

IONIAN UNIVERSITY
DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY

“Microbial Production and Applications of Carotenoids”

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

FANI SERETI

ARGOSTOLI 2024

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**“Παραγωγή και Εφαρμογές Καροτενοειδών Μικροβιακής
Προέλευσης”**

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΦΑΝΗ ΣΕΡΕΤΗ

ΑΡΓΟΣΤΟΛΙ 2024

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Η έγκριση της διδακτορικής διατριβής από την επταμελή εξεταστική επιτροπή και από το Τμήμα Επιστήμης και Τεχνολογίας Τροφίμων του Ιονίου Πανεπιστημίου δεν υποδηλώνει απαραίτητως και αποδοχή των απόψεων του συγγραφέα.

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Fani Sereti

Η παρούσα διατριβή αποτελεί πρωτότυπο ερευνητικό έργο και δεν περιλαμβάνει στοιχεία προσβολής πνευματικής ιδιοκτησίας.

Οποιαδήποτε αναπαραγωγή, διανομή και παρουσίαση του έργου, και μόνο για μη εμπορικούς σκοπούς, προϋποθέτει τη μη-τροποποίηση του περιεχομένου και την ανάλογη αναγνώριση του συγγραφέα.

Φανή Σερέτη

Abstract

This thesis focuses on the production of microbial carotenoids using novel red yeast strains, coupled with efficient and environmentally friendly recovery methods, targeting high-value applications in food and pharmaceuticals. Carotenoids are bioactive compounds known for their vibrant color range and beneficial biological properties, making them especially valuable for the rapidly evolving food industry. Microbial production of carotenoids presents a stable, efficient, and environmentally friendly approach to carotenoid synthesis, prioritizing human health and sustainability. Additionally, microbial synthesis offers a broad spectrum of color options. To this end, four unexplored *Rhodospiridium* strains were initially evaluated for their ability to grow and synthesize carotenoids while assimilating various carbon sources.

More specifically, the first part of this thesis investigates the ability of two *Rhodospiridium kratochvilovae* isolates (Y-42 and Y-43) to produce carotenoids under different experimental conditions, such as carbon source and carbon-to-nitrogen (C/N) ratio. Among the tested conditions, glucose and a glucose-galactose mixture proved to be the most promising substrates not only for carotenogenesis but also for the synthesis of microbial oil, another valuable metabolite. Carotenoid synthesis was highly affected by C/N ratio in both strains, while a competitive trend with microbial oil accumulation was also observed. HPLC-DAD analysis of the obtained carotenoid extracts indicated that the main molecule was β -carotene (55-85%) regardless the fermentation conditions or the strain-employed.

The fermentation efficiency on glucose: galactose mixtures led to their replacement with hydrolyzed cheese whey permeate (CWP), as an alternative carbon source. Prior to fermentation, CWP was hydrolyzed either with commercial β -galactosidase or with a crude enzyme consortium. When CWP was hydrolyzed with β -galactosidase, strains *Rhodospiridium kratochvilovae* Y-42 and Y-43 were able to produce 1.87 and 1.64 mg/L of total carotenoids, respectively, while a microbial oil content of 19.7% and 23.9% (w/w) was also obtained. HPLC-DAD analysis of the chemical profile

indicated that β -carotene was the predominant carotenoid. Conversely, when CWP hydrolysis was carried out with crude enzyme consortia using fermented wheat bran, which also served as source of free amino nitrogen (FAN), both yeasts synthesized almost solely lycopene, and at the same time low levels of microbial oil were accumulated. To further elucidate the metabolic behavior of each strain, lycopene production was investigated under different C/FAN ratios and incubation temperatures. Under the optimized culture conditions (C/FAN 40 and 33 °C) yeast strain Y-42 produced 36.4 mg/L carotenoids, composed of more than 97% of lycopene, highlighting the substantial role of fermentation media in carotenoids synthesis and composition. Moreover, the lycopene-rich carotenoid extract exhibited antioxidant activity (IC_{50} equal to 0.89 mg/mL), signifying also the possibility to be further utilized as a functional additive in novel food formulations.

Another set of experiments was then carried out investigating the unexplored marine yeasts *Rhodospiridium paludigenum* 2663 and 2664 on different carbon sources (glucose, fructose, sucrose, mixture of glucose-galactose). Strain 2663 produced the highest titer of total carotenoids (2.21 mg/L) when cultivated on sucrose, together with 4 g/L microbial oil (30% w/w content). Exopolysaccharides (EPS) production was also detected when sucrose was used as the sole carbon source. In the case of strain 2664, glucose favored the production of carotenoids, reaching a value of 2.93 mg/L along with 1.57 g/L microbial oil (31.8% w/w content). Bioreactor fermentations were subsequently carried out using *Rhodospiridium paludigenum* 2663 either with sucrose or molasses as substrate to elucidate the feasibility of producing multiple metabolites. The fed-batch strategy with molasses yielded to the highest concentrations of all metabolites, with a carotenoid titer of 3.3 mg/L, microbial oil content of 50.7% w/w (10.9 g/L), and an EPS concentration of 3 g/L. Analysis of the carotenoids chemical profile during fermentation revealed that β -carotene was the predominant carotenoid. On the other hand, oleic acid was the main fatty acid in the produced microbial oil. The obtained carotenoid extracts exhibited antioxidant (IC_{50} 0.14 mg/mL) and antimicrobial activity, against common bacterial and fungal pathogenic strains.

The recovery of microbial oil and carotenoids produced by the yeast strain *Rhodospiridium paludigenum* 2663 was also evaluated using supercritical CO₂ extraction (SFE-CO₂), an environmentally friendly technique. Yeast biomass was subjected to six different cell disruption methods prior to SFE-CO₂ in order to assess the optimal pretreatment method. High speed homogenization emerged as the optimal pretreatment method, which when coupled with SFE-CO₂, resulted in an 80% recovery yield of total carotenoids and an 83% recovery yield of microbial oil. The use of ethanol as co-solvent was imperative for the efficient extraction of both metabolites.

Finally, potential applications of the produced carotenoid-rich microbial oil were unraveled by employing two different processes, namely electrospinning and oleogelation. Regarding encapsulation using electrospinning, the choice of encapsulation matrix significantly influenced the encapsulation efficiency (EE) of both carotenoids and microbial oil. The highest EE, 71.15% for microbial oil and 42.20% for carotenoids, were achieved when the EPS fraction was employed as a natural surfactant and incorporated in the encapsulating matrix. Regarding the oleogel-encapsulated microbial oil, a gradual degradation of carotenoids was observed during the oleogelation process. Ultimately, the oleogel retained 45% of the initial carotenoid concentration. Thermal stability of oleogels was investigated in the range of 50-90 °C targeting to assess the oil oxidation stability and carotenoids degradation. Thermal kinetics revealed that oil oxidation rate and carotenoids degradation rate were significantly lower in the oleogel-encapsulated microbial oil compared to the non-encapsulated oil. This proved that the oleogel served as a protective carrier for microbial oil and carotenoids under thermal processing conditions. Additionally, the physical properties of the oleogel showed that the oleogel had strong gel properties and good spreadability, supporting its potential for use in novel food formulations.

Keywords: *Rhodospiridium kratochvilovae*; *Rhodospiridium paludigenum*; Microbial carotenoids; β -Carotene; Lycopene; Microbial oil; Exopolysaccharides; Cheese whey; Molasses; Supercritical CO₂ extraction; Electrospinning; Oleogels

Περίληψη

Η παρούσα διατριβή εστιάζει στην παραγωγή μικροβιακών καροτενοειδών με τη χρήση νέων στελεχών ζυμών, σε συνδυασμό με αποτελεσματικές και φιλικές προς το περιβάλλον μεθόδους ανάκτησης, στοχεύοντας σε εφαρμογές υψηλής προστιθέμενης αξίας σε τρόφιμα αλλά και σε φαρμακευτικά προϊόντα. Τα καροτενοειδή είναι βιοδραστικές ενώσεις γνωστές για το ευρύ φάσμα χρωμάτων αλλά και για τις ευεργετικές βιολογικές τους ιδιότητες, γεγονός που τα καθιστά ιδιαίτερα πολύτιμα για την ταχέως εξελισσόμενη βιομηχανία των τροφίμων. Η μικροβιακή παραγωγή καροτενοειδών παρουσιάζει μια αποτελεσματική και φιλική προς το περιβάλλον προσέγγιση για τη σύνθεσή τους, δίνοντας προτεραιότητα στην ανθρώπινη υγεία και την περιβαλλοντική βιωσιμότητα. Επιπλέον, η μικροβιακή παραγωγή καροτενοειδών, αναλόγως της σύστασης, δύναται να προσφέρει ένα ευρύ φάσμα επιλογών χρωμάτων. Για το σκοπό αυτό, τέσσερα ελάχιστα μελετημένα στελέχη του γένους *Rhodospiridium* αξιολογήθηκαν αρχικά για την ικανότητά τους να αναπτύσσονται και να συνθέτουν καροτενοειδή αφομοιώνοντας διάφορες πηγές άνθρακα.

Αρχικά, η έρευνα επικεντρώθηκε στη διερεύνηση της ικανότητας δύο στελεχών *Rhodospiridium kratochvilovae* (Y-42 και Y-43) να παράγουν καροτενοειδή υπό διαφορετικές συνθήκες καλλιέργειας, όπως σε διαφορετικές πηγές άνθρακα και σε διαφορετικούς λόγους άνθρακα/αζώτου (C/N). Η γλυκόζη και το μείγμα γλυκόζης-γαλακτόζης αποδείχθηκαν τα πιο υποσχόμενα υποστρώματα για την παραγωγή καροτενοειδών αλλά και μικροβιακού λίπους. Η σύνθεση των καροτενοειδών επηρεάστηκε σε μεγάλο βαθμό από την αναλογία άνθρακα/αζώτου και στα δύο υπό μελέτη στελέχη, ενώ παρατηρήθηκε επίσης μια ανταγωνιστική τάση με τη συσσώρευση μικροβιακού ελαίου. Η ανάλυση των εκχυλισμάτων των καροτενοειδών έδειξε ότι κυρίαρχο ήταν το β-καροτένιο (55-85%), ανεξαρτήτως των συνθηκών ζύμωσης ή του στελέχους που χρησιμοποιήθηκε.

Η αποτελεσματικότητα των ζυμώσεων στο μίγμα γλυκόζης:γαλακτόζης οδήγησε στην μελέτη υδρολυμένου (και αποπρωτεϊνομένου) τυρογάλακτος ως εναλλακτική πηγή άνθρακα. Πριν από τη ζύμωση, το τυρόγαλα υπέστη υδρόλυση είτε με τη χρήση

εμπορικής β-γαλακτοσιδάσης είτε με τη χρήση ακατέργαστων ενζύμων. Στην περίπτωση που για την υδρόλυση χρησιμοποιήθηκε η εμπορική β-γαλακτοσιδάση, τα στελέχη *Rhodospiridium kratochvilovae* Y-42 και Y-43 παρήγαγαν 1,87 και 1,64 mg/L συνολικών καροτενοειδών, αντίστοιχα, με την ταυτόχρονη συσσώρευση 19,7% και 23,9% (w/w) μικροβιακού λίπους. Η ανάλυση του προφίλ των παραγόμενων καροτενοειδών έδειξε ότι το β-καροτένιο ήταν το κύριο καροτενοειδές. Αντιθέτως, όταν η υδρόλυση του τυρογάλακτος έγινε με ακατέργαστα ένζυμα από ζυμούμενο πίτουρο σιταριού, το οποίο επίσης λειτούργησε ως πηγή αζώτου (FAN), και τα δύο στελέχη παρήγαγαν σχεδόν αποκλειστικά λυκοπένιο, ενώ μειώθηκε σημαντικά η σύνθεση μικροβιακών λιπιδίων. Η παραγωγή λυκοπενίου διερευνήθηκε περαιτέρω υπό διαφορετικές αναλογίες άνθρακα/αζώτου και θερμοκρασίες επώασης. Υπό τις βέλτιστες συνθήκες καλλιέργειας (λόγος άνθρακα/αζώτου 40 και 33 °C), το στέλεχος Y-42 παρήγαγε 36,4 mg/L καροτενοειδών, με το 97% να είναι λυκοπένιο, αναδεικνύοντας τον καθοριστικό ρόλο του υποστρώματος στη σύνθεση και σύσταση των καροτενοειδών. Επιπλέον, το εκχύλισμα καροτενοειδών παρουσίαζε υψηλή αντιοξειδωτική δράση, υποδεικνύοντας τη δυνατότητα περαιτέρω χρήσης του ως πρόσθετου σε καινοτόμα τρόφιμα.

Επιπρόσθετα, δύο νέα στελέχη ζυμομυκήτων, τα *Rhodospiridium paludigenum* 2663 και 2664, μελετήθηκαν χρησιμοποιώντας διαφορετικές πηγές άνθρακα (γλυκόζη, φρουκτόζη, σακχαρόζη, μείγμα γλυκόζης-γαλακτόζης). Το στέλεχος 2663 παρήγαγε τη μεγαλύτερη συγκέντρωση καροτενοειδών (2,21 mg/L) όταν αναπτύχθηκε σε σακχαρόζη, ενώ παράλληλα παρήγαγε και 4 g/L μικροβιακού ελαίου (30%, w/w). Μάλιστα, το ίδιο στέλεχος ήταν ικανό να παράγει και εξωπολυσακχαρίτες, όταν χρησιμοποιήθηκε η σακχαρόζη ως πηγή άνθρακα. Στην περίπτωση του στελέχους 2664, η γλυκόζη ευνόησε την παραγωγή καροτενοειδών επιτυγχάνοντας συγκεντρώσεις της τάξεως των 2,93 mg/L μαζί με 1,57 g/L μικροβιακού ελαίου (31,8%, w/w). Ως εκ τούτου, πραγματοποιήθηκαν ζυμώσεις σε βιοαντιδραστήρα χρησιμοποιώντας είτε σακχαρόζη είτε μελάσα με το στέλεχος 2663, για την περαιτέρω διερεύνηση της δυνατότητας παραγωγής των εν λόγω μεταβολιτών. Η στρατηγική καλλιέργειας ημι-συνεχούς λειτουργίας με μελάσα ως υπόστρωμα οδήγησε στην παραγωγή των υψηλότερων συγκεντρώσεων όλων των μεταβολιτών, με τα καροτενοειδή να ανέρχονται στα 3,3 mg/L, ενώ η περιεκτικότητα σε

μικροβιακό έλαιο έφθασε στο 50,7% (w/w), και η συγκέντρωση των εξωπολυσακχαριτών στα 3 g/L. Η ανάλυση του προφίλ των καροτενοειδών έδειξε ότι το β-καροτένιο ήταν το κύριο καροτενοειδές, ενώ το ελαϊκό οξύ ήταν το κύριο λιπαρό οξύ του παραγόμενου μικροβιακού ελαίου. Τα εκχυλίσματα καροτενοειδών που προέκυψαν έδειξαν υψηλή αντιοξειδωτική δράση, καθώς επίσης και αντιμικροβιακή δράση έναντι κοινών βακτηριακών και μυκητιακών παθογόνων στελεχών.

Στη συνέχεια, αξιολογήθηκε η ανάκτηση του μικροβιακού ελαίου και των καροτενοειδών από το στέλεχος *Rhodospiridium paludigenum* 2663 χρησιμοποιώντας εκχύλιση με υπερκρίσιμο CO₂ (SFE-CO₂), μια μέθοδο φιλική προς το περιβάλλον. Πριν από την εκχύλιση, η βιομάζα υποβλήθηκε αρχικά σε έξι διαφορετικές μεθόδους διάρρηξης των κυττάρων, με την ομογενοποίηση υψηλών ταχυτήτων να αναδεικνύεται ως η βέλτιστη μέθοδος προ-επεξεργασίας, η οποία σε συνδυασμό με τη μέθοδο (SFE-CO₂), οδήγησε σε αποδόσεις ανάκτησης των καροτενοειδών της τάξεως του 80% και σε ανάκτηση 83% του μικροβιακού ελαίου. Η χρήση αιθανόλης ως συν-διαλύτη ήταν καθοριστικής σημασίας για την αποδοτική ανάκτηση και των δύο μεταβολικών προϊόντων.

Στο τελευταίο στάδιο της παρούσας διδακτορικής διατριβής εξετάστηκαν πιθανές εφαρμογές του πλούσιου σε καροτενοειδή μικροβιακού ελαίου, μέσω της αξιολόγησης δύο μεθόδων ενθυλάκωσης: της ηλεκτρονηματοποίησης και των ελαιοπηκτών. Όσον αφορά τη διαδικασία ηλεκτρονηματοποίησης, η επιλογή των συστατικών της μήτρας ενθυλάκωσης επηρέασε σημαντικά την αποδοτικότητά της ενθυλάκωσης. Οι μεγαλύτερες αποδόσεις ενθυλάκωσης για το μικροβιακό έλαιο και τα καροτενοειδή ήταν 71,15% και 42,20%, αντίστοιχα, όταν χρησιμοποιήθηκαν οι εξωπολυσακχαρίτες στη μήτρα ενθυλάκωσης. Όσον αφορά την ελαιοπηκτή που αναπτύχθηκε με το μικροβιακό έλαιο, παρατηρήθηκε σταδιακή μείωση της περιεκτικότητας σε καροτενοειδή κατά τη διαδικασία της παραγωγής της ελαιοπηκτής, η οποία τελικά διατήρησε το 45% της αρχικής συγκέντρωσης των καροτενοειδών. Η μελέτη της θερμικής σταθερότητας του μικροβιακού ελαίου και των καροτενοειδών διερευνήθηκε στην περιοχή 50-90 °C, με στόχο την αξιολόγηση της σταθερότητάς τους. Η εφαρμογή κινητικών μοντέλων έδειξε ότι οι ρυθμοί οξείδωσης του ελαίου και αποικοδόμησης των καροτενοειδών ήταν σημαντικά χαμηλότεροι στην ελαιοπηκτή σε σύγκριση με το μη ενθυλακωμένο μικροβιακό

έλαιο. Ως εκ τούτου, η ελαιοπηκτή λειτούργησε ως προστατευτικός φορέας τόσο του μικροβιακού ελαίου όσο και των καροτενοειδών υπό συνθήκες θερμικής επεξεργασίας. Επιπλέον, η ανάλυση των φυσικών ιδιοτήτων ανέδειξε την υψηλή ικανότητα επάλειψης και την υψηλή μηχανική αντοχή της ελαιοπηκτής, υποστηρίζοντας περαιτέρω την δυνατότητα εφαρμογής της για την ανάπτυξη καινοτόμων προϊόντων τροφίμων.

Λέξεις-κλειδιά: *Rhodosporidium kratochvilovae*; *Rhodosporidium paludigenum*; Μικροβιακά καροτενοειδή; β-Καροτένιο; Λυκοπένιο; Μικροβιακό έλαιο; Εξωπολυσακχαρίτες; Τυρόγαλα; Μελάσα; Εκχύλιση με υπερκρίσιμο CO₂; Ηλεκτρονηματοποίηση; Ελαιοπηκτές

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CHAPTER 1

Introduction

1 Introduction

The field of industrial biotechnology deals with the utilization of biotechnological tools to enhance industrial processes and to produce bio-based products, including fuels, chemicals, and plastics from renewable resources (Erickson et al., 2012). Microorganisms constitute the base of this field to create a range of innovative technologies and processes. These advancements are being actively pursued by companies, researchers, and scientists, targeting commercial applications. There are currently numerous examples of industrialized microbial processes for the production of different goods belonging to the food, chemical and pharmaceutical sectors. The more the knowledge on microbial metabolic systems is expanding, the broader the variety of products, that can be developed. Microbial processes confer multiple advantages when compared to chemical routes, offering faster and environmentally sustainable routes for production, while minimizing the dependence on fossil resources. In addition, biotechnological products are either recyclable or biodegradable and their synthesis leads to the production of less toxic waste while using milder conditions. Carotenoids are molecules of broad color spectrum naturally synthesized by many living organisms (*e.g.* plants, microorganisms) or can be synthesized by chemical processes (Papapostolou et al., 2023). According to Markets and Markets, (2024), the global carotenoid market is projected to grow by 6.5% during the forecast period of 2023-2029. The carotenoids market was valued at USD 2.3 billion in 2023, increased to USD 2.5 billion in 2024, and is anticipated to reach USD 3.4 billion by 2029. Increasing consumer awareness of health and wellness, combined with the rising demand for natural, clean-label products, is driving the continued expansion of the carotenoid market (Alexandri et al., 2022). Based on findings from Sensient's U.S. an online consumer study conducted from 2015 to 2020, indicated that approximately one-third of consumers preferred natural foods. This tendency increased to 50% within a single year (2020), probably due to COVID-19 pandemic. Additionally, nearly half of European consumers prefer to avoid products containing artificial ingredients (Sencient Food Colors, 2020). **Figure 1.1** illustrates the global demand for carotenoids from 2023 to 2029. Specifically, while Europe is currently the largest carotenoid market (42.1%), the Asia-Pacific region is expected to see the highest growth in carotenoid demand, with an anticipated CAGR of 6.7% until 2029 (MarketsandMarkets, 2024). β -carotene is projected to hold the largest revenue

share among carotenoids, accounting for 24.7% in 2023 (Grand View Research, 2023a).

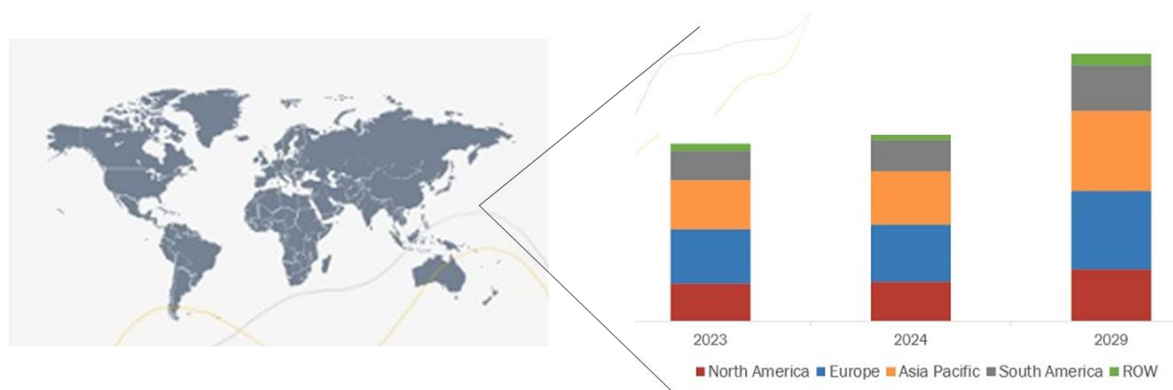


Figure 1.1 Global carotenoid market demand (MarketsandMarkets, 2024).

To date, synthetic carotenoids are still utilized in food applications. However, the growing demand for functional foods is expected to drive the incorporation of natural carotenoids into food formulations. More specifically, while the current market value of natural carotenoids stands at USD 878 million, it is projected to reach USD 953 million by 2034 (Fact.MR, 2023). **Figure 1.2** presents the global distribution of functional foods across various food application sectors. Functional dairy products accounted for the largest market share in 2021 (39%), followed by the bakery sector. The incorporation of carotenoids into food systems is projected to grow at a CAGR of 7.4% by 2034. Nevertheless, regulations play a crucial role in governing the inclusion of carotenoids in food applications. In the European Union, the use of carotenoids in food and feed is controlled by Regulation (EC) No 1333/2008 on food additives, which stipulates strict safety assessments and labeling requirements.

