosterol productions were recorded in strains of *Sporidiobolus pararoseus* on the GLY (F.H.) medium (14.365 mg/g) and *Rhodotorula kratochvilovae* in the F + 10% GLY + F.H. medium (11,584 mg/g). In the *Rhodotorula kratochvilovae* strain, the most significant production of ubiquinone was observed in GLY + F.H. (F.H.) (15.505 mg/g) and GLY (F.H.) (13.149 mg/g) media. A significant accumulation of lipids and the percentage content of lipids in the biomass at the same time were observed in the strain *Rhodotorula mucilaginosa* (CCY 19-4-25) in the F + 10% GLY + F.H. medium (38.33% with a lipid production of 10.62 g/L). Another important producer of lipids was the strain *Rhodotorula kratochvilovae* on the F + 25% GLY + F.H. medium (with lipid accumulation 37.87%). The second most significant value of lipid production was recorded for the strain *Sporidiobolus pararoseus* in the F + 10% GLY + F.H. (10.32 g/L) medium.

The *Rhodosporidium toruloides* strain was selected for cultivation in the bioreactor. This strain was used as a comparative culture in the study [12] where the effect of the use of lipidic waste substrates on the growth of biomass in a bioreactor was tested. Similar conditions as in the study were induced in our bioreactor cultivation to provide the possibility to compare the behavior of the same genus of yeast on a different carbon and nitrogen substrate at the same C/N ratio. The study shows that the final biomass growth during cultivation in the bioreactor was 10.46 g/L after 96 h (the carbon source consisted of 25% hydrolysate from spent coffee grounds and 75% from coffee oil, with urea used as a nitrogen source). In the second case, where a mixture containing 25% of spent coffee ground hydrolysate and 75% of waste frying oil was used as a carbon source, the biomass growth was 11.75 g/L after 96 h of cultivation [12]. In the case of cultivation on glycerol and feather hydrolysate, the maximum growth in the biomass achieved at the same time interval as in the comparative study (0–96 h) was at the 72nd h of cultivation, with a growth of 20.51 g/L, which is 96.08% more compared to the first value and 74.55% more compared to the second value. The continuation of this cultivation up to 144th h resulted in a final biomass yield of 34.92 g/L. The better growth in our induced conditions may be explained by the easier utilization of glycerol by yeasts compared to the more complex sugar–lipid substrate. This experiment could serve as model for cultivation on F.H. with waste glycerol, which is by-product in biofuel processing.

The main goal of the work was to find out whether carotenogenic yeasts are capable of utilizing a complex substrate in the form of feather hydrolysate and poultry fat. This theory was confirmed, while very interesting results were achieved both from the point of view of the accumulation of intracellular metabolites and biomass growth, but also from the point of view of a new approach to these types of waste, which could be biotechnologically used to produce feed biomass, food, or food supplements using carotenogenic yeast cultivation in the future.

5. Conclusions

Feeding of poultry with red yeast biomass cultivated on waste poultry fat as the only carbon source and poultry feather hydrolysate as the only nitrogen source fully accepts the conception of the circular economy. Animals supplemented with enriched carotenogenic yeast biomass achieved better body growth and condition than animals whose diet was not supplemented with yeasts. Biomass or its components have the potential to be used not only in poultry biorefinery, but also in supplements for human nutrition, cosmetics, or pharmacy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11020321/s1, Tables: Table S1: Fatty acids content of poultry waste fat; Table S2: Results from analysis of biomass from first inoculum of yeast *strain Rhodosporidium toruloides*; Table S3: Results of cultivation of *Rhodotorula kratochvilovae* (1A) and *Cystofilobasidium macerans* (1B); Table S4: Results of cultivation of two different strains of *Rhodotorula mucilaginosa*: strain CCY 19-4-25 (2A) and strain CCY 20-9-7 (2B); Table S5: Results of cultivation of two different species of the *Sporidiobolus* genus: *Sporidiobolus metaroseus* (3A) and *Sporidiobolus pararoseus* (3B).

Author Contributions: Conceptualization, I.M., M.S. and J.H.; methodology, J.H., M.S., O.C. and P.S.; software, M.S. and J.H.; investigation, I.M., M.S., J.H. and P.S.; resources, I.M., M.S. and J.H.; data curation, M.S., J.H., P.S., O.C. and D.Š.; writing—original draft preparation, J.H., writing—review and editing, J.H. and I.M.; supervision, I.M. and M.S.; project administration, J.H. and P.S.; funding acquisition, I.M. All authors have read and agreed to the published version of the manuscript.