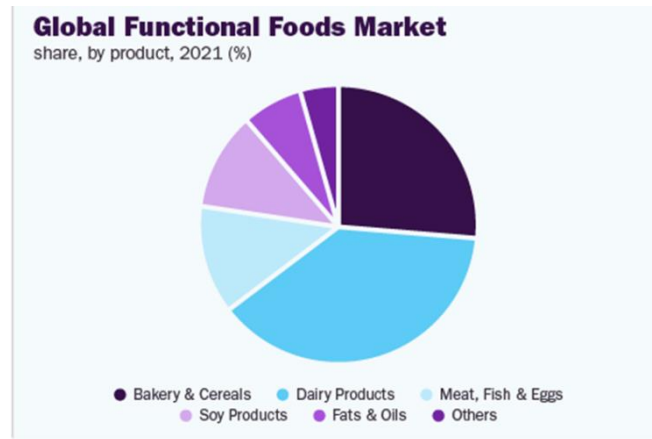


Figure 1.2 Global functional foods market share by food sectors (Grand View Research, 2023b).

The utilization of renewable resources could further enhance the production of natural carotenoids by improving the cost-competitiveness of their production while also minimizing the environmental impact associated with food waste disposal (Mehri et al., 2021). It is estimated that one-third of all food produced, equivalent to 1.3 billion tons, results in food loss and waste caused about USD 940 billion per year in economic losses. Therefore, global regulations have emerged to reduce the disposal of food waste (European Commission, 2019). More specifically, the Sustainable Development Goals (SDGs), also referred to as the Global Goals, are a set of objectives established through global agreements to safeguard the planet's habitability, reduce poverty, and ensure that people can live in peace and prosperity, both now and in the future (Morton et al., 2017). SDG 12 focuses on promoting sustainable consumption and production patterns, with Target 12.3 emphasizing that “By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses”.

CHAPTER 2

Natural Carotenoids

2 Natural Carotenoids

2.1 Introduction

Carotenoids are regarded as the second most abundant pigments in nature, after chlorophyll, displaying a wide color spectrum ranging from yellow to red. It was not until the early 19th century, however, that carotenoids were recognized as the compounds responsible for the coloration of many food materials (Britton, 2020). In 1817, the first carotenoid was discovered in paprika. The term 'carotenoid' was initially used following the discovery of carotene in carrots in 1831 (Maoka, 2020; Zia-Ul-Haq et al., 2021). To date, over 1,200 different carotenoids have been documented, derived from more than 722 distinct sources (Yabuzaki, 2017).

2.2 Structure

Carotenoids are bioactive compounds that constitute one of the most various classes of secondary metabolites called tetraterpenoids. They consist of eight isoprene units forming a 40-carbon skeleton ($C_{40}H_{64}$); thus carotenoids belong to the isoprenoid subfamily (Antolak et al., 2017). According to their chemical composition, carotenoids are classified in carotenes and xanthophylls. Carotenes are solely non-oxygenated hydrocarbons, like lycopene, β -carotene and torulene whereas xanthophylls contain oxygen in their skeleton such as lutein, astaxanthin and torularhodin. Their structure of long polyene chain contains 3-15 conjugated double bonds. Depending on the presence of end rings, carotenoids are also classified as cyclic such as β -carotene and astaxanthin or acyclic like lycopene (Yabuzaki, 2017).

Figure 2.1 presents the structure of several carotenoids.

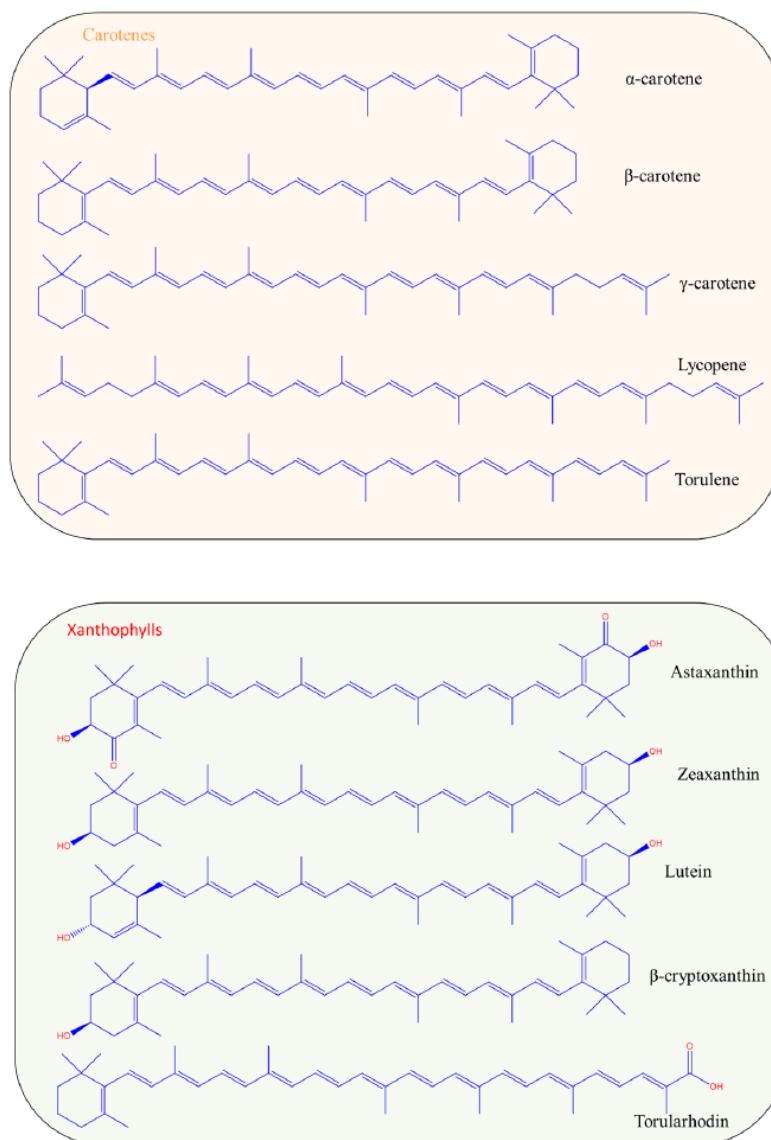


Figure 2.1 Chemical structure of some natural carotenoids (Papapostolou et al., 2023).

2.3 Natural role of carotenoids

Carotenoids are one of the most widespread lipid-soluble pigments in nature. Plants are considered to be the dominant source of natural carotenoids with occurrence in leaves, roots, seeds, fruits and vegetables. However, a plethora of carotenoids could also be biotechnologically produced from microorganisms. Microbial carotenoids could be synthesized from microalgae, bacteria, fungi and yeasts (Langi et al., 2018). The main natural role of carotenoids is to act as antioxidants due to the presence of conjugated double bonds on their skeleton. In photosynthetic organisms like plants, microalgae and phototropic bacteria, carotenoids absorb visible light resulting in a

wide coloration from yellow to red. Moreover, they act as a first line defense mechanism protecting the cells by scavenging singlet oxygen species within the chloroplast (Sun et al., 2022). Regarding the non-photosynthetic microorganisms, carotenoids provide a photoprotective effect against oxidative damage caused by unfavorable conditions (Mata-Gómez et al., 2014).

2.4 Biosynthesis

Biosynthesis of carotenoids occurs through two different metabolic pathways depending on the substrate provided by the central carbon pathway. Plants and most bacteria produce pyruvate and glyceraldehyde-3-phosphate (G3P) which are utilized as substrates for the 2-C-methyl-D-erythritol-4 phosphate (MEP) pathway (Sathasivam et al., 2021), while the mevalonic (MVA) pathway requires acetyl-CoA derived from the metabolism of eukaryotes and archaea (Mata-Gómez et al., 2014) (**Figure 2.2**). Both biosynthetic pathways include a range of enzymatic reactions leading to the production of the universal isoprene (C₅) precursor of carotenogenesis; the isopentyl pyrophosphate (IPP). The final steps are identical between these two metabolic routes. Three IPP molecules and one molecule of its allylic isomer dimethylylallyl pyrophosphate (DMAPP) are required for the formation of geranylgeranyl pyrophosphate (GGPP) which is considered as the second precursor during carotenoid synthesis (Papapostolou et al., 2023). Two molecules of this compound lead to the formation of the C₄₀ carotenoid phytoene. After four steps of dehydrogenation catalyzed by phytoene desaturase, lycopene is finally formed (Hong et al., 2019). A combination of different enzymatic reactions could result in a wide range of carotenoids depending on the natural source and/or the strain of each microorganism. For instance, the addition of one or two β - end rings through the activity of lycopene β -cyclase converts lycopene either to γ -carotene or β -carotene (Kot et al., 2018). It is worth mentioning that the utilization of yeasts as carotenoid producers offers an additional route for lycopene towards two “unusual” carotenoids: torulene and torularhodin (Kot et al., 2018).

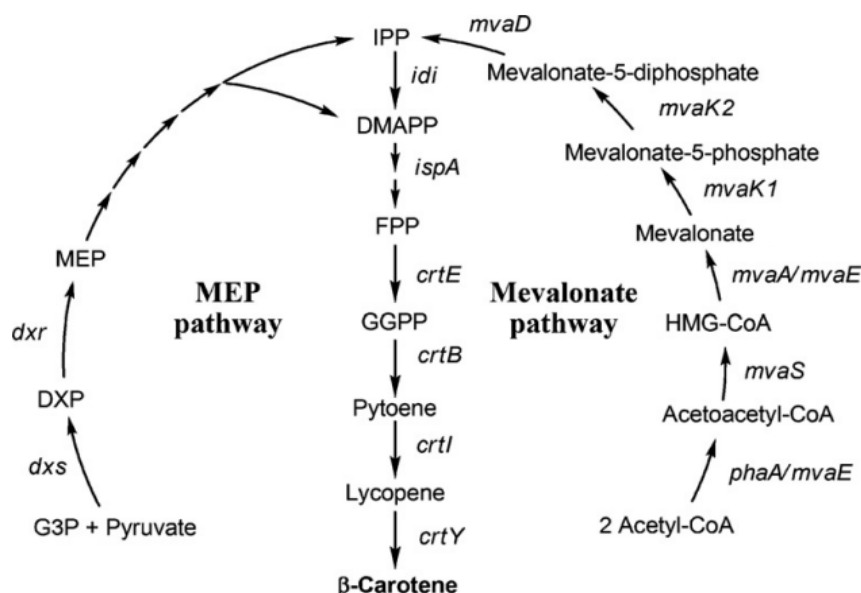


Figure 2.2 Biosynthesis of β -carotene via both the MEP and MVA pathway (Yoon et al., 2009).

2.5 Properties

2.5.1 Vitamin A activity

Vitamin A consists of an unsaturated hydrocarbon group containing retinol and its derivatives like retinoic acid, retinaldehyde and retinyl ester and is considered as an essential vitamin (Han et al., 2022). Besides the beneficial effect on eyesight, vitamin A also promotes embryonic development via modulating gene expression and could also influence both cellular growth and differentiation (Khan et al., 2023). Vitamin A must be obtained from the diet due to human's body incapability to synthesize retinol. Natural carotenoids are known for their vitamin A activity as they are retinol precursors (Kelly et al., 2018). Carotenoids possessing provitamin A activity are characterized by the presence of at least one β -ionone ring in their polyene skeleton along with the appropriate number and position of methyl groups. β -carotene, α -carotene and β -cryptoxanthin are the main retinol precursors as, human intestinal cells are able to convert them into two retinol molecules by the action of β - β -carotene-15,15'-monooxygenase 1 (BCMO1) in the presence of oxygen (Eroglu and Harrison, 2013). β -carotene, which accounts for the main vitamin A precursor, is abundant in natural foods such as carrots, spinach, broccoli, sweet potatoes and cantaloupe (Gul et al., 2015; Li et al., 2023).

2.5.2 Antioxidant activity

Natural carotenoids, as it is already mentioned, possess a key role as antioxidants due to their ability to diminish the damage induced by reactive oxygen species (ROS). The oxidative stress could lead to a variety of chronic and inflammatory diseases by affecting the cell cycle's regulation steps and as a consequence, cell proliferation (Ribeiro et al., 2018). Lycopene, astaxanthin and β -carotene are considered as strong free radical scavengers due to their higher amount of double conjugated bonds on their skeleton (Rizzardi et al., 2022). Mussagy et al., (2022) indicated that 100 $\mu\text{g/mL}$ astaxanthin synthesized by *Phaffia rhodozyma* NRRL Y-17268 exhibited strong antioxidant capacity as indicated by the DPPH free radical method. Additionally, pumpkin peel extract consisting mainly of β -carotene also presented great antioxidant activity with a value of 1049.47 μg Trolox equivalent per g of dry weight (Pinna et al., 2023). Elkholy et al., (2023) measured the antioxidant activity of β -carotene extracted from carrots and a value of 4.06 μM Trolox equivalent per μM carotenoid was obtained. Recent studies designate that the presence of different carotenoids on the extract could further enhance their ability to function as antioxidants (Böhm, 2017). The combination of β -carotene and astaxanthin extracted from *Rhodotorula mucilaginosa* CCT 7688 strain presented strong antioxidant activity (Cipolatti et al., 2019).

2.5.3 Anti-inflammatory activity

Another critical function of natural carotenoids is their interaction with anti-inflammatory defense mechanisms, which might contribute to the inhibition of various acute and chronic inflammatory diseases in humans, including asthma, atherosclerosis, Type 2 diabetes, and rheumatoid arthritis. The regulation of inflammatory processes is largely dependent on the balance between M1 and M2 macrophage phenotypes, which exerts opposing functions (Aharoni et al., 2015). M1 macrophages are activated by interferon γ (INF- γ) and lipopolysaccharides (LPS), leading to the production of pro-inflammatory cytokines such as interleukin 1 β and 6 (IL-1 β , IL-6) and tumor necrosis factor α (TNF- α) as well as the inflammatory mediators prostaglandin E_2 (PGE $_2$) and nitric oxide (NO), synthesized by cyclooxygenase (COX) and inducible nitric oxide (iNOS), respectively (Bai et al., 2005). Additionally, nuclear factor κB (NF κB) further promotes inflammation by

enhancing the production of pro-inflammatory cytokines in macrophages. Alternatively, M2 macrophages are responsible for the production of anti-inflammatory cytokines, such as interleukin 10 (IL-10) (Farruggia et al., 2018). Moreover, nuclear factor E2-related factor 2 (NFR2) is responsible for the inhibition of NFκB (Gorrini et al., 2013). The presence of carotenoids has been associated with the activation of the NFR2 factor, as well as with the suppression of several pro-inflammatory cytokines and inflammatory mediators. Farruggia et al., (2018) demonstrated that astaxanthin could enhance the nuclear translocation of NFR2 in LPS-stimulated macrophages, thereby promoting the switch of macrophage phenotypes from M1 to M2 in obese mice. Astaxanthin exhibited an inhibitory effect on inflammatory regulators iNOS and COX when added to LPS-stimulated BV-2 microglial cells (Park et al., 2015). It also contributed to the reduction of NFκB and the pro-inflammatory cytokines TNF-α and IL-1β, leading to decreased liver injury in rats (Zhang et al., 2020). According to Hazewindus et al., (2012), lycopene derived from tomato-based foods could effectively reduce inflammation by decreasing nuclear NFκB and inhibiting TNF-α, while simultaneously stimulating IL-10. Furthermore, both lycopene and β-carotene were shown to inhibit the expression of LPS-induced *cox* mRNA in RAW 264.7 macrophages (Kawata et al., 2018). Additionally, Bai et al., (2005) demonstrated that β-carotene could inhibit the expression of pro-inflammatory cytokines, including IL-1β and TNF-α, as well as the activation of NFκB.

2.5.4 Anti-cancer activity

Most notably, natural carotenoids seem to present a positive correlation also on cancer prevention, especially against gastric (Kim et al., 2018), colon (Cheng et al., 2021), prostate (Van Hoang et al., 2018), breast (Sinha et al., 2023) and liver cancer (Thomas et al., 2020). Their anti-cancer effect is based on their ability to regulate the expression of proteins involved in cell proliferation by altering several signaling pathways (Gong et al., 2018). According to Sowmya et al., (2017) the majority of MCF-7 breast cancer cells were rested at G0/G1 phase when treated with a mixture of natural carotenoids composed of astaxanthin, β-carotene and lutein. The presence of carotenoids was able to downregulate the cell phase-related protein cyclin D1 and the Bcl-2 protein which is responsible for the suppression of programmed cell death.

Concurrently, the apoptosis of MCF-7 cells was triggered by inducing the expression of p53 and Bax proteins which refer to apoptosis cytokines. Similar pattern was observed by Han et al., (2019) when the growth of AGS gastric cancer cell line was reduced after treatment with lycopene. The cell proliferation was eliminated by the decrease of Bcl-2 protein while the rate of cell apoptosis was enhanced through the increase of cleaved caspase-3 and caspase-9 and of the protein Bax concentration. The A549 lung cancer cell line was also arrested at G0/G1 phase after treatment with lutein, while the p53 signaling pathway was upregulated (Zhang et al., 2023). Zeaxanthin could provoke the arrest of AGS gastric cancer cells at G2/M phase as well as the enhancement of their apoptotic rate by inducing the proteins Bax, p38 and cleaved caspase-3 and suppressing the Bcl-2, STAT3 and NF- κ B proteins (Sheng et al., 2020).

2.6 Industrial utilization of natural carotenoids

In view of the aforementioned profits of natural carotenoids, their ever growing market demand in various applications is evident. Total carotenoids are anticipated to yield 3.4 Billion USD until the end of 2032 while the estimated CAGR would be around 6.3% during the forecasted period of 2023-2032 (Market.us Report, 2023). Carotenoids could be utilized as food and feed additives, as ingredients of pharmaceutical and cosmetic products as well as dietary supplements. The food and beverage industry has already been emerged as the prevailing field for carotenoids applications considering the ever increasing market demand for functional food products (Custom Market Insights, 2023).

Chemically synthesized carotenoids have been widely produced in order to meet market demands for colorants. Even though the technology for their chemical synthesis is inexpensive and efficient, synthetic carotenoids have been accused of causing various health implications (Mussagy et al., 2019). Moreover, artificial pigments provoke substantial and hazardous waste when disposed of; causing environmental pollution (Santos Ribeiro et al., 2019). Nevertheless, the industrial exploitation of carotenoids from plant-based sources presents several limitations. The cultivation process of plants requires large areas, specific seasonal conditions and manipulation of numerous parameters during growth, like the soil content or the

temperature, in order to achieve high production yields (Bogacz-Radomska et al., 2019; Igreja et al., 2021). Additionally, the dependence on geographical variability also complicates the production process which results to a time consuming, not viable strategy for industrial scale application (Paul et al., 2023). Considering all these restrictions, in the last decades many researchers have focused on the biotechnological production of these molecules (Elfeky et al., 2019; Qi et al., 2020). Microbial carotenoids offer a viable strategy for stable, efficient and safe carotenoids production with regard to human health and the environment. Another advantage of microbial synthesis includes the ability of high product yields together with vast color diversity (Mussagy et al., 2019; Wang et al., 2021). Therefore, further research into the production of biotechnologically produced carotenoids has the potential to facilitate their industrial application as a replacement of their synthetic counterparts.

CHAPTER 3

Biotechnological production of value-added compounds

3 Biotechnological production of value-added compounds

3.1 Introduction

The biotechnological production of valuable compounds is steadily increasing. Environmental and economic concerns, climate change, and the depletion of fossil fuel sources have led to a shift toward more eco-friendly and sustainable sources for valuable compound production (Gordillo Sierra and Alper, 2020; Miller and Alper, 2019). Microbial production of value-added compounds could contribute to reducing the environmental footprint while synthesizing compounds with potential nutritional benefits (Barzee et al., 2021). The microbially-derived compounds produced might exhibit antioxidant and anti-inflammatory properties, promoting human health compared to synthetic compounds, which might present toxic effects (Wu et al., 2020; Rani et al., 2021; Alamgir, 2018). Additionally, many microorganisms are able to utilize food industry waste and by-product streams as renewable resources for the production of high-value metabolites. This potential highlights their role in advancing the Sustainable Development Goals (SDGs) by addressing global challenges related to resource depletion and environmental sustainability (Qumsani, 2024).

3.2 Microorganisms as producers of valuable compounds

Depending on the targeted compound, strain selection is crucial for its efficient production. Among the various biotechnological products, carotenoids, microbial oil and exopolysaccharides (EPS) constitute compounds of high technological interest especially for the food industry. Numerous microorganisms exhibit high production capacities, driving continuous advancements in associated technologies (Alexandri et al., 2022). The growing number of studies towards their biotechnological production highlights the increasing interest in this field. With respect to the capabilities of bacterial strains, Liu et al., (2020) reported that the marine bacterial strain *Brevundimonas scallop* efficiently produced astaxanthin, yielding 1.3 µg per gram of dry cell biomass. Vila et al., (2020) demonstrated that the bacterial strain *Flavobacterium* sp. P8 synthesized 0.27 mg/L of zeaxanthin. According to Shields Menard et al., (2015), the bacterial strain *Rhodococcus rhodochrous* was capable of accumulating microbial oil at a content of 43% (w/w). Furthermore, the strain

Lactobacillus rhamnosus LOCK 0943 produced 1.13 g/L of exopolysaccharides, as reported by Oleksy-Sobczak et al., (2020). Microalgae are also potent producers of valuable compounds. Approximately 2.92 mg of lutein per gram of dry cell biomass was extracted from the marine microalga *Chlorella salina* (Gayathri et al., 2018), while *Chaetoceros muelleri* produced 2.92 mg of fucoxanthin per gram of ash-free dry weight (Ishika et al., 2017). Additionally, dos Santos et al., (2016) reported that the strain *Chlorella vulgaris* could reach a microbial oil content of 25.4% (w/w). Microalgae strains were also capable of synthesizing exopolysaccharides, with *Porphyridium purpureum* producing 0.24 g/L, as reported by Ferreira et al., 2021. Regarding the fungal strains, they have also proved to be efficient producers of the aforementioned metabolites. *Phaffia rhodozyma* PR106 could synthesize 54.45 mg of total carotenoids per g of dry cell biomass (Zhang et al., 2019). Michelon et al., (2012) reported that the fungal strain *Phaffia rhodozyma* NRRRL Y-17268 could produce 190.65 µg of total carotenoids per g of dry cell biomass. Additionally, an enhanced microbial oil content of 83.3% (w/w) in the dry biomass of *Mordellistena isabellina* ATHUM 2935 was reported by Papanikolaou et al., 2017. Moreover, fermentation using the fungal strain *Aureobasidium pullulans* spi 10 yielded 4.53g/ 100 mL of the exopolysaccharide pullulan (Gaikwad et al., 2022).

3.3 Red yeasts as multi-product cell factories

Among microorganisms, yeasts, especially red yeasts, have arisen as the most promising candidates from both a commercial and economic point of view. High growth rates, the ability of assimilating a variety of substrates, and the fast production rate of secondary metabolites constitute some of the main advantages of yeasts (Fakankun and Levin, 2023). Carotenoids, microbial oil and exopolysaccharides are exemplifying compounds synthesized by red yeasts.

3.3.1 Carotenoids

Numerous studies are focusing on yeasts, with particular emphasis on *Rhodotorula* and *Rhodospiridium* species, commonly referred to as red yeasts, owing to their ability to accumulate high amounts of carotenoids like β-carotene, torulene and torularhodin (Kot et al., 2018). Cultivation parameters including temperature, pH,

agitation, and the medium composition (carbon and nitrogen sources) highly affect carotenoid synthesis (**Table 3.1**).

Table 3.1 Recent advances in the biotechnological production of carotenoids by red yeasts under various culture conditions.

Strain	Medium composition	Fermentation parameters			Carotenoid concentration	Reference
		Temp (°C)	pH	Agitation (rpm)		
<i>Rhodospiridium toruloides</i> Y27012	40 g/L Glucose, Yeast extract, C/N 20	30		200	1001.51 µg/g dcw Total carotenoids	Alakraa et al. (2020)
<i>Rhodotorula</i> sp. RY1801	10 g/L Glucose, 10 g/L Yeast extract	28	5.0	120	0.987 mg/L Total carotenoids	Zhao et al. (2019)
<i>Rhodospiridium diobovatum</i> IMBY-5023	10 g/L Glucose, 5g/L Maltotriose, 5g/L Peptone, 25 g/L Malt extract, 3g/L Yeast extract, C/N 12	25	5.0		24.98 mg/g dcw Total carotenoids	Ielchishcheva et al. (2018)
<i>Sporidiobolus salmonicolor</i> CCY 19-4-7	20 g/L Glucose, 4g/L Yeast extract, 10 g/L (NH ₄) ₂ SO ₄ , H ₂ O ₂ , Zn (II) ions	22		80	1100 µg/g dcw β-Carotene, 360 µg/g dcw Torulene	Breierová et al. (2018)
<i>Rhodotorula glutinis</i> CC 20-2-33					470 µg/g dcw Torularhodin	
<i>Rhodotorula rubra</i> PTCC 5255	18.75 g/L Glucose, 5 g/L (NH ₄) ₂ SO ₄ , C/N 5	30	6.5	150	6.84 mg/g dcw Total carotenoids	Varmira et al. (2016)
<i>Rhodospiridium paludigena</i> TL35-5	10 g/L Glucose, 1.92 g/L Potassium nitrate, 20 °C	20	6.5	200	3.69 mg/L Astaxanthin	Hoondée et al. (2024)
<i>Rhodotorula sampaioana</i> PL61-2	40 g.L Glucose, 0.67 g/L Urea	20	7.5	200	4.68 mg/L Astaxanthin	
<i>Sporobolomyces pararoseus</i> CJR	20 g/L Glucose, 20 g/L Peptone, 10 g/L Yeast extract, 40 mM H ₂ O ₂	25		180	2.16 mg/g dcw Total carotenoids	Li et al. (2024)

<i>Rhodospiridium toruloides</i> CCT7815	70 g/L xylose: glucose (6:1 ratio), 2 g/L Yeast extract, 0.4 g/L (NH ₄) ₂ SO ₄	28	6.0	700	255 µg/g dcw Total carotenoids	Ribeiro et al. (2023)
<i>Rhodotorula mucilaginosa</i> I2002	50 g/L Glucose, 1.7 g/L Yeast extract, (NH ₄) ₂ SO ₄ , C/N 200	30		210	566.6 µg/g dcw Total carotenoids	Zeng et al. (2023)
<i>Rhodospiridium paludigena</i> SP9-15	40 g/L Glucose, 5g/L Peptone, 3 g/L Malt extract, 3 g/L Yeast extract	25	7.5	200	0.558 mg/g dcw Astaxanthin	Phuengjayaem et al. (2023)
<i>Rhodospiridium toruloides</i> C23	120 g/L Glucose, 0.2 g/L Yeast extract, 0.2 g/L (NH ₄) ₂ SO ₄	28		300	15.5 mg/L Total carotenoids	Xue et al. (2023)
<i>Rhodotorula glutinis</i> ZHK	20 g/L Glucose, 20 g/L Peptone, 10 g/L Yeast extract, 10 mM H ₂ O ₂	25		180	118.86 µg/g dcw Total carotenoids	Zhao and Li (2023)
	20 g/L Glucose, 20 g/L Peptone, 10 g/L Yeast extract, 100 mg/L TiO ₂				23.165 µg/g dcw Total carotenoids	
<i>Rhodotorula</i> sp. strain ATL72	7.7 g/L Fructose, 3.7 g/L Malt extract, 9 g/L Urea, 1 g/L Yeast extract	27.5	6.7	250	21.5 mg/L Total carotenoids	Dyaa et al. (2022)
<i>Rhodospiridiobolus colostri</i> CGMCC 2.5556	20 g/L Glucose, 20 g/L Peptone, 10 g/L Yeast extract	16	6.5	180	29.01 mg/L Total carotenoids	Li et al. (2022)
<i>Rhodotorula mucilaginosa</i> sp.	YM medium with Glucose	28	6.0	200	16.96 mg/L Total carotenoids	Elsanhoty et al. (2017)
<i>Rhodospiridium diobovatum</i> 08-225	40 g/L Glucose, 3 g/L Yeast extract	30	5.5	150 rev/min	324 µg/g dcw Total carotenoids	Nasirian et al. (2018)
<i>Rhodotorula glutinis</i> CCY 20-2-26	50 g/L Glucose, 5 g/L Yeast extract, C/N 50	28		180	12.7 mg/L Total carotenoids	Tkáčová et al. (2017)

Depending on the strain employed, the optimal incubation temperature for carotenogenesis could vary significantly. Li et al., (2022) demonstrated that *Rhodotorula colostri* CGMCC 2.5556 produced 29.01 mg/L of total carotenoids when fermentation was conducted at 16 °C, following tests at various cultivation temperatures. A temperature of 25 °C could also be favorable for carotenogenesis as the yeast strain *Sporidiobolus pararoseus* CJR produced 2.16 µg of total carotenoids per g of biomass (Li et al., 2024). The yeast strain *Rhodospiridium toruloides* Y27012 synthesized 1001.51 µg of total carotenoids per g of dry cell weight at an incubation temperature of 30 °C (Alakraa et al., 2020). The pH employed also influences carotenoid production, as it affects the metabolic flux in red yeasts. The enzymes required for each biosynthetic pathway function optimally at different pH levels. According to **Table 3.1**, pH values of 5.0 or above are preferred for carotenogenesis. Hoondie et al., (2024) indicated that the favorable pH level for the maximum astaxanthin production differed between the two yeast strains examined. The yeast strain *Rhodospiridium paludigena* TL35-5 produced 3.69 mg/L of astaxanthin at pH 6.5, whereas *Rhodotorula sampaioana* PL61-2 synthesized 4.68 mg/L at a pH value of 7.5. Furthermore, Elsanhoty et al., (2017) reported that a yeast strain belonging to *Rhodotorula mucilaginosa* sp. was able to accumulate 16.96 mg/L of total carotenoids when the pH was set at 6.0.

Agitation during fermentation is also regarded as a crucial parameter, as red yeasts are aerobic microorganisms. The airflow is influenced by agitation, which in turn affects yeast growth and the production of secondary metabolites (Mata-Gómez et al., 2014). Depending on the aeration requirements of each employed yeast strain, the agitation rate is accordingly adjusted (**Table 3.1**). An agitation of 150 rpm was preferred for the growth of the strain *Rhodotorula rubra* PTCC 5255, which produced 6.84 mg/g of total carotenoids (Varmira et al., 2016). On the other hand, *Rhodotorula* sp. strain ATL72 could synthesize high amounts of carotenoids (21.5 mg/L) at an agitation rate of 250 rpm (Dyaa et al., 2022).

The medium composition, particularly the combination of carbon and nitrogen sources, as well as various stress factors could significantly affect the carotenoid synthesis (**Table 3.1**). Nitrogen sources, including yeast extract, ammonium sulfate and urea, have been utilized separately or in combination to investigate different

carbon to nitrogen ratios (C/N) and their impact on the production of secondary metabolites. Zeng et al., (2023) demonstrated that a C/N ratio of 200 using yeast extract and ammonium sulfate favored carotenogenesis for strain *Rhodotorula mucilaginosa* I2002, resulting in the synthesis of 566.6 µg of total carotenoids per gram of biomass. On the contrary, the optimal C/N ratio for strain *Rhodospiridium toruloides* Y27012 was 20 using only yeast extract as the nitrogen source. Additionally, stress factors such as different concentrations of H₂O₂ could be added to the culture media to induce carotenogenesis. Zhao and Li, (2023) reported that the production of carotenoids by the strain *Rhodotorula glutinis* ZHK was significantly influenced not only by the addition of stress factors but also by their type and concentration (**Table 3.1**). Thus, depending on the strain, the fermentation conditions that enhance carotenoids synthesis can vary significantly. Besides final titers, proper manipulation of key fermentation parameters can lead to the production of specific carotenoids. Depending on the targeted compound, the selection of the appropriate strain can lead to the production of various molecules of high market value. The most important carotenoids synthesized by yeast include β-carotene, astaxanthin, lycopene, torulene and torularhodin (Bertacchi et al., 2020; Qi et al., 2020).

3.3.1.1 β-carotene

β-carotene is one of the most widely used carotenoids, with significant industrial applications such as in food, agriculture, pharmaceutical and in cosmetics. The global β-carotene market is projected to exceed 955 million USD by 2032, with an anticipated CAGR of over 5% during the period 2023-2032 (Global Market Insights, 2023a). In addition to synthetic β-carotene, the European Food Safety Authority (EFSA) has approved the use of biotechnologically produced β-carotene in food applications, derived from the natural algal strains *Dunaliella salina* (or the variety *Dunaliella bardawil*) and from the fungus *Blakeslea trispora* (Aguilar et al., 2008). Given that red yeasts are promising producers of β-carotene, further research, particularly on the toxicological effects of the produced carotenoids upon consumption, could lead to an expansion of the approved microorganisms for β-carotene production.

3.3.1.2 Lycopene

Lycopene has been recognized as class A nutrient from the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) (Liang et al., 2019). Therefore, lycopene's market share is ever-increasing due to its potential usage in industrial scale (Alexandri et al., 2022). According to a recent market report, the value of lycopene would yield above 250 million USD until 2032, while the anticipated CAGR would be over 5.5% globally (Global Market Insights, 2023b). Lycopene could be produced biotechnologically through microbial fermentations. EFSA already allows the production of lycopene from *Blakeslea trispora* and its usage in food applications (Aguilar et al., 2008). Nevertheless, sustainable lycopene production from microbial fermentations has several limitations, since metabolic engineering techniques or blocking agents like imidazole are required in order to prevent the enzyme lycopene cyclase from converting lycopene into β -carotene (Choudhari et al., 2008).

3.3.1.3 Torulene and Torularhodin

Torulene and torularhodin are considered “unusual” carotenoids, particularly produced by red yeasts. Due to their structure, which consists of 13 conjugated double bonds, they exhibit higher antioxidant activity compared to other natural carotenoids, thereby opening new opportunities for industrial applications (Kot et al., 2018). Numerous recent studies have focused on their microbial production (Li et al., 2017; Zeng et al., 2023). However, neither the synthetic forms nor those produced biotechnologically have been approved for use in industrial-scale, as the EFSA and the FDA (Food and Drug Administration) have not yet authorized their inclusion as supplements. Investigating both the toxicological effects and benefits of torulene and torularhodin on human health is crucial for their further evaluation as potential food additives.

3.3.2 Microbial oil

Red yeasts are also considered as oleaginous due to the fact that they are able to accumulate lipids up to 70% of their total dry cell weight (Kot et al., 2019). Likewise, lipid accumulation could also occur through red yeast fermentations, utilizing a wide

range of substrates (Patel et al., 2015). The produced oil is comprised of a fatty acid profile which is similar to plant-derived oil and is mainly characterized by the presence of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) (Wu et al., 2023). Recent studies have focused on optimization of yeast fermentation conditions in order to enhance the lipid accumulation by directing their metabolic pathway to fatty acid synthesis instead of carotenogenesis. Byrtusová et al., (2020) demonstrated that the culture conditions, particularly the C/N ratio, could impact the lipid production. The strain *Rhodospiridium toruloides* CCY-62-2-4 achieved over 45% (w/w) microbial oil content when glucose was used as the carbon source with a C/N ratio of 200. On the other hand, a C/N ratio equal to 70 was optimal for the yeast strain *Rhodospiridium kratochvilovae* CCY 20-2-26, resulting in 37.92% (w/w) microbial oil content, while Filippousi et al., 2022 demonstrated that the optimal C/N ratio for microbial oil production from *Rhodospiridium toruloides* NRRL Y-27012 was 100, yielding a 50% (w/w) content per dry biomass. Additionally, Jiru et al., (2017) evaluated a wide range of fermentation parameters, including the carbon and nitrogen sources, their C/N ratio, pH, temperature, and agitation rate. Their results indicated that under optimal conditions, *Rhodospiridium kratochvilovae* SY89 accumulated a high microbial oil content of 56.06% (w/w). Moreover, according to Maza et al., (2020) the utilization of glucose along with nitrogen starvation during fermentation could enhance the microbial oil production in *Rhodotorula glutinis* R4, reaching a content of 47% (w/w). Furthermore, Papanikolaou et al., 2017 reported that *Rhodospiridium toruloides* DSMZ 4444 could accumulate microbial oil up to 39.6% (w/w) of the total dry weight using glucose as the carbon source and a C/N ratio of 100.

However, under optimal fermentation conditions, red yeasts could simultaneously produce both carotenoids and lipids, resulting in a carotenoid-rich oil due to the lipid-soluble nature of carotenoids. Dias et al., (2015) suggested that a pH controlled strategy during fermentation could enhance the production of both secondary metabolites. The strain *Rhodospiridium toruloides* NCYC 921 presented a microbial oil content of 27.7% (w/w) at pH 4.0, while the maximum carotenoid concentration of 0.28 mg/g biomass was observed when the pH was increased to 5.0. Another strategy for enhancing carotenoid production and lipid accumulation is the adjustment of the

appropriate amount of nitrogen source in the culture medium to trigger their respective metabolic pathways. Mirzaie et al., (2019) demonstrated that a C/N ratio of 50 was preferred by the strain *Rhodospiridium diobovatum* 08-225 for the production of high amounts of both metabolites. The co-production of carotenoids and lipids might enhance their respective utility. In particular, carotenoids-rich microbial oil has been utilized in the development of novel food formulations such as wax esters and oleogels (Papadaki et al., 2019a, 2019b), while their inclusion in novel formulations (e.g. edible films) could enhance their bioactivity, increasing their potential applications (Papadaki et al., 2024, 2023).

3.3.3 Exopolysaccharides

Exopolysaccharides (EPS) are extracellular polymers primarily composed of sugar residues such as mannose, glucose, galactose, xylose, fucose, and rhamnose (Saadat et al., 2021). Yeasts, including red yeasts, have emerged as promising candidates for EPS production. Yeasts offer advantages for their industrial-scale production due to their simpler and faster isolation compared to bacterial strains. Additionally, yeasts-derived EPS exhibit a variety of biological activities, which are closely linked to their structure and specific characteristics, such as monomer composition, the presence of functional groups, and their molecular weight (Seveiri et al., 2020). Their potential antioxidant, antiviral, and antitumor properties render these polymers as promising additives both in foods and pharmaceuticals. Additionally, they could serve as thickeners or stabilizers, owing to their enhanced emulsifying properties and water-holding capacity (Ma et al., 2018; Singh and Kaur, 2018). The properties of yeast-derived EPS are influenced by both the strain employed and the fermentation conditions. According to the comprehensive review of Gientka et al., (2015), optimal EPS production from yeasts could be achieved by utilizing sucrose as a carbon source, at limiting nitrogen conditions -preferable using inorganic nitrogen sources- and maintaining strong oxygenation during fermentation. Moreover, Saadat et al., (2021) highlighted the importance of utilizing higher C/N ratios to improve EPS production from yeasts. Lower C/N ratios could alter yeast's metabolic pathways, leading to the synthesis of other metabolites, such as fatty acids (Cho et al., 2001).

Red yeasts, particularly those of the *Rhodotorula* genus, are considered promising sources for EPS production. The strain *Rhodospiridium babjevae* IBRG-M 30088 produced 1.6 g/L of EPS, composed of 78% carbohydrates, primarily mannose and glucose. The produced EPS demonstrated higher antioxidant activity compared to hyaluronic acid, a widely used biopolymer in cosmetics. Additionally, they exhibited good emulsifying activity in oil-in-water emulsions (Seveiri et al., 2020). Moreover, Singh and Kaur, (2018) reported that the strain *Rhodotorula paludigenum* PUPY-06 synthesized 2.12% (w/v) pullulan-like EPS, a commercially promising polymer typically produced by *Aureobasidium pullulans*. Pullulan could be utilized as non-polluting wrapping material for food supplements as well as a gelling agent and emulsifier (**Figure 3.1**). Furthermore, the strain *Rhodotorula mucilaginosa* CICC 33013 produced a high quantity of EPS (6.2 g/L) under optimal culture conditions. They were primarily composed of galactose, arabinose, glucose, and mannose, and exhibited significant antioxidant properties (Ma et al., 2018). Therefore, EPS produced from red yeasts could be considered a potential source of biopolymers suitable for industrial-scale applications. The fact that yeast-derived EPS present non-toxicity and biocompatibility renders them as promising candidates for potential applications in food, cosmetic, and pharmaceutical industries (Seveiri et al., 2019).

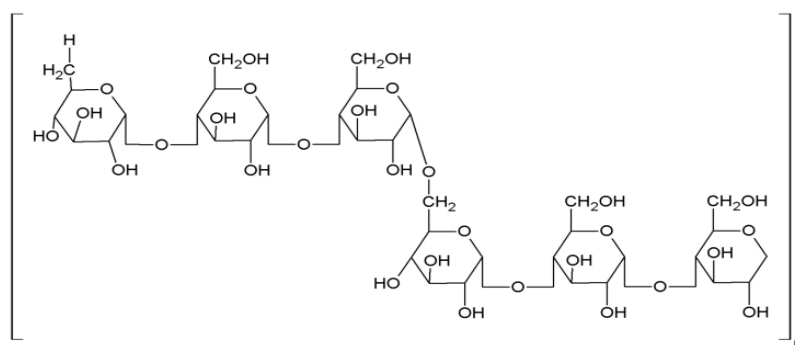


Figure 3.1 Chemical structure of pullulan (Ferreira et al., 2015).

3.4 Food waste and by-product streams as renewable resources for red yeasts

Waste and by-product streams from the agri-food sector constitute one of the largest environmental problems of our era. Numerous studies have elaborated on the utilization of food industry waste and by-product streams to generate high-added value products via biotechnological routes, targeting to mitigate environmental pollution. **Table 3.2** presents studies on the implementation of these streams as low-

cost supplements for bioconversion processes. Sharma and Ghoshal, (2020) reported that strain *Rhodotorula mucilaginosa* MTCC-1403 produced 717.82 µg of total carotenoids per g of biomass when onion peels were used as the carbon source and mung bean husk as the nitrogen source in the fermentation media. Furthermore, a combination of raw glycerol and potato wastewater, serving as the carbon and nitrogen sources respectively, was effectively utilized for the growth of *Rhodotorula gracilis* ATCC 10788, resulting in the production of 6.24 mg/L carotenoids and 4.26 g/L microbial oil (Kot et al., 2020). Two strains were investigated for their ability to produce both carotenoids and microbial oil using a mixture of coffee oil and glycerol (90:10). A study demonstrated that, while *Rhodospiridium toruloides* CCY 062-002-004 and *Rhodospiridium kratochvilovae* CCY 020-002-026 produced similar amounts of carotenoids, the predominant carotenoid differed depending on the strain employed. Moreover, both yeast strains exhibited lipid accumulation exceeding 25% (Szotkowski et al., 2023). Thus, the use of various food waste by-products as renewable resources could effectively support the production of both secondary metabolites by red yeasts.

Table 3.2 Biotechnological production of carotenoids by red yeasts using food waste by-products.

Strain	Food waste by-product	Carotenoid concentration	Microbial oil content	Reference
<i>Rhodotorula mucilaginosa</i> MTCC-1403	Onion peels, Mung bean husk	717.82 µg/g dcw Total carotenoids		Sharma and Ghoshal (2020)
<i>Rhodospiridium toruloides</i> NRRL Y-1091	Wheat straw hydrolysates	24.58 mg/L Total carotenoids	3.99 g/L	Liu et al. (2020)
<i>Rhodotorula mucilaginosa</i> sp.	Olive mill waste	7.3 mg/L Total carotenoids		Ghilardi et al. 2020)
<i>Rhodospiridium toruloides</i> DSM 4444	<i>Camelina sativa</i> meal hydrolysate	16 mg/L Total carotenoids		Bertacchi et al. (2020)
<i>Rhodospiridium paludigenum</i> InaCC Y236	Orange peels	107.63 µg/g dcw Total carotenoids		Warjoto et al. (2020)
<i>Rhodotorula gracilis</i> ATCC 10788	Raw glycerol fraction, Potato	6.24 mg/L Total carotenoids 47% β-Carotene 51% Torulene	4.26 g/L	Kot et al. (2020)

	wastewater			
<i>Rhodotorula mucilaginosa</i> sp.	Glycerol	223.5 µg/g dcw Total carotenoids		Allahkarami et al. (2021)
<i>Rhodotorula mucilaginosa</i> SML	Food industry wastewater	810 µg/g dcw Total carotenoids 54% Torulene		Sundaramahalingam and Sivashanmugam (2023)
<i>Rhodospiridium toruloides</i> CCY 062-002-004	Coffee oil with waste	10.51 mg/g dcw Total carotenoids 8.92 mg/g dcw Torulene	28.46%	Szotkowski et al. (2023)
<i>Rhodospiridium kratochvilovae</i> CCY 020-002-026	Glycerol (90:10)	10.75 mg/g dcw Total carotenoids 10.34 mg/g dcw Torularhodin	25.38%	
<i>Rhodospiridium toruloides</i> L/24-26-1	Sugarcane molasses	715.4 µg/g dcw Total carotenoids 59.5% β-carotene		Ochoa-Viñals et al. (2024)

3.4.1 Cheese whey

The negative effect of the dairy industry effluents to environmental pollution is indisputable. Dairy industry accounts for the largest food wastewater source in Europe, considering that approximately 800–850 kg of whey are produced during cheese manufacturing from 1 ton of milk. Significant quantities of cheese whey are currently disposed in landfills, thereby, strict environmental regulations have emerged to prevent disposal methods, compelling also the establishment of effective and more permanent solutions for whey treatment (Mehri et al., 2021). Whey contains 4.5%-5.0% (w/v) lactose, 0.6%-0.8% (w/v) protein, 0.4%-0.5% (w/v) lipids and a significant amount of minerals and vitamins (Lappa et al., 2019). To this end, whey has been proposed as an alternative carbohydrate source for microbial metabolite synthesis, even if some microbial entities (e.g. the majority of yeasts in nature) do not utilize lactose. Lactose hydrolysis is feasible through diversified strategies like co-cultivation with lactose-positive yeast or bacteria or the addition of β-galactosidase or acid hydrolysis (Frengova et al., 2006; Gervasi et al., 2018; Mata-Gómez et al., 2023; Roukas et al., 2015). Marova et al., (2012) demonstrated that *Rhodotorula glutinis* CCY 20-2-26 produced 1.26 mg of β-carotene per g of dry biomass using deproteinized cheese whey. Additionally, fermentation of *Rhodotorula mucilaginosa* NRRL-2502 with whey lactose resulted in 35 mg of total carotenoids per g of dry cells (Aksu and Eren, 2005). Therefore, cheese whey valorization routes for the

production of value-added compounds have emerged, following the principles of circular economy (Kachrimanidou et al., 2022; Lappa et al., 2019).

3.4.2 Molasses

Molasses is the primary by-product of the sugar production industry. While the sugar beet industry is regarded as the dominant sugar industry in Europe, beet sugar accounts for only 20% of global sugar production, with the remaining 80% derived from sugarcane (Ruggeri and Corsi, 2019, Maitah et al., 2024). Approximately 35 to 45 kg of molasses are produced per ton of sugarcane, whereas 36 to 40 kg are obtained per ton of sugar beet (Jamir et al., 2021, Adbhai et al., 2022). Molasses is characterized as a viscous, dark, sugar-rich syrup primarily composed of sucrose. Its composition varies depending on the source (Solomon, 2011). Molasses derived from sugarcane typically contain 30-35% sucrose, 10-25% reducing sugars (such as glucose and fructose), along with proteins and various minerals. In contrast, molasses from sugar beet contain a higher sucrose content (approximately 60%), while reducing sugars constitute less than 1% (Jamir et al., 2021, Sjolín et al., 2020). Its high sugar content, combined with its relatively low market price, positions molasses as an alternative feedstock for fermentation processes aimed at producing valuable compounds (Cazetta et al., 2007). Red yeasts have already been evaluated for their ability to produce metabolites such as carotenoids, microbial oil and EPS using molasses as a carbon source. Rodrigues et al., (2022) demonstrated that the strain *Rhodotorula mucilaginosa* CCT 7688 produced 1.79 mg/L of total carotenoids using sugarcane molasses as a substrate while, *Rhodotorula mucilaginosa* URM 7409 achieved 2.95 mg/L of carotenoids (Machado et al., 2022). Additionally, Boviatsi et al., (2020) reported that *Rhodospiridium toruloides* NRRL Y-27012 could accumulate microbial oil up to 61% (w/w) of the total dry biomass using sugarcane molasses as the carbon source. Lakshmidevi et al., (2021) evaluated the synthesis of both carotenoids and microbial oil using molasses as a substrate, reporting yields of 0.9 mg/L for carotenoids and 7.55 g/L for microbial oil, respectively. Research on the potential of red yeasts to produce exopolysaccharides using molasses is limited. Grigorova et al., (1999) demonstrated that the strain *Rhodotorula acheniorum* MC was capable of producing 2.64 g/L of EPS in shake-flask fermentations.