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Attachment 3

HOLUB, J.; CHUJANOV, O.; SZOTKOWSKI, M.; ŠIMANSKÝ, S.; DZURICKÁ, L.; MÁROVÁ, I.; SCHILDOVÁ, V. Optimization of cultivations of carotenogenic yeasts on poultry waste substrates. Proceedings of the 9 th International Conference on Chemical Technology. 1. Prague: 2022. p. 356 ISBN: 978-80-88307-11-2

OPTIMIZATION OF CULTIVATIONS OF CAROTENOGENIC YEASTS ON POULTRY WASTE SUBSTRATES

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Abstract

Carotenogenic yeast, which are also called oleaginous, are microorganisms capable of accumulating significant amounts of carotenoids, lipids, and glucans into their biomass. Their ability of processing waste materials and different carbon sources from various industries, such as agricultural industry, provides the possibility to use them for waste material treatment. This waste treatment process then could be used as a part of circular economy cycle. This research is focused on optimization of cultivation process in Erlenmeyer flasks by changing cultivation conditions and media composition as well. Research is focused on carotenogenic yeasts, especially the genus *Rhodotorula*, *Cystofilobasidium* and *Rhodosporidium* which have achieved significant results with interesting biomass growth (above 20 g/l). The optimization process is focused predominantly on mineral media with addition of animal by-products, namely poultry waste feathers and fat. Hydrolysed feathers play a major role as nitrogen source and waste fat serves as carbon source.

Introduction

Ensuring food security for the growing human population requires a continuous increase in the volume and efficiency of agricultural production and in the processing of food raw materials. One of the ways to make the food production process more efficient is to seek new methods of processing waste materials from food and agriculture industries via processes of circular economy[1]. The concept of circular economy within the food industry and agriculture includes more efficient handling of biomass and its reuse, for example in the form of nutritional substrates for animal and microorganisms in order to valorise food waste into valuable products.

Carotenogenic yeasts are heterotrophic, mainly aerobic ubiquitous microorganisms that contain many biologically active compounds within their cells, and they possess extracellular lipase activity, which allows them to process various sources of waste greasy substrates[3]. The concept of circular economy is applied to reuse waste substrates such as waste poultry fat or poultry feathers in pure or pre-treated form by cultivation of carotenogenic yeasts. Yeasts are capable of incorporating macroelements and microelements from complex waste substrates into biomass and through the biotransformation processes are capable of production of valuable metabolites such as carotenoids, ergosterol, ubiquinone, glucans, and lipids[4,5].

Study objectives

Study objectives were aiming on obtainment of information from cultivation of three carotenogenic yeasts strains *Rhodotosporidium toruloides*, *Rhodotorlula kratochvilovae* and *Cystofilobasidium macerans* on media with content of model and waste substrates with various ratios between them.

Those substrates were:

- Waste poultry fat
- Waste poultry feathers
- Purified glycerol (model for waste glycerol)

Materials and methods

Waste poultry feathers were pre-treated by organic solvent where the lipid content was extracted into nonpolar dissolvent. Feathers stripped of lipid content were dried and then hydrolysed by alkaline hydrolysis using 1% sodium hydroxide solution at the temperature 100 °C. Feather hydrolysate (FE) was added into cultivation media in crude form. Waste poultry fat did not require special pre-treatment due to the fact that carotenogenic yeasts possess natural lipase activity.

Carotenogenic yeasts were cultivated from stock cultures via solid YPD agar on Petri dishes and then via double inoculation in submerse YPD media in Erlenmeyer flasks. Main test cultivations were performed in Erlenmeyer flasks filled with artificial mineral media with addition of waste substrates to adjust to required value of C/N ratio. Glycerol added in media served as control carbon source and as model for usage of waste glycerol for cultivation purposes. Inoculation ratio from inoculation media into production media was 1:5. Production media were designed to contain the same mineral base but differed in types of carbon and nitrogen substrates while maintaining the same C/N ratio at the start of cultivation. All combinations of used media are described in Table I. Production cultivations persisted 96 hours and then were terminated. Biomass obtained from cultivations was subjected to gravimetric analysis to acquire information about its growth.