Therefore, red yeasts have the capacity to produce various valuable metabolites, including carotenoids, microbial oil, and exopolysaccharides. Their ability to assimilate a wide range of carbon sources, including food waste and by-product streams, makes them highly favorable for further exploitation.

CHAPTER 4

Green extraction of microbially produced carotenoids and lipids

4 Green extraction of microbially-derived value-added compounds

4.2 Green extraction techniques

In recent years, the utilization of green extraction processes for the recovery of bioactive compounds has gained prominence. This shift stems from the limitations of conventional extraction methods, which rely on organic solvents and energy, making them unsuitable for industrial applications. High energy consumption is a critical concern for industries, due to increased production costs, reduced profit and significant environmental impact (Boukroufa et al., 2017). Therefore, one of the principles of green extraction is the reduction of energy consumption through energy recovery and the implementation of innovative technologies (Rukavina et al., 2021). Green extraction techniques could yield high-quality and safer products that are free from hazardous organic solvents (Morón-Ortiz et al., 2024). Furthermore, the temperature-controlled environment or the absence of heat during the process is advantageous for the extraction of thermo-sensitive compounds (Anticono et al., 2020). As depicted in **Figure 4.1**, among green extraction technologies, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pulsed electric fields (PEF) and supercritical fluid extraction (SFE) have been widely employed for the extraction of bioactive compounds, and particularly carotenoids (Morón-Ortiz et al., 2024).

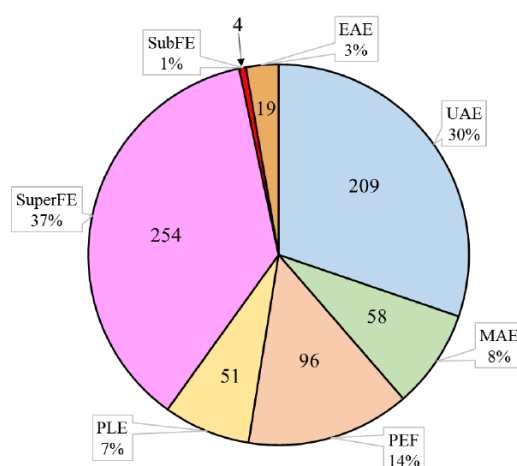


Figure 4.1 Number (inside the chart) and percentage (outside the chart) of published research articles related to carotenoid extraction using the respective methodologies. UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; PEF: pulsed electric field; PLE: pressurized liquid extraction; SuperFE: supercritical fluid extraction; SubFE: subcritical fluid extraction; EAE: enzyme-assisted extraction (Morón-Ortiz et al., 2024).

4.2.1 Green solvents

Microbially produced carotenoids and oil are conventionally extracted with organic solvents such as dimethyl sulfoxide (DMSO), petroleum ether, acetone and chloroform (Papapostolou et al., 2023). However, if the extracted product is intended for incorporation into food formulations, the use of chemicals is prohibited according to the European Guidelines (Directives, 2009/32). To this end, combinations of green solvents with green extraction technologies have been developed as environmentally friendly alternatives to minimize or replace the use of toxic solvents (Morón-Ortiz et al., 2024).

Green extraction solvents are employed to avoid the use of their organic counterparts, thereby minimizing impurities and denatured chemicals in the final extracted product (Yara-Varón et al., 2016). The choice of solvent is a key factor in achieving improved extraction yields. The selection of the appropriate solvent depends on the complexity of the matrix and the polarity of each compound targeted for extraction (Meléndez-Martínez et al., 2022; Morón-Ortiz et al., 2024). Green solvents could efficiently be used for the extraction of bioactive compounds, such as carotenoids. Ionic liquids and

natural deep eutectic solvents are among the most commonly used green solvents reported in the literature.

4.2.1.1 Ionic Liquids (ILs)

Ionic liquids (ILs) are defined as salts composed of an organic cation and an organic or inorganic anion (Lim et al., 2022). They have a melting point below 100 °C and remain in a liquid state at room temperature. Their unique physicochemical properties, non-flammability, and high stability have led to their extensive use in the extraction of carotenoids (Viñas-Ospino et al., 2023). Mussagy et al., (2022) demonstrated that both β -carotene and astaxanthin derived from *Phaffia rhodozyma* were extracted more efficiently- 1.9 and 1.7 times, respectively- using cholinium-based ILs compared with acetone. The utilization of water-based ILs instead of methanol increased the extraction efficiency of lutein from *Chorella saccharophila* by 20.5 times (Paliwal et al., 2022). Moreover, the extraction yield of total carotenoids from orange peels was enhanced by approximately 2.9 times when using ILs compared to acetone (Murador et al., 2019). However, concerns have emerged regarding the recognition of ionic liquids (ILs) as eco-friendly solvents, as their high stability renders them resistant to decomposition. Consequently, their prolonged lifespan could lead to soil and groundwater contamination (Morón-Ortiz et al., 2024).

4.2.1.2 Natural Deep Eutectic Solvents (NADES)

Natural deep eutectic solvents (NADES) are a type of deep eutectic solvent (DES) synthesized from natural components, designed to be more environmentally friendly. NADES are mixtures of two or more compounds that associate through hydrogen bonding, resulting in a low melting point, often below ambient temperature (Morón-Ortiz et al., 2024; Panić et al., 2019). Their properties make them highly desirable for extraction compared to other green solvents, as they are non-toxic, biodegradable, inexpensive, with possible recyclability (Chemat et al., 2019). Moreover, their simple preparation, combined with the efficiency in purifying extracted products, also enhance their preference for compounds extraction. However, their high viscosity might lead to mass transfer issues, thereby limiting extraction efficiency (Gullón et al., 2020). Recent studies have focused on use of NADES for the extraction of carotenoids. Stupar et al., (2021) demonstrated that 90% of the β -carotene in pumpkin

extract could be efficiently extracted using NADES composed of C8 and C10 fatty acids in a ratio 3:1. Additionally, Kyriakoudi et al., (2022) indicated that the extraction of lycopene from tomatoes using NADES, composed of C10 and C12 fatty acids in a ratio 1:2, was 1.8 times more efficient compared to extraction with acetone. Furthermore, a significant improvement in astaxanthin recovery from *Haematococcus pluvialis* was achieved using NADES composed of menthol and C14 fatty acid in a ratio of 8:1, compared to Soxhlet extraction (Rodrigues et al., 2020).

4.2.2 Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction technology employs waves with frequencies exceeding 10 MHz able to penetrate cell membranes, thereby facilitating interaction with the solvent and enhancing the extraction of bioactive compounds (Montero-Calderon et al., 2019). More specifically, the phenomenon known as “cavitation” occurs, resulting in the formation and implosion of small bubbles in a liquid, which disrupts cellular structures (Morón-Ortiz et al., 2024). **Figure 4.2** presents the equipment for the extraction using ultrasounds.

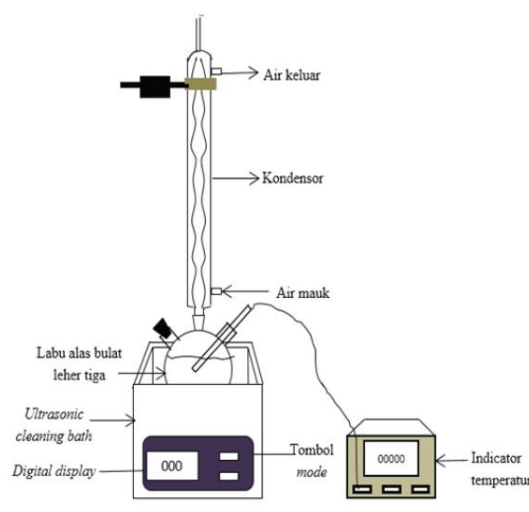


Figure 4.2 Ultrasound-assisted extraction equipment (Qadariyah et al., 2018).

UAE exhibits enhanced extraction yields while utilizing lower energy levels and reducing extraction time compared to conventional methods. However, the high ultrasound intensity could lead to the formation of hydroxyl radicals and hydrogen atoms, which can contribute to the degradation of antioxidant compounds such as carotenoids (Kumari et al., 2018). Selvakumar and Sivashanmugam, (2019)

demonstrated a recovery of over 90% of microbial oil from *Naganishia liquefaciens* NITTS2 using UAE as green extraction method. Meanwhile, Coelho et al., (2022) reported that that UAE resulted in a 1.4-fold increase in the concentration of total carotenoids extracted from yellow cashew apples compared to conventional methods.

4.2.3 Microwave-Assisted Extraction (MAE)

The microwave-assisted extraction method utilizes microwave heating through irradiation to enhance the disruption of cell walls, facilitating the extraction of bioactive compounds (Bagade and Patil, 2021) (**Figure 4.3**). The electromagnetic energy from microwave irradiation is converted to thermal energy through ionic conductivity or dipole polarization (Papapostolou et al., 2023). The high temperatures generated during the process may negatively affect compounds such as carotenoids. Therefore, the selection of an appropriate green solvent is crucial for controlling both the efficiency and the temperature of the MAE (Geow et al., 2021). Elik et al., (2020) reported that, under optimal conditions (165 W of microwave power and 9.39 min of extraction time), 77.48% of the total carotenoids could be extracted from carrot juice using ethanol as a green solvent through MAE. Moreover, Leema et al., (2022) demonstrated that the extraction of lutein from *Chlorella sorokiniana* was 3.3 times higher with MAE compared to conventional methods. Additionally, microbial oil derived from *Saccharomyces cerevisiae* could also be efficiently recovered using MAE, achieving extraction yields comparable to those of conventional methods in a significantly reduced extraction time (Khoomrung et al., 2013).

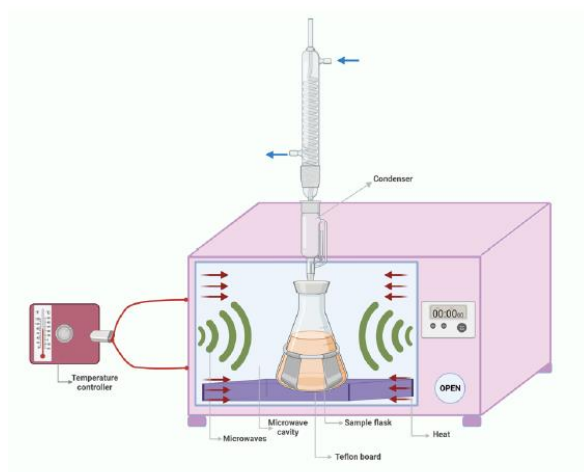


Figure 4.3 Microwave-assisted extraction (Fărcaș et al., 2022).

4.2.4 Pulsed Electric Fields (PEF)

Pulsed electric fields are regarded as an environmentally friendly extraction process, in which the application of electric fields induces electroporation in cell membranes. This process destabilizes the bilipid layer of biological membranes, promoting the release of intracellular compounds (Luengo et al., 2014). PEF is advantageous as it is a non-thermal method that requires low energy levels to permeabilize cell membranes (Morón-Ortiz et al., 2024). Thus, PEF could be used for the extraction of value-added compounds and particularly carotenoids. The main drawback of this technique is that the conductivity of the environment could alter its effectiveness, as high conductivity leads to the conversion of electrical energy into heat (Bocker and Silva, 2022). **Figure 4.4** depicts a diagrammatic representation of PEF extraction. Siddeeg et al., (2019) demonstrated that the application of PEF significantly increased the total carotenoid content extracted from date palm fruits relative to untreated samples when the frequency was 10 Hz and the electric field strength was 3 kV/cm. Additionally, Pataro et al., (2020) reported that both the extraction rate and yield of lycopene from tomato by-products were significantly improved with PEF treatment when the energy field strength was 5 kV/cm and the energy input was 5 kJ/kg. Furthermore, enhanced recovery of microbial oil from *Saitozyma podzolica* DSM 27192 was achieved using PEF treatment (electric field strength of 40 kV/cm), following an additional washing step to reduce conductivity (Gorte et al., 2020).

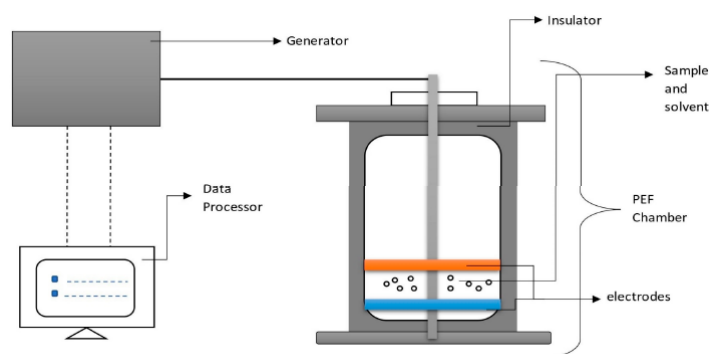


Figure 4.4 A diagrammatic representation of pulsed electric field extraction (Kumar et al., 2023).

4.2.5 Supercritical Fluid Extraction (SFE)

The extraction of value added compounds could be efficiently achieved using supercritical fluids. Supercritical fluids are compounds that, when subjected to pressures and temperatures exceeding their critical point, exhibit characteristics of both gases and liquids (**Figure 4.5**). Particularly, they could simultaneously dissolve materials like liquids and diffuse through solid matrices as gases (Papapostolou et al., 2023). The extraction of bioactive compounds using SFE enables the application of low temperatures, making them suitable for thermo-sensitive compounds such as carotenoids. Additionally, the simplicity of removing the supercritical fluids from the extracted products comprises another advantage (Bubalo et al., 2018).

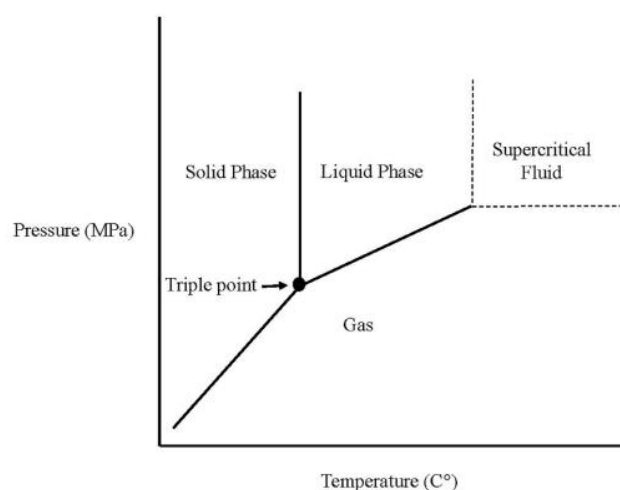


Figure 4.5 Schematic diagram showing the supercritical fluid region (Ahangari et al., 2021).

Another positive characteristic of this technique is its low energy consumption. Carbon dioxide is the most commonly used solvent, with SFE-CO₂ to be considered as an economically viable method due to the feasibility to recycle the CO₂ gas stream throughout the extraction process (Ferreira et al., 2024). Carbon dioxide is a non-flammable, non-toxic solvent and recognized as safe (GRAS). Under supercritical conditions, CO₂ exhibits enhanced diffusivity and low viscosity, resulting in the improved solubility of bioactive compounds and thus in high extraction efficiency and product purity (Uwineza and Waśkiewicz, 2020). Carbon dioxide is preferentially utilized for non-polar, lipophilic substances with small molecular weight such as microbial oil and carotenoids (Lima et al., 2019; Sánchez-Camargo et al., 2019).

Figure 4.6 represents a diagram of SFE-CO₂ equipment. The efficiency of SFE-CO₂ is influenced by factors such as pressure, temperature, CO₂ flow rate, and the use of co-solvents. The addition of a modifier (co-solvent) could increase the polarity of the system and therefore the extractability of polar compounds. Ethanol is the most commonly used co-solvent, mainly due to its GRAS status and “green” nature. Moreover, ethanol residues in the final extract do not pose health risks, as a daily consumption of 50 mg is considered acceptable (European Medicines Agency, 2019).

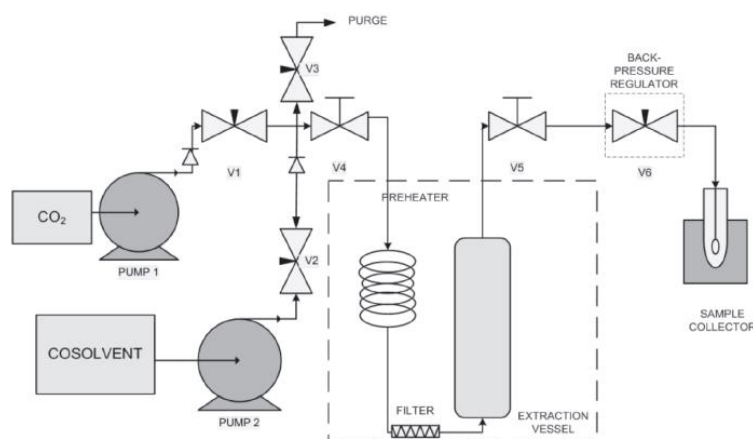


Figure 4.6 Design of the experimental system used for the supercritical fluid extraction system with CO₂ and co-solvent in the work of Radzali et al., (2014).

SFE-CO₂ (with or without the use of co-solvent) has been extensively evaluated for the recovery of bioactive compounds. Lima et al., (2019) demonstrated that using SFE-CO₂ the total recovery of carotenoids from various vegetable waste matrices, including sweet potato peels, peach flesh, and red pepper flesh, ranged from 92.4% to 99.8%. Additionally, after optimizing parameters such as temperature (70 °C), pressure (390 bar), and CO₂ flow rate (35 mL/min), a recovery of 90.12% of β -carotene from ripe bitter melon pericarp was achieved by Patel et al., (2019). Couto et al., (2010) reported that nearly 50% of the total microbial oil was extracted from the microalga *Cryptocodinium cohnii* under optimal conditions through SFE-CO₂ (pressure at 300 bar and temperature at 50 °C). Moreover, Molino et al., (2018) achieved 92% recovery of astaxanthin from the microalga *Haematococcus pluvialis* while the yield of lutein extracted from *Scenedesmus almeriensis* was around 98% (Mehariya et al., 2019).

CHAPTER 5

Encapsulation of carotenoids

5 Encapsulation of carotenoids

5.1 Introduction

Recently, there has been a noticeable trend towards functional foods, due to their potential health benefits. Consumer preferences have shifted, showing an increasing demand for products that offer protection against various chronic illnesses, including diabetes, obesity and cardiovascular diseases. Consequently, consumers seek functional, clean-label, and non-genetically modified products (Alexandri et al., 2022; Topolska et al., 2021). This trend is highly reflected by the growing market of functional products, that is projected to reach 586.1 billion USD by 2030, while the anticipated CAGR would exceed 8.5% globally (Grand View Research, 2023). The European Union dictates that a product could be classified as functional if it demonstrates a proven positive effect on health, either due to its nutrient-rich ingredients or through fortification with additional beneficial components (European Commission, 2021). Furthermore, to qualify as “natural”, a product must be free from artificial or synthetic substances, devoid of chemical additives, and composed of natural ingredients (FSIS, 2020).

Natural carotenoids have the potential to serve as functional compounds in various food applications. However, their sensitivity to light and temperature, combined with poor water solubility and gastrointestinal instability, results in low bioavailability and bioaccessibility, restricting their industrial implementation (Chen et al., 2023; Han et al., 2023). For that purpose, encapsulation methods such as electrospinning, micro-/nano- capsules and liposomes were proposed as means to create novel functional delivery systems in which the shelf-life of carotenoids as well as their physicochemical properties could be ameliorated. In this way, carotenoids could be effectively utilized as bioactive ingredients in a plethora of novel applications (Mosaddik et al., 2018).

5.2 Encapsulation processes

Encapsulation is considered as a widely used method for entrapping sensitive compounds such as phenolics and carotenoids in a solid or liquid carrier (Dias et al., 2015). There are four types of carriers employed as wall materials which are classified

based on their nature as lipids- based (Sirikhet et al., 2021), proteins (İnan-Çınkır et al., 2023), carbohydrates (Shi et al., 2023) and gums (Corrêa-Filho et al., 2022). Depending on the application, the carrier could be composed by a sole compound (de Lima et al., 2022; Zhang et al., 2022) or in a combination in order to improve the efficiency of the encapsulation system (Falsafi et al., 2022; Koo et al., 2023). The evaluation of the expedient carrier is dependent on the nature and the chemical properties of the core material, the encapsulation process applied and the final application of the product (Saifullah et al., 2019).

The selection of the appropriate encapsulation technique is based on both the features of the internal phase as well as on the applicability (Tolve et al., 2016). Despite the plethora of the different encapsulation methods that have emerged during the previous decades, the major objectives remain similar and are associated with the protection of the core materials from deterioration, the preservation of their properties as well as the controlled release of the encapsulated ingredient during food processing and/or transition through the gastrointestinal track. The implementation of an encapsulation process could lead to the formation of new chemical interactions between the encapsulated compounds and the carrier which subsequently could enhance their stability and functionality (Chen et al., 2023; Foo et al., 2024). A crucial factor that significantly affects the function of the encapsulated product is the final particle size (Stinco et al., 2020). Particle sizes between 1 to 1000 μm are referred as microparticles while sizes below 100 nm are called nanoparticles. Depending on the particle size, the encapsulation methods are separated in two main classes: the microencapsulation and the nanoencapsulation processes (de Queiroz et al., 2022).

5.2.1 Microencapsulation of carotenoids

Microencapsulation is widely considered a frequently employed strategy for packaging bioactive compounds such as carotenoids in liquid, solid or gaseous phases in a continuous coating-like film in order to achieve microcapsule formation (Siregar and Margareta, 2019). The food industry has the potential to use microcapsules; however, it is essential to limit the particle size to 80 μm or less to prevent any adverse effects on the product's sensory attributes including rheological, texture or flavor changes (Tyle, 1993). Typically, the production of microcapsules is a cost-

effective process that enables efficient protection of heat-sensitive compounds such as carotenoids, as well as their controlled release (Dias et al., 2015). However, through microencapsulation the core materials could be susceptible to temperature and pH changes during the process; hence, selecting the appropriate wall materials for microencapsulation poses a challenge (Pang et al., 2014).

5.2.1.1 Spray drying

Spray drying is considered the most commonly employed encapsulation method, involving the conversion of a liquid phase into dry powder through a hot gas stream (Rajabi et al., 2015) (**Figure 5.1**). The technique's simplicity and cost-effectiveness have led to its widespread application. The main drawbacks of the method involve the use of high temperatures that could deteriorate heat-sensitive compounds, as well as the uncontrolled droplet size, resulting in the formation of irregular particles (Both et al., 2020). Spray drying process has already been applied to encapsulate carotenoids (Correâ-Filho et al., 2019; Griep et al., 2023); however, the authors found that the technique negatively impacts their encapsulation efficiency. Due to their heat-sensitive nature, carotenoids might be degraded by the elevated temperatures during the process (Janiszewska-Turak, 2017).

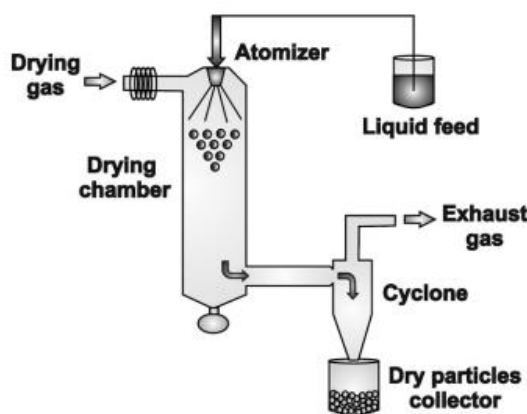


Figure 5.1 Diagram of the process of spray drying technique (Sosnik and Seremeta, 2015).

5.2.1.2 Freeze drying

Freeze drying process is based on the solidification of a solution of interest through exposure on a colder environment. In particular, the frozen water phase of the sample

undergoes sublimation, being directly transformed into vapor without transitioning through the liquid state (Foo et al., 2024). Recent studies have focused on improving this technique by adjusting the encapsulation matrix in order to enhance the quality of the final product, minimizing several limiting factors of the process, such as its lengthy processing time, high cost, and the production of dispersed particles (Anandharamakrishnan et al., 2010). Freeze drying could be utilized for encapsulating carotenoids as it does not cause thermal degradation (Vulić et al., 2022). Sharayei et al., (2024) encapsulated astaxanthin derived from shrimp shells using freeze drying, demonstrating enhanced encapsulation efficiency and high stability.

5.2.1.3 Microemulsion

In addition, emulsification is relied on the formation of droplets within a two-phase liquid system where two immiscible solvents are thoroughly blended (Tsogtoo et al., 2020). There are two primary types of emulsions, the oil-in-water (O/W) emulsions and the water-in-oil (W/O) emulsions with the former being the prevalent type (Chaari et al., 2018; Gomes et al., 2023). Considering that microemulsions have a tendency to separate into two phases due to interfacial tension, the incorporation of artificial or natural surfactants is often required (Cabezas-Terán et al., 2023; Lara-Abia et al., 2023). The development of microemulsions has the potential to enhance several key aspects of the final product, including stability and solubility, by resolving common issues such as droplet aggregation, flocculation, and gravitational separation (McClements, 2015). Even though microemulsions are considered thermodynamically stable, their chemical stability is easily affected by environmental conditions like temperature or pH changes (Aswathanarayan and Vittal, 2019). The emulsification method has also been examined for its effectiveness in encapsulating carotenoids. Microemulsions containing either synthetic β -carotene or natural carotenoids could positively influence both their encapsulation efficiency and *in vitro* bioaccessibility (Guo et al., 2022; Han et al., 2023; Lara-Abia et al., 2023).

5.2.1.4 Liposomes

Liposomal microcapsules are recognized as artificial vehicles consisting of bilayers which were mainly composed of phospholipids (Esposito et al., 2022). Liposomes present similarity with natural membranes, possessing a hydrophilic head and a

hydrophobic tail (Gopi et al., 2018). The physicochemical properties of liposomes contribute to distinctive biological characteristics, including their interaction with a variety of cell membranes (Nasr et al., 2020). Their amphiphilic nature is also responsible for their ability to entrap a plethora of bioactive compounds like the hydrophobic carotenoids (Chaves et al., 2018). They are highly valued by the food, pharmaceutical and cosmetic industries since they could release encapsulated compounds at specific targets, enabling the creation of a controlled delivery system (Zhao et al., 2017). However, the high cost of the process, coupled with their instability during storage, compromises their suitability for industrial applications (Varma et al., 2021). Liposomal microcapsules are also regarded as efficient carriers of carotenoids resulting in improved bioavailability (Ji et al., 2023; Liu et al., 2022; Xu et al., 2022) (**Figure 5.2**).

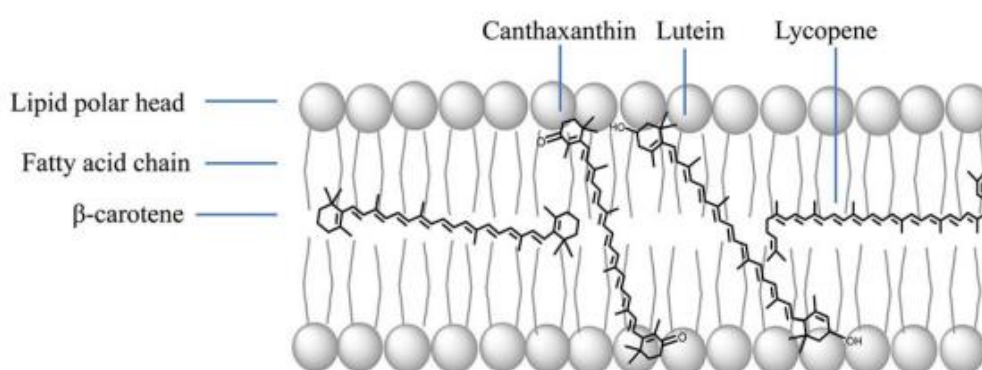


Figure 5.2 Molecular structure and location in the lipid bilayer of different carotenoids (Tan et al., 2014).

5.2.1.5 Oleogels

Oleogelation is a process in which networks are formed through the self-assembly of lipophilic -amphiphilic molecules, enabling the entrapment of an organic liquid within a thermo-reversible, three-dimensional gel matrix (Ramezani et al., 2024). The primary objective in developing this technique was to eliminate *trans* fatty acids and reduce the levels of saturated fatty acids in food applications (Puscas et al., 2020); however oleogels can also act as protective carriers of bioactive compounds. Oleogels could serve as physical barriers against environmental factors, such as oxygen and light, thereby protecting bioactive compounds from degradation and enhancing their

bioavailability. Their solid-like structure might also facilitate the controlled release of the entrapped compounds (Martins et al., 2020; Perța-Crișan et al., 2023) (**Figure 5.3**). Moreover, chemical interactions between oil and oleogelators, such as proteins, polysaccharides, and waxes, could enable the development of tailor-made oleogels with specific, desirable properties (Papadaki et al., 2019b). Among different oleogelators, proteins have gained the attention and they could be an ideal oleogelator candidate, acceptable by consumers due to their high nutritional value (Scholten, 2019). When proteins are used as oleogelators, two main methodologies are commonly applied: the aerogel method and the emulsion-templated method. In the emulsion-templated method, polar and non-polar compounds, such as protein and oil, are processed in three steps: first, forming a hydrogel; then, creating emulsions; and finally, applying a drying technique to form oleogels. Whey protein isolate or concentrate has been widely applied for the production of whey protein-based oleogels via the emulsion-templated technique.

In the context, oleogels could also be utilized as a novel food system for the delivery and protection of carotenoids. In fact, it has been reported that carotenoids could be efficiently encapsulated within oleogels, leading to their improved bioavailability and bioaccessibility (Chen et al., 2016; Ramezani et al., 2024; Zhao et al., 2023).

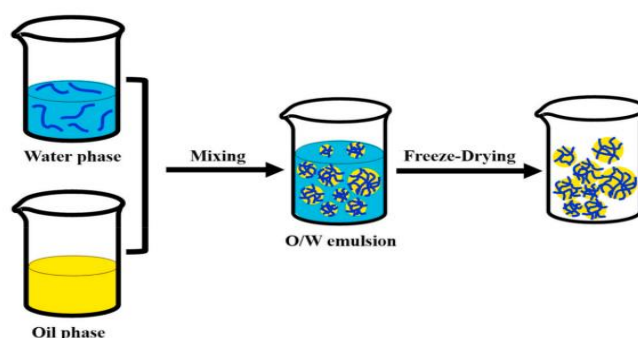


Figure 5.3 Schematic representation of the preparation of oleogel via the emulsion-templated approach (Baraki et al., 2021).

5.2.2 Nanoencapsulation of carotenoids

Research advancements in the field of nanotechnology have enabled development of functional compounds in nanoscale. The principle behind nanoencapsulation methods is similar to microencapsulation where a sensitive compound is coated with a wall

material (Quilaqueo et al., 2019). Nowadays, the direction of research in encapsulation processes is towards achieving increasingly smaller particle sizes. Reducing the particle diameter could offer an improvement on adhesive properties of the final product, potentially leading to enhanced bioavailability as well as optimized site specific delivery, especially during gastrointestinal transit (Human et al., 2019). Furthermore, the stability of the bioactive compounds is ameliorated with the decrease of the particle size (Hidalgo et al., 2017). Moreover, the release of these compounds exhibits a linear dependence on surface area, with smaller particle sizes resulting in increased surface area and therefore, a proportional enhancement of release. The increased surface area could also positively influence the solubility of the encapsulated compounds (Du et al., 2019). Considering the importance of reducing the final particle size, a variety of nanoencapsulated methods have already been emerged. Electrospinning, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) as well as some microencapsulation procedures modified in nanoscale such as nano-emulsification and nano-liposomal formation have been thoroughly investigated.

5.2.2.1 Electrospinning

The electrospinning process involves the production of a polymer solution, which is then injected through a nozzle. Due to electrostatic force applied, a charged polymer droplet is shaped which leads to the formation of a Taylor cone, resulting in the production of nanofibers (Ramos et al., 2022) (**Figure 5.4**).

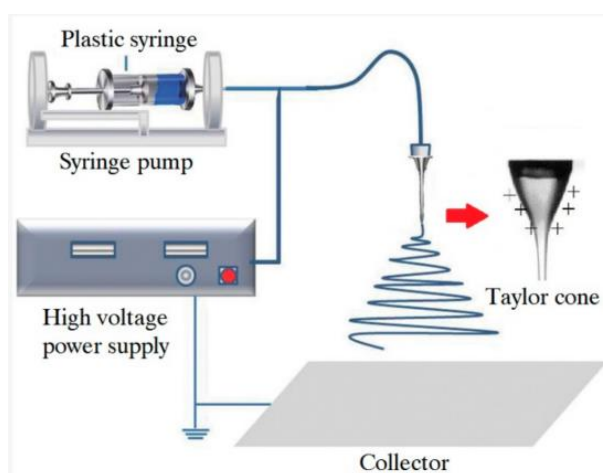


Figure 5.4 Diagram of electrospinning process (Chen et al., 2019).

Besides the smaller particle diameter compared with the microcapsules, the improved stability of nanoparticles is relied on the addition of synthetic surfactants like Tween-80 or natural compounds such as polysaccharides and proteins (Boonpratum et al., 2022; Chen et al., 2023). The increased surface area of nanofibers leads to enhanced solubility, bioavailability and released rate, allowing further applications of the encapsulated bioactive compounds compared with microencapsulated ones (Yildiz et al., 2023). Despite the notable benefits of this method, electrospinning is regarded as a technique with slow production rate which poses limitations for broader commercialization (Muniz et al., 2020). Carotenoids could be efficiently encapsulated through an electrospinning process, which operates at moderate temperatures, thus minimizing the risk of thermal degradation (Xiang et al., 2022). Notably, the encapsulation efficiency of carotenoids exceeded 90%, while the retention rate of the encapsulated carotenoids decreased under various storage conditions compared to unencapsulated carotenoids, demonstrating the effectiveness of the process (Horuz and Belibağlı, 2019; Tian et al., 2024).

5.2.2.2 Solid lipid nanoparticles

The first generation of lipid nanoparticles is known as solid lipid nanoparticles (SLN) which are prepared using lipids that remain solid at room temperature. The formation of crystals could efficiently entrap bioactive compounds between the fatty acid chains offering enhanced protection from environmental conditions (Pink et al., 2019) (**Figure 5.5**). The nature of SLNs allows the encapsulation of both hydrophobic and hydrophilic compounds resulting in nanoparticles with increased bioavailability through a cost-effective process (Rizvi et al., 2019). However, they present low loading capacity as well as decreased stability in comparison with other nanoencapsulation techniques due to sudden expulsion of the encapsulated compound (Montoto et al., 2020). Several studies have investigated the encapsulation of carotenoids using solid lipid nanoparticles; however, this method has shown reduced encapsulation efficiency compared to other encapsulation processes (Dutta et al., 2024; Jain et al., 2019).

5.2.2.3 Nanostructured lipid carriers

A second generation of lipid nanoparticles has been developed, the nanostructured lipid carriers (NLC). The main difference with SLNs is relied on their composition where the inclusion of varying ratios of liquid lipids (oils) at room temperature results in reduced crystalline degree (Salvi and Pawar, 2019) (**Figure 5.5**). The structural changes in NLCs improve their physical and chemical stability, preventing expulsion and increasing both encapsulation efficiency and loading capacity (Katouzian et al., 2017). The formation of nanostructured lipid carriers resulted in enhanced encapsulation efficiency and increased loading capacity (Zardini et al., 2018; Osanlou et al., 2022). Additionally, nanostructured lipid carriers provide enhanced physical stability of carotenoids over time and improved bioaccessibility compared to free carotenoids (Sirikhet et al., 2021; Teeranachaideekul et al., 2022).

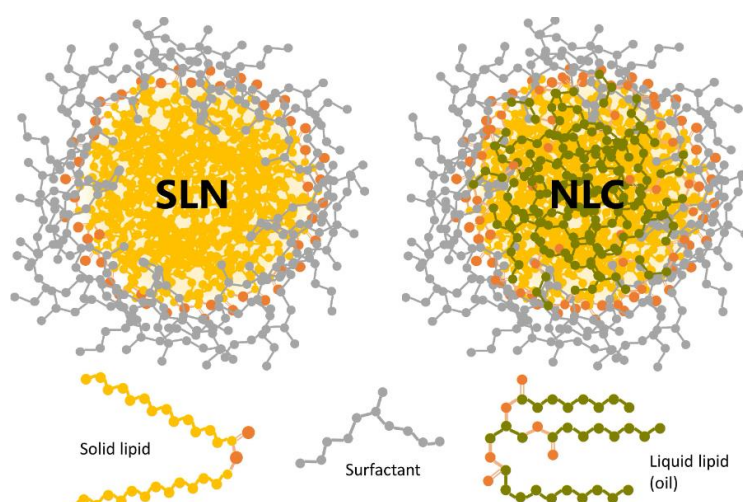


Figure 5.5 Schematic representation of SLN and NLC (Montoto et al., 2020).

5.2.2.4 Nanoemulsion

Several microencapsulation techniques have been adapted in nanoscale. The structure development of nanoemulsions remains similar with microemulsions consisted of an oil phase, a water phase and surfactant (Bi et al., 2023). It is worth mentioning that in nanoemulsions, the amount of emulsifiers is reduced due to improved physical stability against gravitational separation and droplet aggregation. This enhanced stability is achieved through the development of new forces, including Van der Waals, as a result of smaller particle sizes (Kharat et al., 2020). Nanoemulsions present

higher surface area compared with microemulsions resulting in improved release rates and bioaccessibility of the encapsulated compound (McClements and Rao, 2011). However, in contrast with microemulsions, they are characterized as thermodynamically unstable (Aswathanarayan and Vittal, 2019). In the nano-emulsification process, an enhanced encapsulation efficiency of carotenoids is observed. However, the nanoparticles appear to exhibit low stability, as evidenced by a decrease in entrapment efficiency and an increase in particle size during storage at moderate temperatures (approximately 40 °C) when compared to other encapsulation techniques (Ali et al., 2022; Liu et al., 2022).

5.2.2.5 Nanoliposomes

The development of nanoliposomes follows a similar strategy to that used for conventional liposomes. These nanoscale bilayer vesicles, which are also mainly composed of phospholipids, exhibit enhanced physicochemical properties. Nevertheless, main impediments of the method lies in the difficulty of controlling nanoliposomes' particle size along with their limited shelf life (Demirci et al., 2017; Esposto et al., 2022). In previous years, the utilization of nanoliposomes was limited due to their characterization as thermodynamically unstable. In addition to their high encapsulation efficiency, nanoliposomes demonstrate significant stability under low temperature storage conditions, which appears to be correlated with the concentration of the entrapped carotenoids (Chang et al., 2022; Hamadou et al., 2020).

5.2.3 Combination of encapsulation techniques

Aiming to further improve encapsulation efficiency and the physicochemical properties of the final product, the implementation of combined encapsulation techniques is currently being intensively investigated. Emulsification could be employed as a preliminary step in conjunction with other methods to incorporate an oil phase, independent of the technique used, thereby providing additional protection to carotenoids, which are lipophilic compounds. Considering that spray drying is regarded as the least favorable technique for carotenoid encapsulation due to their thermo-sensitive nature, the most common combination among the microencapsulation processes is emulsification followed by spray drying. Recent studies have shown that this combination significantly enhances the encapsulation

efficiency of carotenoids compared to the efficiency observed after spray drying (Li et al., 2023; Nunes et al., 2024). The combination of emulsification and freeze drying process has also been evaluated to further enhance the final characteristics of microparticles. Notably, the entrapment efficiency did not follow the same trend as described above; instead, it showed a slight decrease (Šeregelj et al., 2021; Shi et al., 2023). However, recent research indicates that the bioaccessibility of carotenoids could be significantly improved through this combination in comparison with other encapsulation techniques (Meira et al., 2023).

Currently, two experimental approaches have been developed to combine nanoencapsulation with microencapsulation techniques. One approach involves developing nanoparticles prior to employing a microencapsulation method, while the other approach follows the reverse route. Both of these strategies result in reduced particle sizes. After the formation of nanoparticles, freeze drying is most commonly employed. Besides the enhanced encapsulation efficiency achieved through this method (Assunção et al., 2021), Oliveira et al., (2021) demonstrated that freeze drying also enhances storage stability. Taking into account the alternative approach of combining the micro- and nano- encapsulation methods, emulsification serves as the primary method preceding the formation of nanoparticles. The most commonly reported combination in the literature involves microemulsion followed by the electrospinning process (Drosou et al., 2022; İnan-Çınkır et al., 2023; Xiang et al., 2022). Chen et al., (2023) demonstrated that the nanoparticles formed with the aforementioned technique presented improved encapsulation efficiency of lycopene and enhanced photostability and thermostability. Moreover, both the release rate of lycopene and its bioaccessibility were significantly increased compared with the free form. It is noteworthy that, depending on the coating matrix, the developed nanoparticles might release the encapsulated carotenoids at specific targets, such as colon (Chen et al., 2023; Liu et al., 2023).

5.3 Applications in food and limitations

Numerous studies have focused on the production of encapsulated carotenoids to evaluate their potential across various fields at an industrial scale. Among the various applications of carotenoids, food and feed sectors represent the largest market

demand; therefore, research on the potential use of encapsulated carotenoids as food ingredients is of great importance (Markets and Markets, 2024). Recent studies have targeted food applications, with special focus on dairy products and particularly yogurt. Horuz and Belibağlı, (2019) encapsulated a carotenoid extract derived from tomato peels using the electrospinning process, and 0.1% of the nanofibers were incorporated into a yogurt which was then stored for 15 days at 4 °C. Yogurt properties, including pH, acidity, and syneresis, were then evaluated. The tested parameters showed no significant alterations compared to the control sample, indicating that the presence of nanofibers did not negatively affect the final product. Additionally, synthetic lycopene was encapsulated using nanoemulsions, and 1% (w/v) was mixed into a yogurt product, which was then stored under refrigeration for 4 weeks. Parameters such as pH, acidity, and water-holding capacity were evaluated throughout this time frame. The addition of nanoparticles did not significantly impact the product, except for the water-holding capacity, which was enhanced when encapsulated carotenoids were utilized. This demonstrates the ability of nanoemulsion to improve water retention (Himanath et al., 2021). Another dairy product used for the application of encapsulated carotenoids is paneer, a soft cheese from India. Lutein was extracted from *Citrus reticulata* (kinnow), encapsulated using the nanoemulsion method, and then paneer was dipped into it. The product was stored at 4 °C for 8 days, during which textural and sensory evaluations were conducted. The texture of the final product was negatively affected by the presence of the nanoemulsion, resulting in decreased cheese hardness. However, the sensory evaluation did not significantly differ from the control sample, and the overall acceptability of the final product at the end of the storage period showed a notable improvement compared to the control (Saini et al., 2022).

Beyond dairy products, Zardini et al., (2018) incorporated 0.5% (w/w) lycopene-loaded nanoparticles derived from SLN technique, into an orange drink. A sensory evaluation of the final product revealed no significant changes. However, overall acceptability decreased compared to the control drink, as the nanoparticles reduced the orange odor of the sample. In another study, 20 g of nanoparticles derived from a microemulsion, followed by electrospinning and containing synthetic β -carotene, were incorporated into meal replacement bars. A sensory score rating for the sample

showed results comparable to the control with respect to color, mouth feel, and texture (Niu et al., 2020). Although the incorporation of nanoencapsulated carotenoids into food products has been already demonstrated research on the retention rate of encapsulated carotenoids during storage, as well as their release rate and bioaccessibility after consumption, remains limited. In addition, the consumer perceptions about nanotechnology might further hinder the commercialization progress of functional foods containing nanoparticles (Siegrist and Hartmann, 2020). The limited consumers' knowledge on food production and nutrition, coupled with their misconceptions about the environmental impact of nanotechnology, contributes to food technology neophobia, leading to a tendency to reject unfamiliar food products (Cavaliere and Ventura, 2018; Hartmann and Siegrist, 2017).

More importantly, it is essential to adhere to the regulations of the relevant authorities pertaining to food products before launching the final product in the market on an industrial scale, (María and Del, 2010). Particularly in the food industry, EFSA regulations require separate physicochemical characterization and risk assessment of each nanomaterial, as well as their combinations. The implementation of toxicological tests is essential as the nanoscale particle size might influence their toxicokinetic behavior. Besides their smaller particle size, changes in encapsulation processes could also result in different or/and new characteristics. Hence, the nanomaterials could interact differently with tissues and organs, so their passage through the GI tract could result in translocated nanoparticles that might cause local adverse effects (More et al., 2021). Few studies evaluate the cytotoxic effects of nanoencapsulated carotenoids, and there is a lack of research addressing their impact when added to food products. Chen et al., (2023) evaluated the cytotoxic effect of synthetic lycopene encapsulated in nanofibers, produced through microemulsion followed by an electrospinning process, on CaCo-2 cells. Their results demonstrated that both the cytotoxicity of free lycopene and of lycopene-loaded micelles were dose-dependent, with concentrations ranging from 0 to 5 $\mu\text{g}/\text{mL}$ showing no adverse effects on the cell line, nor any disruption to the integrity of the cytomembrane. The toxicity of astaxanthin encapsulated in nanosized liposomes was also evaluated by Chang et al., (2022) through testing the viability of RAW 264.7 macrophages and 7F2 osteoblast cells. The liposomal formulation exhibited reduced cytotoxicity compared to the astaxanthin

extract, with a dose of 0.51 $\mu\text{g/mL}$ maintaining 80% cell viability in RAW 264.7 macrophages. In the case of 7F2 osteoblasts, 0.25 $\mu\text{g/mL}$ of astaxanthin-loaded liposomes not only reduced the cytotoxicity of the astaxanthin extract but also demonstrated potential to stimulate cell proliferation. Additionally, nanofibers containing fucoxanthin, produced through the electrospinning process, were examined for their effects on the cell viability of HepG2 cells. Their results indicated that 2 $\mu\text{g/mL}$ of nanofibers maintained cell viability at 85%, and the fucoxanthin-based delivery system demonstrated efficient biocompatibility, supporting its potential for future cellular applications (Tian et al., 2024). Moreover, nanoparticles produced via SLNs containing synthetic β -carotene were evaluated for their cytotoxic effects on MCF-7 cells by Jain et al., (2019). Neither free β -carotene nor the encapsulated form showed any inhibitory effect on cell viability at concentrations below 10 $\mu\text{M/mL}$. Conversely, Dutta and Dutta, (2024) investigated the *in vitro* cytotoxicity of nanoparticles produced using SLNs technique containing β -carotene on the MCF-7 cell line. The study found that cell viability was significantly reduced with the nanoparticles (IC_{50} of 14.89 $\mu\text{g/mL}$), compared to free β -carotene, which had an IC_{50} value of 182.6 $\mu\text{g/mL}$. Therefore, the encapsulated matrix may be crucial for the cytotoxic effects of nanoencapsulated carotenoids on different cell lines.