Table I
Distribution of experiments described by contain of carbon and nitrogen source

		1st series			
shortcut	GLY	FAT	0,1 GLY	0,25 GLY	GLY+FE
carbon source	100 % glycerol	100 % fat	90 % fat + 10 % glycerol	75 % fat + 25 % glycerol	100 % glycerol + FE
		2nd series			
shortcut	GLY (FE)	FAT+FE	0,1 GLY+FE	0,25 GLY+FE	GLY+FE
carbon source	100 % glycerol	100 % fat + F.H	90 % fat + 10 % glycerol + F.H	75 % fat + 25 % glycerol + F.H	100 % glycerol + F.H

Results and discussion

Rhodosporidium toruloides

The first of cultivated strain was yeast *Rhodosporidium toruloides* which, as it is showed on Figure 1., has significant growth of biomass on glycerol media in the first (20.0 g/l) and in the second series (17.8 g/l). This phenomenon could be used in cultivations with carbon source based on waste glycerol. In the second series of experiments (with FE) an increasing trend can be observed in the biomass growth based on increasing concentration of glycerol and on decreasing amount of added poultry fat.

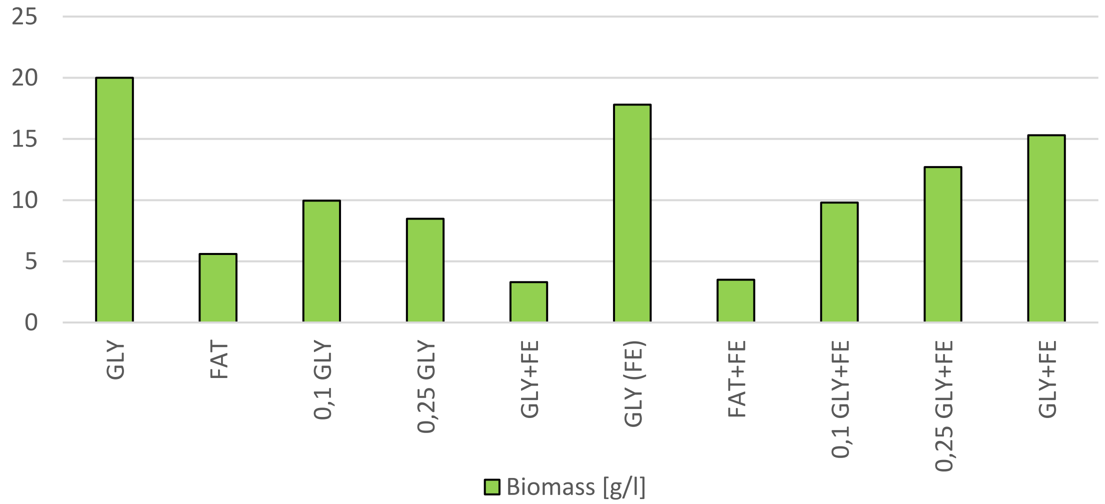

Figure 1. Biomass growth of strain *Rhodosporidium toruloides*

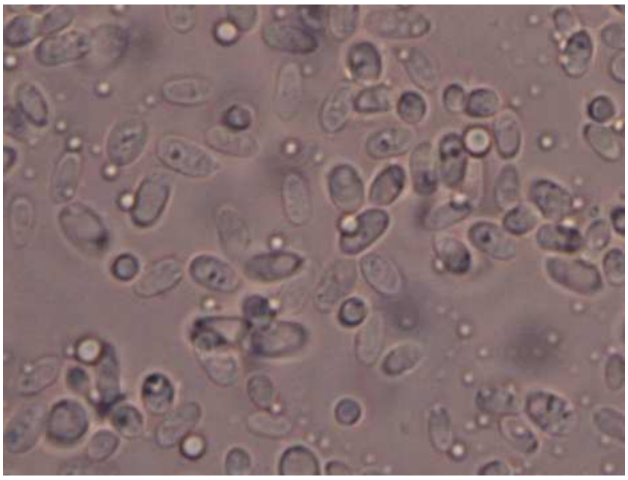

Figure 2. Microscopic image of yeast *Rhodosporidium toruloides* cultivated on waste fat

Rhodotorula kratochvilovae

The second cultivated strain was yeast *Rhodotorula kratochvilovae*. Growth and production parameters of the strain are showed in Figure 2. The largest biomass growth was observed on the medium with 25 % glycerol content and 75 % poultry fat content, with a value of 19.2 g/l followed the medium with 90 % poultry fat content where the biomass growth reached 17.7 g/l.

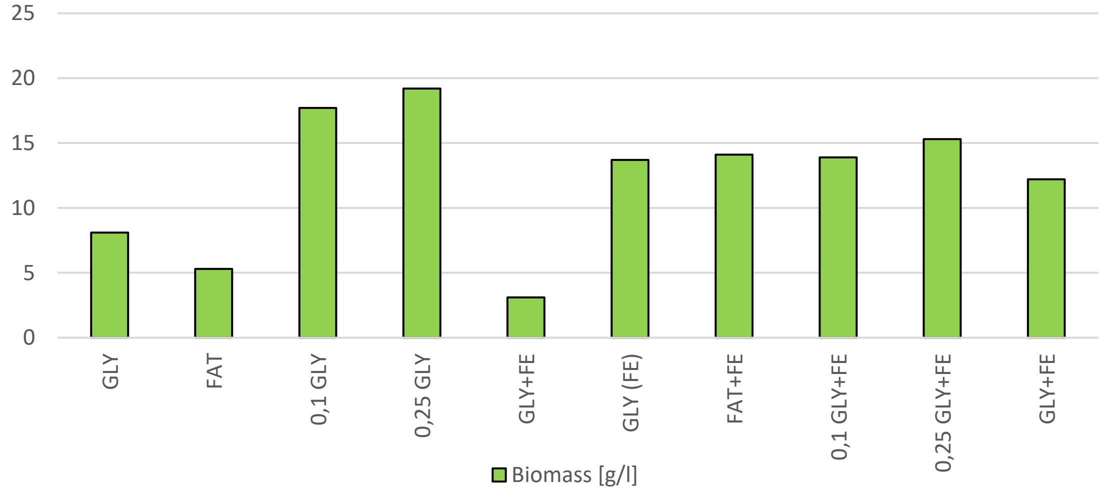

Figure 3. Biomass growth of strain *Rhodotorula kratochvilovae*

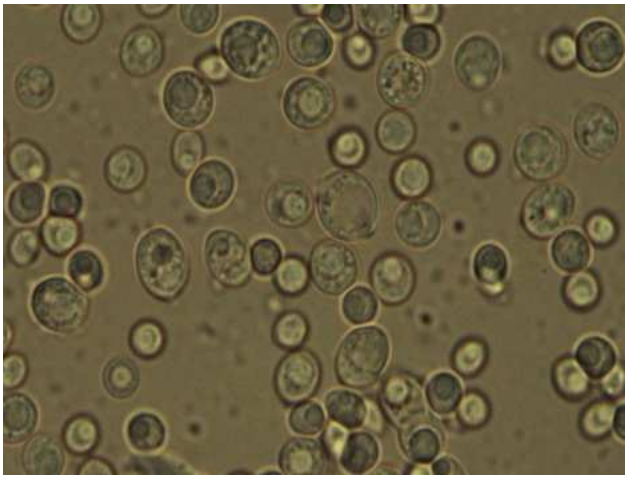

Figure 4: Microscopic image of yeast *Rhodotorula kratochvilovae* cultivated on waste fat

Cystofilobasidium macerans

The third cultivated strain was yeast *Cystofilobasidium macerans*, the results of which are presented in Figure 3. An increasing trend in biomass production can be observed in fat-based media of both series, depending on the decreasing concentration of waste fat in media. The highest biomass yields were achieved in glycerol media with the addition of feather hydrolysate in both series, in the 1st series 14.6 g/l, in the 2nd series 16.6 g/l.