It is therefore essential to evaluate the physicochemical properties of encapsulated carotenoids to develop potential new functional food products. The additional evaluation of the toxicological effects of nanoencapsulated carotenoids is of greater importance and is required to ensure the safety of the final products. This will provide regulatory agencies with the necessary evidence to amend existing food product regulations.

CHAPTER 6

Objectives

6 Objectives

Rising market demand for carotenoids across diverse applications, along with concerns over the potential toxicity of synthetic carotenoids, has driven the search for safer, natural alternatives. In the context of the bio-economy era, producing valuable compounds like carotenoids through microbial processes using renewable resources could support the shift to a circular bio-economy, reducing environmental impact and enabling cost-competitive production. The incorporation of biotechnologically produced carotenoids into functional food systems requires green extraction methods and encapsulation techniques to prevent degradation from environmental factors.

Under this prospect, the present thesis has focused on the development of bioprocesses for the efficient production of microbial carotenoids from both commercial and renewable resources, aiming their utilization in novel food applications. More specifically, various yeast strains were evaluated for their ability to synthesize carotenoids while assimilating different carbon sources and under different fermentation conditions (carbon-to-nitrogen ratio (C/N), temperature). Additionally, the evaluation and optimization of a green extraction process using supercritical CO₂ extraction method was also conducted. Finally, the potential application of carotenoids was evaluated through the production of oleogel and electrospun as novel food formulations.

The main objectives of the experimental work are presented below:

- ❖ Evaluation of four yeast strains belonging to *Rhodospiridium* genera to produce value-added compounds such as carotenoids in shaking flasks. At the same time the effect of different carbon sources and C/N ratio on carotenogenesis was evaluated.
- ❖ Chemical characterization of the biotechnologically produced carotenoids along with the analysis of their antioxidant and antimicrobial properties.
- ❖ Evaluation of cheese whey as fermentation feedstock for the biotechnological production of carotenoids and microbial oil in shaking flasks. The effect of

temperature and C/N ratio was also evaluated and the conditions were optimized for maximum carotenoid synthesis.

- ❖ Evaluation of molasses as fermentation feedstock for the biotechnological production of carotenoids and microbial oil in bioreactor scale.
- ❖ Development of an efficient recovery process of carotenoid-rich microbial oil using supercritical CO₂ extraction. Cell disruption techniques were also evaluated in order to optimize the extraction process. The kinetic behavior of both bioactive compounds throughout the extraction process was also examined.
- ❖ Study on the application of electrospinning as a suitable encapsulation process for the production of novel electrosprayed particles containing the carotenoid-rich microbial oil. The feasibility of incorporating the microbially-produced EPS in the encapsulating matrix was also assessed. Electrospinning process was evaluated through the analysis of microbial oil and carotenoids encapsulation efficiency.
- ❖ Development of novel oleogels using carotenoid-rich microbial oil through the emulsion-templated approach. Oleogelation process and potential applications of the formed oleogel were evaluated through the analysis of its physical properties and thermal stability of carotenoids and oil.

CHAPTER 7

Materials and Methods

7 Materials and Methods

7.1 Microorganisms, maintenance and growth conditions

7.1.1 Yeast strains

Rhodospiridium kratochvilovae strains (FMCC Y-42 & Y-43) were kindly provided by Prof. E. Panagou of the Laboratory of Food Microbiology and Biotechnology (Agricultural University of Athens, Greece). *Rhodospiridium paludigenum* NCYC 2663 and NCYC 2664 were purchased by the National Collection of Yeast Cultures (NCYC, UK). Yeast strains were maintained at - 80 °C, in cryovials filled with 20% glycerol in yeast, peptone, dextrose (YPD) medium (10%, w/v). Yeast propagation was performed on YPD agar plates at 28 °C for two days and then maintained at 4 °C up to two weeks. A single colony from agar plates was used to inoculate the pre-cultures, consisting of 50 mL YPD medium in 250 mL Erlenmeyer flasks. Pre-cultures were incubated at 28 °C, 180 rpm for 27 h.

7.1.2 Fungal strains

The strains *Aspergillus niger* FMCC F-7 and *Aspergillus flavus* FMCC F-8 were kindly provided by the Laboratory of Food Microbiology and Biotechnology (Agricultural University of Athens). All fungal strains were maintained at - 80 °C in cryovials filled with 20% (v/v) glycerol. Pre- culture preparation of fungal strains was carried out by incubating 10 µL inoculum on MEA (malt extract agar) plates at 25 °C for 7 days. Subsequently, the fungal spores were collected in 10 mL of 0.5% Tween 80 and further diluted to obtain fungal spore suspensions of 10⁶ cfu/mL.

7.1.3 Bacterial strains

The strains *Listeria monocytogenes* FMCC B-124, *Staphylococcus aureus* FMCC B-134, *Pseudomonas aeruginosa* FMCC B-223 and *Salmonella enteritidis* FMCC B-286, were kindly provided by the Laboratory of Food Microbiology and Biotechnology (Agricultural University of Athens), while the strains *Staphylococcus epidermidis* FBMP_121, *Staphylococcus saprophyticus* FBMP_111, *Klebsiella oxytoca* FBMP_1333 and *Clostridium difficile* were kindly provided by the Laboratory of Applied Microbiology & Biotechnology, Department of Molecular

Biology & Genetics (Democritus University of Thrace). All bacterial strains were maintained at -80 °C in cryovials filled with 20% (v/v) glycerol. Reactivation of the bacterial pathogens was performed twice by adding 100 µL inoculum in 10 mL Tryptic Soy Broth (TSB) followed by incubation at 37 °C for 24 h. The final inoculum was adjusted at 10⁶ cfu/ mL.

7.2 Microtiter experiments

Microtiter experiments with the strains *Rhodospiridium kratochvilovae* (FMCC Y-42 & Y-43) were performed using a 96-well microtiter plate system (EPOCH2 Microplate reader, BioTek, USA). Final volume was 200 µL, with an inoculum size of 10% (v/v), which was previously diluted to a final optical density OD_{620nm} = 0.200. Three carbon sources, namely glucose, galactose and glucose-galactose (1:1) and six different C/N ratios (40, 60, 80, 100, 120 and 140) were used as substrates. The C/N ratios were calculated as grams of carbon per gram of nitrogen. The concentration of all carbon sources was 50 g/L in all experiments and yeast extract was added at a concentration of 0.5 g/L, whereas different concentrations of (NH₄)₂SO₄ were applied to adjust the C/N ratio (**Table 7.1**). A mixture of trace elements was also used, with the following composition (g/L): KH₂PO₄ (7.0), Na₂HPO₄ (2.5), MgSO₄·7H₂O (1.5), CaCl₂·2H₂O (0.15), FeCl₃·6H₂O (0.15), MnSO₄·H₂O (0.06), ZnSO₄·7H₂O (0.02). Incubation took place at 28 °C with interval shaking of 2 s at 200 rpm every 20 min.

Table 7.1 The composition of substrate in each C/N ratio.

C/N (g/g)	Yeast (g/L)	Ammonium sulfate (g/L)
40	0.5	2.08
60	0.5	1.32
80	0.5	0.93
100	0.5	0.7
120	0.5	0.54
140	0.5	0.43

OD measurements were used to estimate microbial growth and the specific growth rate (μ , h^{-1}) was calculated by the OD of three independent replicates within the linear logarithmic phase of cell growth, using the following equation:

$$\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1} \quad (\text{eq.1})$$

7.3 Fermentations in shaking flasks

7.3.1 Fermentation with commercial carbon sources

7.3.1.1 Fermentations with yeasts *Rhodosporidium kratochvilovae* FMCC Y-42 & Y-43

Shake flasks fermentations with the strains *R. kratochvilovae* were carried out in 100 mL Erlenmeyer flasks filled with 20 mL of fermentation media, using different commercial carbon sources. Sugar concentration was equal to 50 g/L in all tested conditions. The effect of different C/N ratios (60, 80, 120) was studied in fermentations using glucose or glucose-galactose 1:1 (50 g/L) as carbon sources, while the C/N ratio equal to 80 was also evaluated in fermentations using sucrose and fructose (50 g/L) as carbon sources. The nitrogen sources and trace elements were identical to the fermentation media of microtiter experiments (Section 7.2). Inoculation was performed by addition of 10% (v/v) pre-culture under sterile conditions and the cultures were incubated at 28 °C, under continuous agitation (180 rpm) at pH 5.8 ± 0.2 .

7.3.1.2 Fermentations with yeasts *Rhodosporidium paludigenum* NCYC 2663 & 2664

The effect of different carbon sources (sucrose, glucose, fructose, glucose: galactose 1:1 (w/w)) was evaluated in shaking flasks with 20 mL working volume using the strains *R. paludigenum* NCYC 2663 and 2664. The initial sugar and yeast extract concentrations were 50 g/L and 0.5 g/L, respectively in all cases. Different concentrations of $(\text{NH}_4)_2\text{SO}_4$ were applied to adjust the C/N ratio. A C/N ratio equal to 80 was selected for the strain *R. paludigenum* 2663, and 20 for the strain *R. paludigenum* 2664. The media were also enriched with a mixture of trace elements similar to the one presented in Section 7.2. The pre-culture was in all cases 10% (v/v),

and fermentations were carried out at 28 °C, under continuous agitation (180 rpm) at pH 5.8 ± 0.2 .

7.3.2 Shaking flask fermentations with cheese whey as substrate

7.3.2.1 Cheese whey

Cheese whey, containing approximately 50 g/L of lactose, was used as carbon source in submerged fermentations with strains *Rhodospiridium kratochvilovae* Y42 and Y43. Cheese whey was obtained after mizithra cheese making and was kindly provided by “Galiatsatos” dairy company (Kefalonia, Greece).

7.3.2.2 Pretreatment and Lactose Hydrolysis

Prior to fermentation, cheese whey was treated to generate cheese whey permeate (CWP). Deproteinization of cheese whey was carried out at 121 °C for 15 min after adjusting pH at 4.5 (Kachrimanidou et al., 2022). Precipitated proteins were removed by centrifugation at 4000 rpm for 10 min at 4 °C and the supernatant was stored at -20 °C until further use. CWP was then enzymatically processed with commercial β -galactosidase from *Aspergillus oryzae* (59.4 units/mg solid enzyme). Hydrolysis was carried out at 50 °C, using 2 U/g of lactose for 40 h, resulting in >90% lactose hydrolysis yield. Besides the use of commercial enzyme preparations, CWP was also mixed with fermented wheat bran following the protocols described by Lappa et al., (2021), yielding to almost 91% lactose hydrolysis and to the production of ~500 mg/L free amino nitrogen (FAN). The hydrolysate was further diluted with appropriate amount of cheese whey for the preparation of a solution containing 350 mg/L FAN.

7.3.2.3 Fermentations using hydrolyzed CWP

CWP previously hydrolyzed with commercial β -galactosidase was employed as fermentation substrate in shaking flask experiments with *Rhodospiridium kratochvilovae* strains (FMCC Y-42 & Y-43). The substrate was also enriched with 0.39 g/L $(\text{NH}_4)_2\text{SO}_4$. Inoculation was performed by adding 10% v/v pre-culture, under sterile conditions, in 20 mL final volume incubated at 28 °C under continuous agitation (180 rpm) at pH 5.8 ± 0.2 . These parameters simulated the optimum cultivation conditions using synthetic glucose-galactose 1:1 (50 g/L) as substrate, as

described previously (Section 7.3.1.1). Respectively, CWP hydrolyzed using fermented wheat bran was also employed using the strains *Rhodospiridium kratochvilovae* (FMCC Y-42 & Y-43). The effect of two different C/FAN ratios (40, 60 g/g) and three different temperatures (23, 28, 33 °C) was investigated.

7.4 Bioreactor fermentations with *Rhodospiridium paludigenum* NCYC 2663

7.4.1 Batch fermentation with commercial sucrose

A batch-fermentation was carried out in a 3 L bench top bioreactor (Minifors 2, Infors HT, Bottmingen-Basel, Switzerland) with 1.5 L working volume. Sucrose was used as the sole carbon source at a concentration of 50 g/L. The C/N ratio was equal to 80 and composed of a combination of yeast extract (0.5 g/L) and (NH₄)₂SO₄ (1 g/L). The medium was additionally enriched with a trace element solution, as presented in Section 7.2. Inoculation was carried out by adding 10% (v/v) pre-culture under sterile conditions. The pH was initially adjusted to 6.0 and controlled throughout the process. An antifoam solution (1%, v/v) was added to regulate foam formation. The dissolved oxygen was controlled at 40% with an agitation cascade at 200-400 rpm and an air flow rate cascade from 1.5 to 2.5 L/min. Samples were withdrawn at regular time intervals to monitor cell growth, the production of microbial oil, carotenoids and exopolysaccharides as well as sugar and nitrogen consumption.

7.4.2 Batch fermentation with molasses

7.4.2.1 Molasses

Molasses, containing approximately 750 g/L sucrose, were used as carbon source in batch and fed-batch bioreactor fermentations. Molasses was purchased from a local market (Kefalonia, Greece).

7.4.3 Batch fermentation with molasses

Molasses were also used as the sole carbon source in bioreactor fermentations using the strain *R. paludigenum* 2663. Molasses were diluted to a final concentration of 50 g/L to simulate the experiment using sucrose as substrate. The experimental conditions were as described in Section 7.4.1, without the addition of a trace element solution.

7.4.4 Fed-batch fermentation with molasses

Molasses were also evaluated in a fed-batch fermentation strategy. The medium contained a C/N ratio equal to 40 composed of a combination of yeast extract (1 g/L) and $(\text{NH}_4)_2\text{SO}_4$ (2 g/L). A concentrated molasses solution was used as feeding, added in pulses at specific time intervals. The operating conditions were as described in Section 7.4.1.

7.5 Recovery of microbial oil and carotenoids produced by *Rhodospiridium paludigenum* 2663

7.5.1 Pre-treatment of yeast biomass

Yeast biomass was recovered via centrifugation of the fermentation medium (4000 rpm, 20 min, 4 °C), washed at least twice with distilled water and then freeze-dried for 48h (BK-FD12P, Biobase Biodustry Co Ltd., Jinan, China). Six different pre-treatments were applied to biomass (20 g each) to achieve cell lysis before extraction of the microbial oil and carotenoids using a supercritical fluid extraction apparatus operating with CO_2 (SFE- CO_2).

Pretreatment using ultrasounds (Process 1)

Yeast cells were dissolved in 150 mL ethanol (98%, v/v) and placed in an ultrasonic bath (40 KHz, 130W, Sonica 2400 M S3, Soltec Milano, Italy) for 2.5 h. Cell lysis was monitored under a microscope after mixing the pretreated yeast cells with tryptan blue (1:2, v/v). Then, the pretreated cell suspension was added into the SFE- CO_2 extraction vessel (Section 7.5.2). Ethanol was also introduced in the system during the SFE- CO_2 (Section 7.5.2). The impact of ultrasound treatment on carotenoid yield was assessed by quantifying the carotenoid content (Section 7.8.6.2) before and after the pre-treatment.

Pretreatment with High Speed Homogenization (Processes 2, 3 and 4)

The use of high speed homogenization as cell disruption method was evaluated as presented below:

Process 2: Yeast cells were dissolved in 150 mL of ethanol and homogenized three times at 13,000 rpm for 5 minutes, with a 5-minute rest between each homogenization (homogenizer HG-15D, Witeg Labortechnik GMBH, Wertheim, Germany). Cell disruption was monitored as previously described (Process 1). The cell slurry was added in the extraction vessel for the SFE-CO₂ process and ethanol was also added in the system as co-solvent (Section 7.5.2).

Process 3: Cell disruption was carried out similarly to Process 2 however the SFE-CO₂ process was performed without extra ethanol addition.

Process 4: Yeast cells were recovered by centrifugation and the cell slurry was subjected to homogenization as described in Process 2 without mixing with ethanol. Cell lysis was monitored as in Process 1 and then freeze-dried prior to SFE-CO₂.

Pretreatment with glass beads and high speed homogenization (Process 5)

Yeast cells were mixed with 150 mL of ethanol and 40 g of glass beads (2.8 mm diameter) and left overnight, at 28 °C and 180 rpm. After incubation, glass beads were removed, and the remaining slurry was further treated by homogenization as described in Process 2.

Pretreatment using decompression (Process 6)

Freeze-dried yeast cells were added in the extraction vessel and subjected to static supercritical CO₂ for 1 h, at 60 °C and 185 bar. Then decompression was applied to 80 bar to disrupt yeasts cell wall (Vladić et al., 2022).

7.5.2 Supercritical fluid extraction with CO₂ of the pretreated biomass

SFE-CO₂ was carried out using an ExtrateX apparatus (Extratex-SFI, France) with a maximum temperature and pressure capacity of 150 °C and 700 bar, respectively. All tests were carried out for 4 h (plus 30 min to exhaust the co-solvent from the system) and the operation conditions were set following a modified protocol based on Larocca et al., (2023). Briefly, the flow rate of high purity CO₂ was set to 20 g/min, while absolute ethanol was employed as co-solvent at a rate of 1.5 mL/min in all cases with the exception of Process 3. The operating temperature was 60 °C and the pressure was

set to 350 bar. Extracts were recovered in amber vials every 30 min, subjected to vacuum evaporation at 40 °C to remove ethanol, weighed and stored at -80 °C for subsequent analysis.

7.5.3 Supercritical CO₂ extraction kinetics of carotenoids and microbial lipids

To monitor the extraction kinetics of both carotenoids and lipids, 20 g of pre-treated cells using the most efficient process (Process 2) were added in the extraction vessel and SFE-CO₂ was performed as described in Section 7.5.2. The extracts were collected in amber vials every 15 min for the first 2 h and every 30 min for the last 2 h. Co-solvent addition was stopped after 4 h of operation and the final sample was collected 30 min later. Then, the extracts were separately evaporated to remove ethanol, weighed and stored at -80 °C until further analysis. The recovery yields of total carotenoids and oils were calculated using the value of each component divided by the yield corresponding at 270 min (end of the process).

7.6 Encapsulation of carotenoids-rich microbial oil using an electrospraying process

7.6.1 Preparation of emulsions

Whey protein concentrate (WPC) was dissolved in distilled water (10%, w/v) and continuously stirred overnight. The pH of the solutions was then adjusted to 8, depending on the experimental conditions, under stirring. Following pH adjustment, the WPC solution was heated at 80 °C for 40 min and subsequently stored at 4 °C. Emulsifiers were added to the solution at varying concentrations under continuous stirring. The concentrations of Tween-80 used were 0.05% and 0.5% (w/w), Triton-X was used at 0.5% (w/w), and exopolysaccharides (EPS) were added at concentrations of 0.05% and 1% (w/w). Microbial oil containing carotenoids derived from *Rhodospiridium paludigenum* NCYC 2663 was then mixed to WPC solution at 3% (w/w), under magnetic stirring. The mixed solutions were homogenized using a high-speed homogenizer at 11,000 rpm for 5 min. The emulsions were subsequently placed to ultrasonic bath for 15 min, followed by cooling under refrigeration conditions prior to further use.

7.6.2 Electrospinning conditions

The encapsulation process was performed on the emulsions containing carotenoids enriched microbial oil using an electrospinning device (Fluidnaked LE-50, Bionicia). The emulsions were loaded into a 10 mL syringe with a diameter of 14.43 mm, equipped with a 20-gauge steel needle (0.8 mm inner diameter). The distance between the needle tip and the collector was at 19 cm. Temperature was controlled to approximately 10 °C. Electrospinning was conducted at an applied voltage of 24 kV, while a voltage of -2 kV was set to the collector. The syringe pump, containing the emulsions, was set to a feed rate of 0.3 mL/h. The resulting electrospun was collected and stored at -20 °C until further use.

7.7 Oleogelation

7.7.1 Preparation of oleogels

The oleogels were prepared following the protocol of Plazzotta et al., (2020), with some modifications. Briefly, WPC was dissolved in distilled water at a concentration of 20% (w/v) and continuously stirred overnight at 4 °C. Following pH adjustment to 5.7, the solution was heated to 85 °C for 15 min then cooled to 4 °C. The WPC hydrogel was subsequently homogenized using a high-speed homogenizer at 13,000 rpm for 5 min. Then, carotenoids-rich microbial oil was added to the hydrogel and further homogenized for 30 sec. The emulsion was then stored overnight at -80 °C and subsequently freeze-dried. The obtained oleogel was kept at -80 °C until its characterization.

7.7.2 Stability of carotenoids during oleogelation

The carotenoid concentration was evaluated during the three preparation steps of the oleogel: homogenization, freezing at -80 °C, and freeze-drying, and compared with the initial concentration of carotenoids of the microbial oil. Briefly, three replicates of precisely weighed samples were withdrawn after each preparation step and were used for the determination of carotenoids. Carotenoid extraction was carried out following the protocol of Guo et al., (2022), with some modifications. Briefly, precisely weighed amount of emulsion or oleogel was combined with a mixture of 4 mL of ethanol: hexane (2:3 ratio). The mixture was vigorously vortexed and centrifuged

(4800 rpm, 30 min, 4 °C). The upper hexane phase was collected and evaporated under vacuum. Carotenoid concentration was evaluated as described in Section 7.8.6.2. The degradation rate of each carotenoid was mathematically expressed using a zero-order kinetic model.

7.7.3 Thermal stability of oleogel

The stability of the oleogels was assessed at the temperature range of 50, 70, and 90 °C. Glass tubes containing a specific quantity of oleogel were placed in a waterbath and samples were withdrawn at several time intervals (0, 10, 30, 60 and 90 min). The samples were analyzed concerning the peroxide value of oil and carotenoids concentration to evaluate the oil oxidative stability and carotenoids degradation. The extraction of carotenoids was performed as described in Section 7.7.2 while the carotenoid concentration was evaluated following the protocol of Section 7.8.6.2. The extraction of microbial oil was employed according to Section 7.7.2 and the peroxide value as described in Section 7.8.9. The results were compared with those obtained from non-encapsulated carotenoid-rich microbial oil. Both the oxidation kinetics of microbial oil and the degradation kinetics of carotenoids were evaluated with a first order kinetic model (Xiao et al., 2018) following the equation :

$$\log \left(\frac{PV \text{ or } C}{PV_0 \text{ or } C_0} \right) = -k \times t \quad (\text{eq.2})$$

where, PV or C are the oil oxidation or carotenoid concentration, PV_0 or C_0 are the initial oil oxidation or carotenoid concentration, k is the reaction rate constant at each temperature and t is the heating time (min).

The obtained k values for each temperature were fitted to the Arrhenius equation to determine the dependence of oil oxidation and carotenoids degradation on heating temperature:

$$k = A \times e^{-\frac{E_a}{RT}} \quad (\text{eq. 3})$$

The equation was linearised by applying the logarithm:

$$\ln k = -\frac{E_a}{RT} + \ln A \quad (\text{eq. 4})$$

where, k is the reaction rate constant, E_a is the activation energy (J/mol), R is the universal gas constant (8.314 J/mol·K), T is the absolute temperature (K) and A is the pre-exponential factor. The E_a was estimated based on linear regression analysis of the plot of $\ln k_i$ vs $1/T_i$ where $i = 50, 70$ and 90 °C.

7.8 Analytical methods

7.8.1 Determination of dry cell weight (DCW)

Yeast cells were recovered from the fermentation medium by centrifugation (Rotina 420R, Hettich, Germany) at 4000 rpm (10 min, 4 °C). Precipitated cells were washed twice with distilled water and then dried at 60 °C until a constant weight was obtained. Biomass was expressed as grams of total dry weight per liter of fermentation (g/L).

7.8.2 Determination of free amino nitrogen (FAN) and inorganic nitrogen (IN) content

Free amino acid analysis during fermentations was determined following the ninhydrin colorimetric method (Lie, 1973). Briefly, 1 mL of adequately diluted sample was mixed with 0.5 mL of color reagent with the following composition (in g/L): 39.63 Na_2HPO_4 , 5 ninhydrin, 3 fructose and 40 KH_2PO_4 (pH at 6.8). The mixture was heated at 100 °C for 16 min and then cooled for 20 min in a water bath. Then, 2.5 mL of dilution reagent (composed of 2 g KIO_3 in 616 mL dH_2O and 384 mL ethanol) were added and the absorption was measured at 570 nm. Different concentrations of glycine were utilized in order to prepare a standard curve.