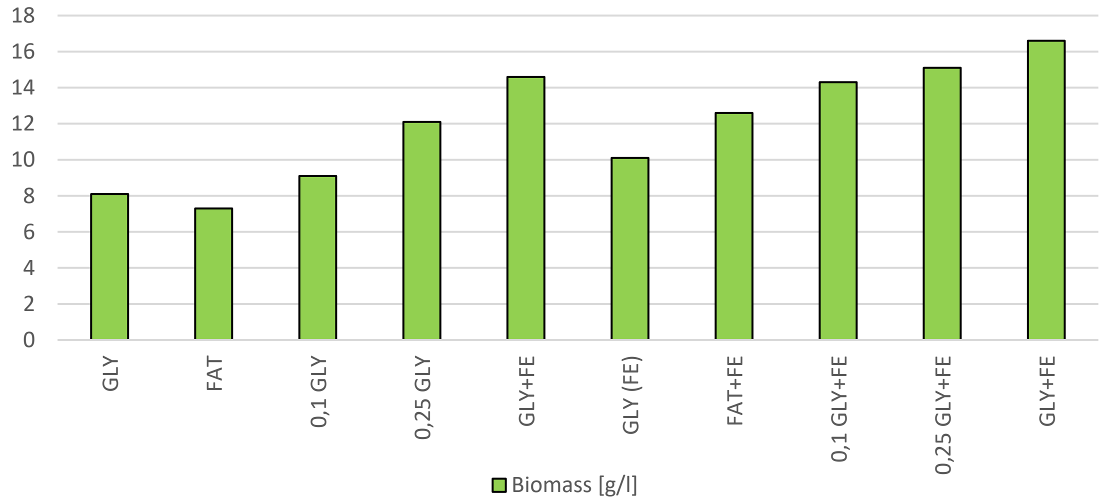

Figure 5. Biomass growth of strain *Cystofilobasidium macerans*

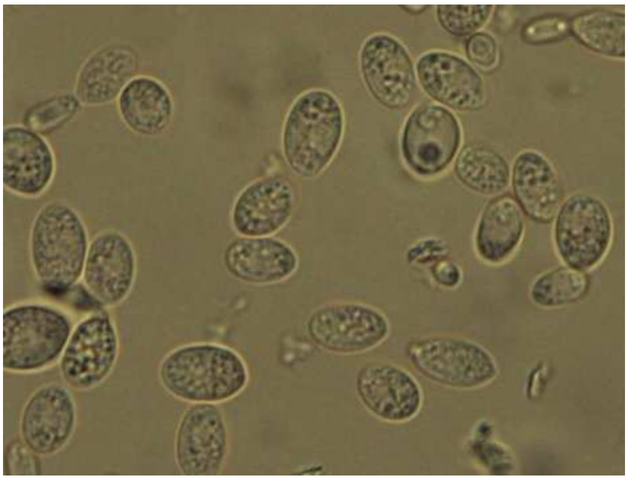

Figure 6. Microscopic image of yeast C*ystofilobasidium macerans* cultivated on waste fat

Conclusion

Cultivations of carotenogenic yeasts on media with added poultry waste materials showed interesting result with significant biomass growths (19.2 or 17.7 g/l). Cultivations on glycerol control media also indicated good results in biomass growth (up to 20.0 g/l) which could serve as a model for cultivations on waste glycerol from biofuels production.

In fat-based media, a favourable effect of reducing the ratio of fat:glycerol on biomass production was observed in almost all strains. This trend was mainly observed in cultivations with the addition of feather hydrolysate. In cultivation of strain *Cystofilobasidium macerans,* the addition of hydrolysate to the media had a significant beneficial effect on biomass production.

In summary, waste poultry fat and feathers can be valorised by carotenogenic yeasts to obtain valuable biomass. By using these waste materials for cultivation purposes, new possibility arises of using these materials for industrial optimization and production valuable biomass and metabolites.

According to results that were obtained from methods used in previous studies, further study is required for optimization of biomass growth and advanced methods implementation[3,5]. In the process of implementation of new methods, such as HPLC-PDA for carotenoid, ergosterol and ubiquinone detection and GC-FID for lipid detection, the preliminary data showed good results in carotenoid production (up to 10 mg/g of biomass) and significant lipid accumulation (up to 40 % of dry biomass).

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Attachment 4

HOLUB, J.; CHUJANOV, O.; ŠPAČKOVÁ, D.; ŠIMANSKÝ, S.; OBRAČAJ, J.; MÁROVÁ, I. CULTIVATION OF CAROTENOGENIC YEAST ON VARIOUS FOOD INDUSTRIAL WASTE SUBSTRATES. Vyšehradská 320/49 128 00 Prague 2 Czech Republic: AMCA, spol. s r.o., 2024. p. 201-201.