Inorganic nitrogen during fermentations was analyzed according to the method proposed by Scheiner, (1976). Briefly, 50 μL of adequately diluted sample was mixed with 2 mL of dH_2O and 1 mL of 5% phenol diluted in buffer solution (1.3 g Na_3PO_4 , 3 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and 0.3 g EDTA, pH 12). The mixture was vigorously shaken and 1.5 mL of alkaline solution (2 mL of NaClO in 80 mL NaOH and 120 mL dH_2O) were added. The absorption of the mixture was measured at 635 nm after incubation for 1 h at 25 °C. A standard curve was obtained using different concentrations of $(\text{NH}_4)_2\text{SO}_4$.

7.8.3 Determination of sugars

The sugar consumption during fermentations was determined by the analysis of the supernatant using High Performance Liquid Chromatography (HPLC) (Agilent) equipped with a Rezex ROA-organic acid H⁺ column (300 mm length × 7.8 mm internal diameter, Phenomenex) coupled to a differential refractometer. Operating conditions were as follows: sample volume 10 µL; mobile phase 0.010 M H₂SO₄; flow rate 0.6 mL/ min and column temperature set at 65 °C or 35 °C depending on the analyzed carbon source. Samples were adequately diluted and filtered through Whatman®, 0.2 µm prior to analysis.

7.8.4 Extraction of carotenoids

Extraction of carotenoids was performed following the protocols of Buzzini et al., (2007) and Lopes et al., (2017) with some modifications. Briefly, wet yeast cells (2 mL) were harvested by centrifugation (4000 rpm, 10 min, 4 °C) and washed twice with distilled water. Then, cells were re-suspended in 3 mL dimethyl sulfoxide (DMSO), vortexed vigorously and incubated for 1 h in a water bath at 40 °C for cell lysis. The colored solution was separated by centrifugation (4000 rpm, 5 min, 4 °C) and the DMSO liquid fraction was collected. Subsequently, 3 mL of acetone were added to the precipitated cell pellets and the mixture was vortexed and centrifuged (4000 rpm, 5 min, 4 °C). The colored phase was collected and the procedure was repeated until the cell pellets became colourless. The pigmented phases were then pooled and carotenoids were extracted with 5 mL of light petroleum ether (boiling range 40–60 °C), whereas phase separation was facilitated by adding 5 mL NaCl 20% (w/v). The polar (upper) phase was collected and utilized for the spectrophotometric quantification. All steps were carried out under minimal-light conditions to avoid carotenoid degradation.

7.8.5 Spectrophotometric quantification of total carotenoids

The total carotenoids concentration was analyzed by a spectrophotometer (Helios Omega UV/Vis, Thermo scientific, USA) at 450 nm. Results were expressed as β-carotene equivalents per g of dry biomass using the equation (Goodwin, 1965):

$$TC (\mu\text{g/g}) = \frac{A \times V \times 10^6}{A_{1\text{cm}}^{1\%} \times 100 \times m_{\text{sample}}} \quad (\text{eq. 5})$$

where TC is the total carotenoid content or carotenoid yield (mg total carotenoids/g dry biomass), A is the absorbance (at 450 nm), V is the volume of petroleum ether (mL), 103 and 100 are unit conversion factors, $A_{1\text{cm}}^{1\%}$ is the specific absorptivity (2592 for petroleum ether) and m_{sample} is the dry biomass (g). The concentration of carotenoids was also expressed in mg/L using the equation above, where the sample volume (in L) was employed instead of m_{sample} following the adequate unit conversions.

7.8.6 Chromatographic analysis of carotenoids

7.8.6.1 Analysis with Thin Layer Chromatography analysis (TLC)

Thin layer chromatography (TLC) was performed for carotenoid analysis using silica gel 60 aluminum plates (Kieselgel 60 F254, Sigma-Aldrich, Canada) following the protocol described by Wei et al., (2020). Specifically, carotenoid extracts were loaded on the TLC plates using a mixture of acetone and hexane (3:7, v/v) as the developing solvents. The bands were identified from their retention factors (R_f), using standard compounds of β -carotene, torulene, torularhodin, γ -carotene and lycopene.

7.8.6.2 Analysis with High Performance Liquid Chromatography (HPLC)

The major carotenoids were identified and quantified through high performance liquid chromatography analysis (HPLC) coupled with a diode array detector (Agilent 1200 Series Gradient HPLC System, Germany) according to the method proposed by Bushway, (1986) with some modifications. Carotenoids were separated along a reverse-phase column (Eclipse XDB-C18, 5 μm , 4.6 $\times$ 150 mm, Agilent, Germany) and the mobile phase consisted of a mixture of acetonitrile, methanol and tetrahydrofuran (stabilized with 0.025% BHT) (40:56:4, v/v/v). The flow rate was 1 mL/min and the detection range was set from 400 to 600 nm. Column temperature was set at 30 °C and the injection volume was 10 μL . Carotenoids were identified on the basis of the external standards retention time and corresponding spectrum; β -carotene at 450 nm, γ -carotene at 460 nm, lycopene at 470 nm, torulene at 480 nm and torularhodin at 500 nm (Britton et al., 2004).

7.8.7 Extraction of microbial oil

The extraction of total cellular oil was conducted using a modified protocol based on the methodologies described by Folch et al., (1987) and Patel et al., (2015). Initially, dried biomass was mixed with 20 mL of chloroform: methanol (2:1, v/v) and remained in a dark and dry place for 72 h. Then, the sample was filtered and centrifuged at 4000 rpm for 8 min. The supernatant was filtered with sintered glass funnel (No. 3, porosity 40 mm) and subsequently with a syringe filter (pore size 0.45 μm , Whatman). Afterwards, 5 mL of 0.072% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were added to the sample and centrifuged at 4000 rpm for 5 min. The bottom organic phase was collected and washed at least three times with 1 mL of 2 N KCl/methanol (4:1, v/v). The bottom layer was transferred to a pre-weighed round-bottom flask and the oil extract was evaporated under vacuum. Total microbial oils were determined gravimetrically and the results were expressed as grams per liter and the lipid yield as the percentage of grams of lipid produced per gram of consumed substrate. Lipid content (% w/w) was expressed as grams of lipids produced per grams of dry cell weight.

7.8.8 Analysis of fatty acid methyl esters

The analysis of fatty acid methyl esters was performed following the protocol of Patel et al., (2015) with some modifications. Transesterification of the extracted microbial lipids was an initial step prior to the analysis. Briefly, the samples (4–16 mg) were initially dried under nitrogen and then, 1 mL of boron-fluoride-methanol reagent was added. Afterwards, the samples were heated at 80 °C for 20 min in a water bath. The extraction of methyl esters was achieved by adding 1 vol of distilled water and 2 volumes of n-hexane and vigorous shaking. The upper layer was collected and dried with anhydrous sodium sulfate. The fatty acid methyl esters were analyzed by gas chromatography (GC-2010 Pro, Shimadzu) using a MEGA-10 column with dimensions 30 m $\times$ 0.25 mm and film thickness 0.25 μm . One microliter of the samples was injected in split injection mode with ratio 1:100 using helium as a carrier gas (3 mL/min) and a flame ionization detector (FID). The column temperature was set at 60 °C for the first 5 min of analysis and then increased to 230 °C at a ratio of 8 °C per minute. Injector's and detector's temperature was regulated at 245 °C. The

fatty acid methyl esters were identified through standard FAME mixture (Supelco 37 Component FAME mix) and expressed as FAME's area percentage.

7.8.9 Peroxide value of microbial oil

The peroxide value of the microbial oil was evaluated using the IDF method as described by Shantha and Decker, (1994). Briefly, 0.4 g of barium chloride dehydrate were dissolved in 50 mL of distilled water to prepare an iron (II) chloride solution. The solution was gradually added under continuous agitation to an iron (II) sulfate solution containing 0.5 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 50 mL of distilled water. Following the subsequent addition of 2 mL of 1 M HCl to the mixture, filtration was performed to obtain a clear iron (II) solution, which was then stored in the dark. Then, 30g of ammonium thiocyanate were dissolved in 100 mL of distilled water to prepare the ammonium thiocyanate solution. Subsequently, 0.01 to 0.3g of sample was added in 9.8 mL of chloroform: methanol (7:3 ratio) and vortexed for 2 to 4 seconds. Afterwards, 50 μL of the ammonium thiocyanate solution and 50 μL of the iron (II) solution were added sequentially. The absorbance of the mixture was measured at 500 nm using a spectrophotometer after a 5 min incubation at room temperature. The peroxide value was calculated following the formula:

$$\text{Peroxide value (meq of peroxide/kg of sample)} = \frac{(A_s - A_b) * m}{55.84 * m_0 * 2} \quad (\text{eq. 6})$$

where, A_s is the absorbance of the sample, A_b presents the absorbance of the blank, m is equal to 41.52 (slope, obtained from the calibration curve), m_0 is the mass in grams of the sample and 55.84 is the atomic weight of iron.

7.8.10 Antimicrobial activity of carotenoids

The antimicrobial activity of the carotenoids extracts was evaluated on pathogenic bacteria and fungi utilizing a 96-well microtiter plate system (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, USA) (Karpiński and Adamczak, 2019). Briefly, the carotenoid extract was completely dried under nitrogen and a specific amount was dissolved in 1% antifoam in order to prepare the selected concentration of carotenoids in the wells. The examined carotenoid concentrations against the bacterial strains were 1.8, 4.5, 6, 9, 18, 36, 54, 72, 90, 180 and 360 mg/L

and for the fungal strains were 9, 18, 36, 54, 72, 90, 180 and 360 mg/L. Then, 2.5% (v/v) pre-culture of each pathogenic strain was added in a final volume of 100 µL TSB for bacterial strains or malt extract for fungal strains. The samples were incubated at either 37 °C or 25 °C depending on the examined pathogenic strains (bacteria and fungi, respectively) for 24 h. The optical density (OD) was measured at 600 nm on a regular timeline (Moreira et al., 2018). The growth of the pathogenic strains solely on 1% antifoam without the addition of the carotenoids was used as the control. The inhibition of microbial growth because of the antimicrobial activity of carotenoids was calculated as follows (Metwally et al., 2022):

$$Inhibition (\%) = \frac{OD_{control}(t_{24}-t_0) - OD_{sample}(t_{24}-t_0)}{OD_{control}(t_{24}-t_0)} * 100 \quad (eq. 10)$$

where, $OD_{control}$ was the difference between the final and the initial optical density of the control sample and the OD_{sample} the respective optical densities of each examined sample. The lowest required concentration of carotenoids in order to inhibit the visible growth of a microorganism after 24 h incubation (minimum inhibitory concentration, MIC) was determined following the above equation. One hundred microliter of the samples that presented complete inhibition of the pathogenic strains after 24 h were further incubated in TSB/ malt extract plates for 24 h to investigate their minimum bactericidal/ fungicidal concentration (MBC/MFC).

7.8.11 Antioxidant activity

The antioxidant activity of carotenoid extracts was determined by the ABTS• [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging radical method (Re et al., 1999). Briefly, 7 mM aqueous solution of ABTS• and 2.4 mM aqueous solution of potassium persulfate were stored as stock solutions at - 20 °C. Before analysis a mixture of equal amounts of these solutions was kept for 16 h at room temperature (25 °C) in the dark. Then, ethanol was added to the mixture until the absorbance reached a value of 0.7 ± 0.2 at 734 nm (UV-visible spectrophotometer, Helios Omega, ThermoScientific, USA). Carotenoid extracts were dissolved in dichloromethane and 10% (v/v) of the sample was added in 1 mL ABTS• ethanol solution. The mixture was vigorously shaken and measured at 734 nm after exactly 1, 3 and 6 min. As control

sample, dichloromethane (10%, v/v) was added in 1 mL ABTS• ethanol solution. The percentage inhibition of free radical formation by ABTS• was calculated as follows:

$$ABTS \bullet \text{ scavenging activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100 \quad (\text{eq.11})$$

where, Abs_{control} was the absorbance of the control and Abs_{sample} was the absorbance of the sample. The IC₅₀ value was also calculated, corresponding to the concentration of carotenoid's extract or standard compound required to achieve 50% inhibition of the ABTS radical.

7.8.12 Precipitation of exopolysaccharides (EPS)

Exopolysaccharides (EPS) production was evaluated following the protocol of Seveiri et al., (2020) with some modifications. More specifically, wet yeast cells were centrifuged (4000 rpm, 10 min, 4 °C) and the supernatant was collected. A mixture of the supernatant with ice cold ethanol (1:2 v/v ratio) was stored for 24 h at 4 °C. The exopolysaccharides were precipitated and collected from the liquid using centrifugation (4500 rpm, 15 min, 4 °C). An additional purification step was performed prior to precipitation, in which the exopolysaccharides were mixed with a 20% (w/v) TCA solution in a 1:1 ratio while stirring at room temperature for 1 h following centrifugation (4500 rpm, 20 min, 4 °C). The precipitated exopolysaccharides with ethanol were recovered through centrifugation (4500 rpm, 15 min, 4 °C) and lyophilized (BK-FD12P, Biobase Biodustry Co Ltd., Jinan, China). A further purifying step of exopolysaccharides prior to lyophilization was the re-suspension of the centrifuged pellet in adequate amount of distilled water and its filtration through dialysis membranes (CelluSep® molecular weight cutoff 6–8 kDa) for 24 h at 4 °C. EPS production was expressed as g of lyophilized mass per L of fermentation broth.

7.8.12.1 Analysis of EPS

7.8.12.1.1 Acid hydrolysis

EPS hydrolysis was performed following a protocol of Koutinas et al., (2019) with some modifications. Briefly, a concentration of 100 mg of EPS diluted in distilled

water was mixed with 100 μ L trifluoroacetic acid (TFA). The mixture was treated for 2 h at 120 $^{\circ}$ C and stored at 4 $^{\circ}$ C until further use.

7.8.12.1.2 Chromatographic analysis of EPS

The EPS fraction before and after acid hydrolysis was further analysed by HPLC and TLC analysis. HPLC analysis of the monosaccharide monomers was carried out as described in Section 7.8.3. TLC analysis was conducted to detect sugar monomers as well as impurities of protein and lipoic nature. Briefly, 5 μ L of 100 mg/L hydrolyzed EPS sample were applied on silica gel 60 aluminum plates. The selection of mobile phases and visualization reagents varied depending on the specific target of identification and based on modified protocols. More specifically, the mobile phases used were chloroform: methanol: acetic acid: dH₂O in a ratio of 25:15:4:1 (Silva et al., 2014), isopropanol: dH₂O in a ratio of 7:3 (Toschkova, 2022) and hexane: diethyl ether: acetic acid in a ratio of 42.5:7.5:0.5 (Patel et al., 2014) for the detection of sugars, proteins and lipids, respectively. Three different spray reagents were used: a mixture of 20% H₂SO₄ with 5% phenol solution (Patel et al., 2014), a ninhydrin solution prepared by dissolving 0.2 g of ninhydrin in 100 mL of acetone (Zampolli et al., 2022) and iodine vapors (Ghazala et al., 2017). The bands of sugar monomers were identified from their retention factors (R_f), using standard compounds.

7.8.12.1.3 Oil Holding Capacity (OHC) of EPS

The OHC of EPS was evaluated following the protocol of Lin et al., (2021) with some modifications. Briefly, 0.2 g of EPS were mixed with 20 mL of soybean oil and vigorously vortexed for 30 sec. The mixture was centrifuged (4800 rpm, 20 min, 10 $^{\circ}$ C) and the supernatant was removed. The EPS residue was weighed and expressed as gram of oil per gram of EPS following the formula:

$$OHC(g/g) = \frac{M_2 - M_1 - M_0}{M_0} \quad (\text{eq. 7})$$

where, M_0 is the weight of the dry sample, M_1 is the weight of the used tube and M_2 is the weight of the tube with the residue.

7.8.12.1.4 Surface tension of EPS

The surface tension of EPS was evaluated using the Wilhelmy plate method with a tensiometer (K20 Krüss tensiometer, KRÜSS GmbH, Germany) equipped with a 1.9 cm platinum plate (wetted length: 40.20 mm) (Kachrimanidou et al., 2022). Each EPS sample (0.1 g), recovered using different methods as described in Section 7.8.12, was diluted in 10 mL distilled water and the samples were measured at room temperature. Before each measurement, the plate was cleaned with isopropanol, which was evaporated by passing the plate through a flame. Each sample was analyzed in triplicates, and the reported values represent the mean value of these measurements.

7.8.12.1.5 Emulsification index of EPS

The emulsifying properties of EPS were evaluated through the emulsification index (E_{24}) following the protocol of Vecino et al., (2015). One mL of each EPS sample, precipitated using different methods as described in Section 7.8.12 at a concentration of 10 g/L, was mixed with 1 mL of paraffin oil and vigorously vortexed for 1 min. The total height of the sample as well as the height of the emulsion was measured after 24 h. The emulsification index was determined using the following equation:

$$E_{24}(\%) = \frac{H_{Emulsion}}{H_{Total}} \times 100 \quad (\text{eq. 8})$$

where, $H_{Emulsion}$ is the height of the emulsion and H_{Total} is the total height of the mixture after 24 h.

7.8.13 Oil Binding Capacity (OBC) of the produced oleogel

The OBC was evaluated the protocol of Ropciuc et al., (2023) with some modifications. Briefly, 0.5 g of oleogel were centrifuged at 4800 rpm for 1 h at a temperature of 20 °C. The samples were inverted for 10 min on a paper towel to remove the liquid fraction. The following calculation formula was used:

$$OBC (\%) = \frac{A-B}{C-B} \times 100 \quad (\text{eq. 9})$$

where, A is the weight of the used tube with the sample mass after centrifugation and after the removal of the liquid fraction, B is the weight of the used tube and C is the weight of the used tube and the sample.

7.8.14 Encapsulation efficiency

The encapsulation efficiency (EE) of both carotenoids and microbial oil of the obtained electrosprayed particles were evaluated following a modified protocol (Mahalakshmi et al., 2020). Briefly, for measuring surface microbial oil content, 200 mg electrosprayed particles were mixed with 20 mL hexane, vortexed for 30 sec and centrifuged (4500 rpm, 5 min, 4 °C). The upper hexane phase was collected and evaporated under vacuum.

For measuring total microbial oil content, 200 mg of electrospun were initially mixed with 2 mL of distilled water and vortexed for 5 min. Subsequently, 18 mL of ethanol were added and vortexed for an additional 5 min, followed by the addition of 20 mL of hexane, which was vortexed for 30 sec. The upper phase of hexane was collected after centrifugation (4500 rpm, 5 min, 4 °C) and evaporated under vacuum. Both the surface and total microbial oil content were determined gravimetrically, with the results expressed in grams.

For extracting surface and total carotenoid content, similar procedures were followed. However, the carotenoid concentration was measured described in Section 7.8.6.2. The total carotenoid content was expressed as electrospinning yield (%), whereas the encapsulated carotenoids content was expressed as EE and it was calculated as follows:

$$EE (\%) = \frac{A-B}{A} \quad (\text{eq. 12})$$

where A is the total content and B is the surface content.

7.8.15 Texture analysis of oleogels

The textural properties of the oleogels were evaluated using a texture analyzer (FRTS, IMADA Co., Ltd., Japan). The firmness was measured at 20 cm depth after plunging a cylindrical probe into sample with a penetration speed of 100 mm/min. The

maximum force was reported as a penetration force (N). The analysis was performed in duplicates.

7.8.16 Rheological analysis of oleogels

The rheological properties of the oleogels were performed using a Discovery HR 10 Hybrid Rheometer (TA Instruments, New Castle, DE, U.S.A.) equipped with a 40mm plate-plate geometry system and a measuring gap of 1600 μm . The viscosity was evaluated at a shear rate of 0.001-100 1/s at 20 °C. A small amplitude oscillatory shear (20 °C) was performed in the shear strain range from 0.01-100% at a fixed frequency of 1 Hz to establish the linear viscoelastic region (LVR). Then, the viscoelastic properties, storage modulus (G') and loss modulus (G''), of the oleogel were evaluated using a dynamic frequency sweep (1-100 Hz) at a constant strain value within the LVR.

7.8.17 FTIR analysis of EPS, oleogel and electrospun

The FTIR (Fourier- Transform Infrared Spectroscopy) analysis was carried out using a Cary 630 FTIR Spectrometer (Agilent, Santa Clara, U.S.A.). A small amount of the sample was used for the analysis, which was placed on the surface of the device's crystal. The spectra of the samples in the IR range from 650 to 4000 cm^{-1} were obtained with a 2 cm^{-1} resolution. The spectra were processed using Origin Pro 9.

7.9 Statistical analysis

Significant differences between the different experiments were evaluated via analysis of variance (ANOVA) and the Tukey's HSD (honest significant difference) at a level of 5% ($p < 0.05$). The analyses were performed with Microsoft Excel software (Microsoft Office Professional plus 2010, version 14.0.7268.5000, © 2010 Microsoft Corporation). The corresponding results are mean values of three independent experiments or analysis.

CHAPTER 8

Production of carotenoids from *Rhodospiridium kratochvilovae* Y-42 & Y-43 using synthetic substrates

8 Production of carotenoids from *Rhodosporidium kratochvilovae* Y-42 & Y-43 using synthetic substrates

8.1 Introduction

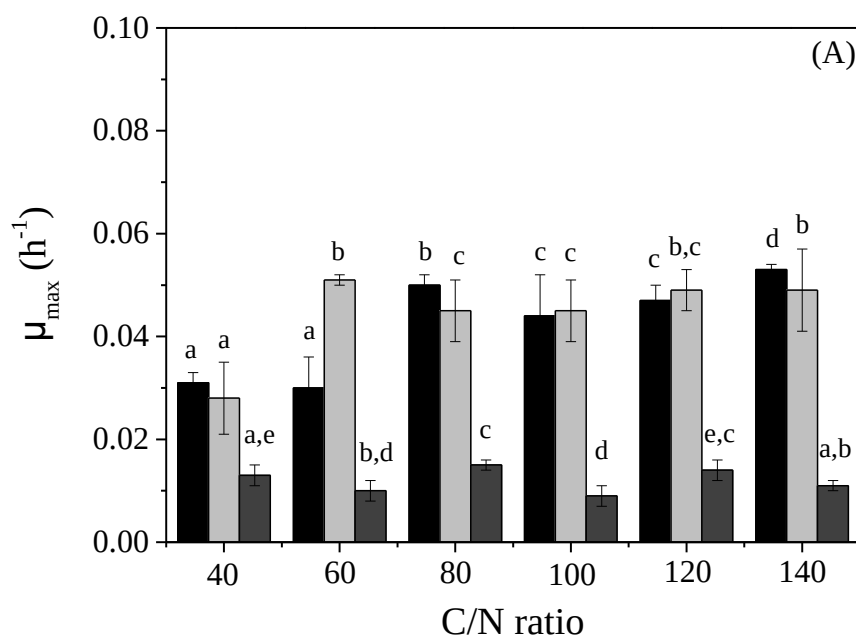
The initial objective of this study was to evaluate the ability of the red yeast strains *Rhodosporidium kratochvilovae* Y-42 and Y-43 to produce carotenoids utilizing different commercial carbon sources. Additionally, the effect of the C/N ratio was examined, as it constitutes a key fermentation parameter influencing both lipid and carotenoid synthesis. The composition of the carotenoid extracts was also determined to assess the impact of the different carbon sources.