Cultivation of carotenogenic yeast on various food industrial waste substrates

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Carotenogenic yeasts are a group of yeasts that produce orange to red pigments in the form of carotenoids. They are also known for their ability to produce a significant amount of other bioactive substances, including ubiquinone, ergosterol, betaglucans, proteins and lipids. Their great biotechnological potential lies in the ability of utilizing many waste materials to produce biomass enriched by the bioactive substances under the biorefinery concept. The adaptability of carotenogenic yeasts allows them to use different waste substrates for their growth, e.g. oils and fats, chicken feather hydrolysate, lignocellulose materials and many others. Specifically, this study focuses on combined utilization of whey, waste poultry fat and waste frying oil as nutrition sources.

In this study, the yeast strains *Rhodotorula toruloides* and *Cystofilobasidium macerans* were studied. Batch cultivations were carried out in artificial media with the addition of waste substrates at different C/N ratios. In these experiments, the best yield was achieved by the strain *Cystofilobasidium macerans* on waste whey with the biomass yield 21.65 g/l. The strain *Rhodotorula toruloides* achieved an increased yield of biomass (14.35 g/l) on glycerol, which can serve as a model for waste glycerol obtained from biofuel production. The main goal of future research is to test also other red yeast strains such as *Phaffia* sp. and *Sporidiobolus* sp. in the presence of waste substrates and to scale-up these cultivations to the laboratory bioreactor.

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Attachment 5

HOLUB, J.; SZOTKOWSKI, M.; ŠIMANSKÝ, S.; HUBAČOVÁ, K.; PLHALOVÁ, Ž.; MÁROVÁ, I. Production of carotenoid and lipid enriched red yeasts biomass on a combination of whey and waste animal fatmal fat. Poland: Sciendo, 2021. p. 90-90.

Production of carotenoid and lipid enriched red yeasts biomass on a combination of whey and waste animal fat

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Carotenogenic yeasts are group of oleaginous microorganisms capable of utilizing various waste substrates from food and agricultural industry. They are well known for the production of carotenoids, lipids and other valuable metabolites, which can be further used in pharmaceutical, food and feed industry. Thus, enriched biomass produced on the waste substrates can be used as a part of circular economy model. In this work, four red yeast strains (Rhodotorula, Cystofilobasidium, and Sporobolomyces sp.) were tested on media containing whey and waste animal fat with different C/N ratios. Results show that all tested strains were able to utilize used waste substrates and produce high amounts of studied metabolites due to stress induction by waste substrates. Result show, that yeasts prefer lower C/N ratio for biomass and carotenoid production. On the other hand, higher lipid content was observed in C/N ratios above 50. In overall the best yeast genera was Rhodotorula, which were able to grow steadily on all media types and all C/N ratios.

Keywords: Red Yeasts, Waste Utilization, Circular Economy, Carotenoids, Lipids, Enriched Biomass

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The study of Obesity and Diabetes within patients with COVID-19 (on the example of Adjara (Georgia) population)

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The outbreak of the coronavirus disease 2019 (Covid-19) until now remains a problematic issue in the world. Obesity and Diabetes are considered significant risk factors for outcomes of COVID 19 Disease. There is a strong association between Diabetes and mortality, also post-infection complications. Therefore, in the present study, we investigated 100 hospitalized patients (above 50 years old, including both sex)with COVID-19. We also include significant clinic-laboratory investigations (including d-dimer, Ferritin, and C-reactive protein as well). For data analysis were used the Fisher's exact test, t-test, $P < 0.05$ consider a statistically significant issue for statistical analysis. Results: Our revealed that in our study population, according to Diabetes (considering both: insulin-independent, insulin-independent), the 89% of men and 74% of women have diabetes. As about differences between men and women, men have a high risk for Diabetes ($p=0.010$). Notably, all diabetic patients have significantly elevated d-dimer, Ferritin, and C-reactive protein levels ($p=0.0001$). As about obesity not revealed a significant difference between men and women. Conclusion. We have thought that understanding the relationships between obesity, Diabetes, and Covid-19 is significant for assessing patients' treatment. In particular, it is helpful to understand more about population risks passement that need more investigations

Keywords: Covid-19, Obesity, Diabetes

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