8.2 Effect of C/N ratios on specific growth rate (μ , h^{-1})

The initial step for investigating the growth patterns of *Rhodosporidium kratochvilovae* Y-42 and Y-43 was to evaluate the effect of various carbon sources on different C/N ratios. To this end, the specific growth rate (μ , h^{-1}) for both strains was evaluated in microtiter experiments using glucose, galactose and a mixture of glucose-galactose (1:1) along with different C/N ratios (60, 80, 120 and 140). These parameters not only affect yeast growth but also modify the metabolic pathway for microbial oil and carotenoid accumulation (Ielchishcheva et al., 2018a). Growth on glucose was evaluated as control experiment since it is the most commonly employed sugar. The selection of the mixture of glucose-galactose and galactose was based on the composition of waste streams, abundant in the area of Kefalonia (and Greece, in general), which are rich in lactose, glucose and galactose (e.g. cheese whey). The fermentability of the pure sugars would indicate the ability of the studied strains to subsequently grow on such waste and by-product streams.

The ability of both strains to consume glucose, galactose and their mixture was evidenced (**Figure 8.1**). Generally, it was observed that both strains presented greater cell proliferation at C/N ratios higher than 60, while strain *Rhodosporidium kratochvilovae* Y-43 exhibited higher μ_{max} values in comparison to *Rhodosporidium kratochvilovae* Y-42. More specifically, when glucose was used as the carbon source, *Rhodosporidium kratochvilovae* Y-42 presented the highest μ_{max} values at C/N ratios of 80 and 140 (0.050 and 0.053 h^{-1} , respectively) whereas when glucose-galactose

(1:1) was the carbon source, growth was favored at C/N ratios of 60, 120 and 140 (0.050, 0.049 and 0.049 h⁻¹, respectively) without statistically significant difference (**Figure 8.1A**). The lowest μ values were observed when galactose was used as carbon source, with an average value of 0.012 h⁻¹. A similar pattern was also displayed by *Rhodospiridium kratochvilovae* Y-43, which achieved the highest $\mu_{\max}$ values in glucose at C/N ratios of 80 and 120 (0.081 and 0.083 h⁻¹, respectively), followed by glucose-galactose at C/N ratios of 80, 100, 120 and 140 (0.064, 0.066, 0.069 and 0.065 h⁻¹, respectively) (**Figure 8.1B**). The C/N ratio 120 was the most favorable when glucose or its mixture with galactose were employed as carbon sources. When galactose was used as sole carbon source, the lowest μ values were obtained (**Figure 8.1B**). Lactose was also evaluated however both strains were unable to grow (data not shown). Similar observations were also carried out by Byrtusová et al., (2021), which reported the inability of *Rhodospiridium kratochvilovae* CCY 20-2-26 to grow utilizing lactose. Red yeasts do not possess the gene to encode the enzyme β -galactosidase, thus to break down lactose into its monomers (Byrtusová et al., 2021). This aspect should be taken into account in future experiments, especially in lactose-rich waste streams.



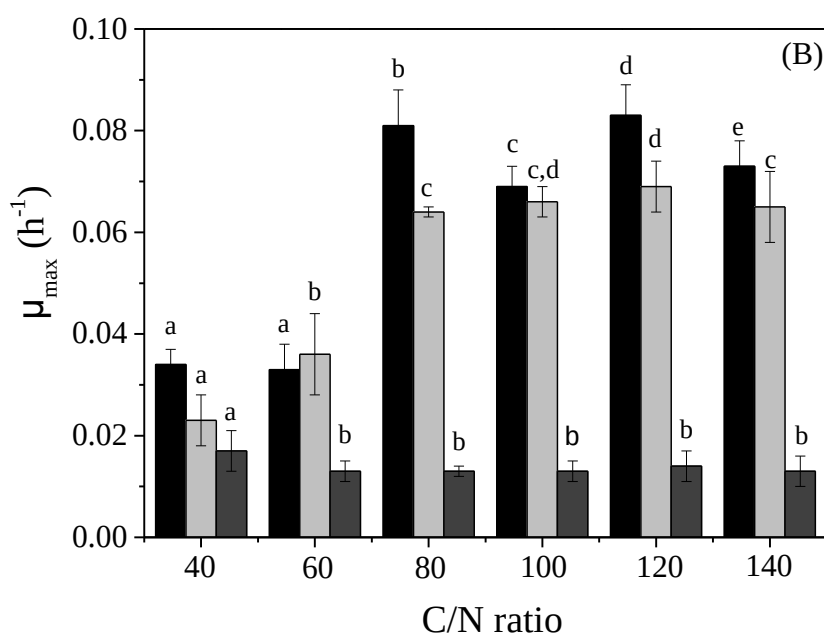


Figure 8.1 Maximum specific growth rate ($\mu_{\max}$, h^{-1}) of (a) *Rhodosporidium kratochvilovae* Y-42 and (b) *Rhodosporidium kratochvilovae* Y-43 at different C/N ratios using synthetic media. Glucose (black bars), glucose-galactose (light gray bars), galactose (dark grey bars). The statistical analysis was taken place between the C/N ratios of each carbon source.

The obtained specific growth rates were in the same range with other *Rhodosporidium* strains. However these values are highly affected by the fermentation substrate and are also strain-dependent (Gosalawit et al., 2021; Shakeri et al., 2021; Tchakouteu et al., 2017). To the best of our knowledge, this is the first study in which the specific growth rate (μ , h^{-1}) was evaluated for *Rhodosporidium kratochvilovae* strains. Since the most promising results were obtained when glucose and its mixture with galactose were employed as carbon sources at C/N ratios of 60, 80 and 120, shake flask fermentations were subsequently carried out to investigate their effect in carotenoid production and oil accumulation.

8.3 Shake flask fermentations

8.3.1 Effect of C/N on cell growth, carotenoid and microbial oil production

It has been well established that the synthesis and the profile of carotenoids strongly correlates with numerous cultivation parameters, including temperature, pH, agitation but also medium composition (Elfeky et al., 2019; Ielchishcheva et al., 2018b; Mata-

Gómez et al., 2014). Hence the following set of experiments targeted to investigate the effect of varying C/N ratios on carotenoid synthesis by *Rhodospiridium kratochvilovae* Y-42 and Y-43.

It was observed that C/N ratio significantly affected the carotenogenesis, revealing C/N 80 (g/g) as the most suitable ratio for both yeast strains in all tested media (**Table 8.1**). Strain Y-42 produced the highest amounts of carotenoids (2.59 mg/L) at 39 h of fermentation, when grown on glucose at a C/N of 80. Carotenoids production decreased by 28%, when the mixture of glucose-galactose was employed under the same C/N ratio (1.86 mg/L), even though the high concentration occurred 5 hours earlier (34 h instead of 39 hours), while almost the same amount of sugars was consumed (**Table 8.1**). Residual FAN was similar in both experiments (24.2 and 25.5 mg/L, respectively), whereas inorganic nitrogen was completely consumed. The obtained results are in accordance with similar studies in the open literature. In the work of Yimyoo et al., (2011), a carotenoid concentration of 2.99 mg/L was obtained by the strain *Rhodospiridium paludigenum* DMKU3-LPK4, when grown on glycerol, with yeast extract as nitrogen source. *Rhodotorula glutinis* NCIM 3353 was able to produce 2.2 mg/L of total carotenoids after 72 h of cultivation utilizing glucose and yeast extract (Bhosale and Gadre, 2001). Alakraa et al., (2020) also underlined the superiority of yeast extract as the most suitable nitrogen source for carotenoid production. On the contrary, Liu and Wu, (2007) concluded that the most efficient conditions for astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* were high C/N ratios at low nitrogen (0.81 g/L ammonium sulphate) and carbon concentrations. Yeast extract and ammonium sulphate had a similar effect on carotenoid synthesis when a newly isolated *Rhodotorula* strain was evaluated in the study of Zhao et al., (2019). It can be concluded that the suitability of the nitrogen source depends highly on the specific strain applied.

At the same time, the C/N ratios resulting in the highest carotenoid production coincided with the lowest microbial oil accumulation, with values of 0.79 g/L and 1.20 g/L, in the cases of glucose and glucose-galactose mixture, respectively. Microbial oil production proceeded and reached its highest concentration close to the end of the fermentation, whereas carotenoids concentration declined. **Table 8.2**

presents the highest values obtained on microbial oil accumulation together with other fermentation parameters such as carotenoids and biomass concentration. Biomass ranged between 7.8-12.4 g/L for Y-42 and from 9.3 to 15.7 g/L for Y-43 (**Table 8.1**). The ratio C/N 80 was the less favorable for microbial oil accumulation, leading to the lowest values regardless the carbon source (2.50 g/L microbial oil concentration). On the contrary, the fermentations performed at C/N 60 and 120 g/g resulted in significantly lower total carotenoids (1.63 and 1.66 mg/L, respectively) but higher microbial oil concentrations (3.4 and 5.7 g/L, respectively), compared to the results of C/N 80 g/g (**Tables 8.1, 8.2**).

It is evident that the lowest (60 g/g) and the highest (120 g/g) C/N ratios favored microbial oil accumulation against carotenoids production. The C/N ratio of 80 (g/g) resulted in the highest carotenoid production (2.07 mg/L) also in the case of *Rhodospiridium kratochvilovae* Y-43 on glucose, after only 39 h of fermentation (**Table 8.1**). Ratios 60 and 120 led to significantly lower amounts of total carotenoids (1.09 mg/L and 1.47 mg/L, respectively), but at higher microbial oil concentrations (**Table 8.1**). In fact, strain Y-43 produced the highest amount of microbial oil (5.48 g/L, 44.8% content on biomass) when grown on glucose at C/N 120 (g/g) (**Table 8.2**). In the mixture of glucose-galactose, strain Y-43 presented a different behavior in comparison to Y-42 as both 80 and 120 (g/g) C/N ratios led to analogous carotenoids production, both in terms of concentration (2.11 and 2.08 mg/L, respectively) and yield (0.200 mg/g for both ratios) after approximately 60 h of fermentation (**Table 8.1**). The combination of the sugar mixture together with the ratio C/N 80 enhanced also microbial oil synthesis, resulting in an accumulation of 48.5% of the total cell biomass at 80 h of fermentation, with a concentration of 5.15 g/L (**Table 8.2**). Patel et al., (2015a) suggested that the combination of easily fermentable and less-fermentable sugars enhances microbial oil accumulation. It seems that the presence of galactose in the fermentation medium prolonged the duration of the cultivation while boosted microbial oil synthesis.

Table 8.1 Maximum carotenoid concentration and other fermentation parameters of *Rhodospiridium kratochvilovae* Y-42 and Y-43 strains using synthetic media at different C/N ratios (60, 80 and 120) and initial total sugar concentration 50 g/L.

Carbon source	C/N (g/g)	Time (h)	Total carotenoids (mg/L)	Carotenoid yield (mg/g biomass)	Sugars consumed (g/L)	FAN content (mg/L)	Inorganic nitrogen (mg/L)	Biomass (g/L)	Microbial oil (g/L)
<i>Rhodospiridium kratochvilovae</i> Y-42									
Glucose	60	55	1.63±0.07 ^a	0.130±0.007	29.4±1.5	27.2±0.3	0.00	12.4±0.3	3.09±0.04 ^a
	80	39	2.59±0.01 ^b	0.310±0.001	24.2±0.8	25.3±0.3	0.00	8.4±0.2	0.79±0.05 ^b
	120	48	1.66±0.25 ^a	0.150±0.020	30.7±1.1	24.4±1.6	0.00	11.3±0.4	3.85±0.56 ^c
Glucose-Galactose (1:1)	60	48	1.61±0.17 ^{a1}	0.150±0.020	42.1±0.7	26.8±0.2	0.0	10.2±0.4	3.05±0.18 ^{a1}
	80	34	1.86±0.08 ^{a1}	0.230±0.020	25.5±2.6	23.0±0.1	1.2±0.1	7.8±0.4	1.20±0.06 ^{b1}
	120	48	1.01±0.07 ^{b1}	0.100±0.001	31.7±0.4	18.1±1.3	0.0	10.1±0.2	1.88±0.24 ^{b1}
<i>Rhodospiridium kratochvilovae</i> Y-43									
Glucose	60	48	1.09±0.12 ^{a2}	0.090±0.010	27.7±1.5	26.9±1.0	0.0	12.4±0.4	2.90±0.14 ^{a2}
	80	39	2.07±0.26 ^{b2}	0.220±0.040	26.1±2.0	25.8±0.2	0.0	9.3±0.6	0.81±0.29 ^{b2}
	120	63	1.47±0.06 ^{c2}	0.150±0.001	35.4±2.1	21.8±2.0	0.0	10.0±0.5	3.18±0.04 ^{a2}
Glucose-Galactose (1:1)	60	72	0.86±0.09 ^{a3}	0.060±0.002	45.9±1.3	27.1±1.2	0.0	15.7±0.1	2.95±0.15 ^{a3}
	80	63	2.11±0.25 ^{b3}	0.200±0.020	40.8±1.2	22.7±0.9	3.1±0.1	10.3±0.9	3.63±0.45 ^{a3}
	120	58	2.08±0.15 ^{b3}	0.200±0.010	35.4±0.3	25.2±1.9	0.6±0.0	10.9±0.1	2.93±0.50 ^{a3}

* Different lowercase letters (a, b, c, a1, b1, c1, a2, b2, c2) indicate significant differences ($p<0.05$) on total carotenoids and microbial oil production between different C/N for the same carbon source.

Table 8.2 Maximum microbial oil production and other fermentation parameters of *Rhodospiridium kratochvilovae* Y-42 and Y-43 strains using synthetic media at different C/N ratios (60, 80 and 120) and initial total sugar concentration 50 g/L.

Carbon source	C/N (g/g)	Time (h)	Microbial oil (g/L)	Microbial oil content (% w/w)	Microbial oil yield (g/g)	Total carotenoids (mg/L)	Biomass (g/L)
<i>Rhodospiridium kratochvilovae</i> Y-42							
Glucose	60	63	3.43±0.48 ^a	25.9±3.2	0.100±0.010	1.24±0.25	13.1±0.2
	80	55	2.52±0.30 ^b	20.6±3.6	0.070±0.004	1.15±0.21	12.3±0.7
	120	80	5.72±0.18 ^c	39.8±1.3	0.130±0.010	1.28±0.03	14.0±0.5
Glucose-Galactose (1:1)	60	55	3.17±0.74 ^{a1}	31.2±5.7	0.08±0.02	0.92±0.09	10.1±0.5
	80	72	2.53±0.65 ^{a1}	18.3±4.0	0.05±0.01	0.64±0.07	13.7±0.5
	120	80	3.51±0.53 ^{a1}	29.0±3.1	0.08±0.01	0.69±0.15	12.0±0.5
<i>Rhodospiridium kratochvilovae</i> Y-43							
Glucose	60	55	3.08±0.15 ^{a2}	24.6±0.6	0.100±0.005	1.05±0.15	12.5±0.1
	80	63	2.03±0.14 ^{a2}	15.2±0.7	0.050±0.010	1.80±0.29	13.3±0.2
	120	72	5.48±0.49 ^{b2}	44.8±1.2	0.120±0.010	1.20±0.03	12.2±0.7
Glucose-Galactose (1:1)	60	55	3.95±0.01 ^{a3}	28.4±0.3	0.090±0.004	0.74±0.05	13.9±0.1
	80	58	5.15±0.26 ^{b3}	48.5±1.2	0.120±0.006	1.60±0.09	10.2±0.5
	120	58	2.93±0.50 ^{c3}	26.8±4.5	0.080±0.010	2.08±0.15	10.9±0.0

* Different lowercase letters (a, b, c, a1, b1, c1, a2, b2, c2) indicate significant differences ($p<0.05$) on total carotenoids and microbial oil production between different C/N for the same carbon source.

Overall, the ability of both strains to produce carotenoids was indicated. The highest concentration of total carotenoids was similar for the two *Rhodospiridium kratochvilovae* strains (~2 mg/L). The kinetics of sugar consumption together with metabolites' production were consequently studied at the most promising ratios for each strain to further understand the trend of carotenoid synthesis under these experimental conditions.

8.3.2 Kinetics of cell growth, carotenoid production, and microbial oil accumulation

The kinetics of sugar and nitrogen consumption as well as the production of carotenoids, microbial oil and cell biomass for both strains under the most promising C/N ratios using glucose or mixture with galactose are presented in **Figures 8.2 and 8.3**. It is apparent, that carotenoids production initiated upon the depletion of both FAN and inorganic nitrogen concentration. Nitrogen excess proliferates cell growth but suppresses enzymes involved in carotenogenesis (Liu and Wu, 2007). Moreover, carotenoids are secondary metabolites synthesized via the mevalonate pathway, meaning that their production is triggered by nutrient limitation or stress conditions (Liu and Wu, 2007). The presence of carotenoids in the yeast cell was detected after 10 h of fermentation almost in all cases (**Figure 8.2B, D, Figure 8.3B, D, F**). Carotenoid synthesis has been mainly observed at the early stationary phase (Byrtusová et al., 2021; Freitas et al., 2014; Singh et al., 2018). For example Singh et al., (2018) reported the production of carotenoids from *Rhodospiridium toruloides* after 48 h of cultivation. On the other hand, in the study of Freitas et al., (2014) carotenogenesis by *Rhodospiridium toruloides* started at around 10 h of fermentation, similarly to our study, while the highest concentration occurred at around 60 h, at the stationary phase. In our study, when carotenoid synthesis started, inorganic nitrogen (in the form of $(\text{NH}_4)_2\text{SO}_4$) was almost depleted, while the concentration of FAN remained stable and approximately 20 mg/L for all the studied cases. Both strains consumed simultaneously the organic (yeast extract) and the inorganic nitrogen source, while some FAN was left unconsumed until the end of the fermentation (**Figure 8.2A, C, Figure 8.3A, C, E**). Cell growth slightly continued even after nitrogen exhaustion. This phenomenon is not unusual and could be attributed to the utilization of metabolites derived from dead cells, or another –possibly intracellular– nitrogen pool (Razavi et al., 2007). Sugar consumption did not follow a similar trend,

with an apparent preference towards glucose, especially by *Rhodospiridium kratochvilovae* Y-43. Strain Y-42 assimilated both sugars simultaneously, although at different rates (**Figure 8.2C**). On the other hand, strain Y-43 started consuming galactose mainly when glucose concentration reached a limiting concentration (around 10 g/L), when a C/N of 80 was employed, whereas in case C/N was 120, galactose consumption started after glucose depletion (**Figures 8.3C, E**). For both strains, the maximum carotenoids production occurred at about 40 h of fermentation, when glucose was the carbon source (**Figures 8.2B, 8.3B**).

Y-42 presented a very abrupt peak on carotenoids accumulation regardless the carbon source, as total carotenoids concentration increased by 54% from 34 to 39 h of fermentation when grown on glucose (**Figure 8.2B**), and by 167% from 29 to 34 h when cultivated on mixture of glucose-galactose (**Figure 8.2D**). The red yeast strain SKK-BM3 followed a similar trend when grown on glycerol, achieving the maximum carotenoids concentration at 36 h of cultivation (1 mg/L) and afterwards their concentration was constantly decreasing (Yimyoo et al., 2011). At this time, nitrogen was already depleted but glucose was in excess (~15 g/L), shifting the metabolism towards carotenoid biosynthesis (Liu and Wu, 2007). During carotenoid production, microbial oil accumulation maintained at low levels and their increase in concentration was observed together with an acute carotenoids decrease.

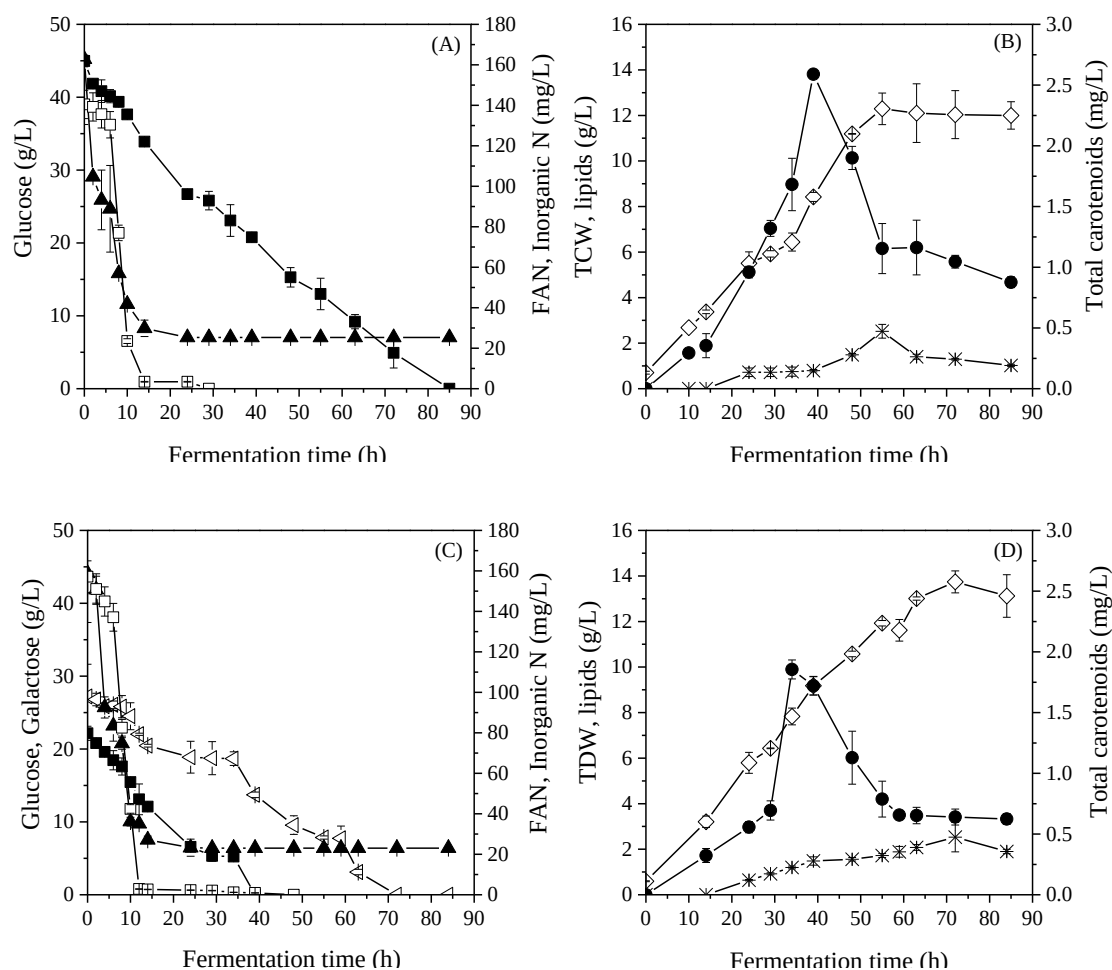


Figure 8.2 Kinetics of glucose (■), galactose (◁), free amino nitrogen (FAN) (▲) and inorganic nitrogen (□) consumption as well as total dry weight (◇), lipids (*) and carotenoids (●) production during batch fermentations of *Rhodosporidium kratochvilovae* Y-42 using glucose (A, B) and glucose-galactose (C, D) at C/N 80. Three replicates of each experiment and analysis were taken place.

Carotenoid synthesis followed a smoother pattern in case of Y-43, significantly differentiating its behavior from Y-42 (**Figures 8.3B, D, F**). The kinetics of Y-43 on glucose (C/N 80) presented some similarities to Y-42, as carotenoids were stably produced upon depletion of the nitrogen sources, reaching a maximum at 39 h. Thereafter their concentration was slowly decreasing, while microbial oil accumulation begun (**Figure 8.3B**). The mixture of glucose and galactose, as previously stated, prolonged carotenoids' production and as shown in **Figure 8.3D**, carotenoids, biomass and microbial oil production kinetics followed a similar trend.

The highest value of total carotenoids (2.1 g/L) was achieved after 63 h, when almost all sugars were completely consumed. In contrast to Y-42, microbial oil accumulation presented a maximum (5.15 g/L), before carotenoids, at 58 h of fermentation. In the case of the sugar mixture at high C/N ratio (120 g/g), the time carotenoids peaked (58 h) coincided with the highest microbial oil content (**Figure 8.3F**). These findings indicate the variations in metabolic behavior of *Rhodospiridium* genera, even between the same species. In general, total carotenoids concentration started to decrease after reaching its highest point, independent of species or cultivation conditions.

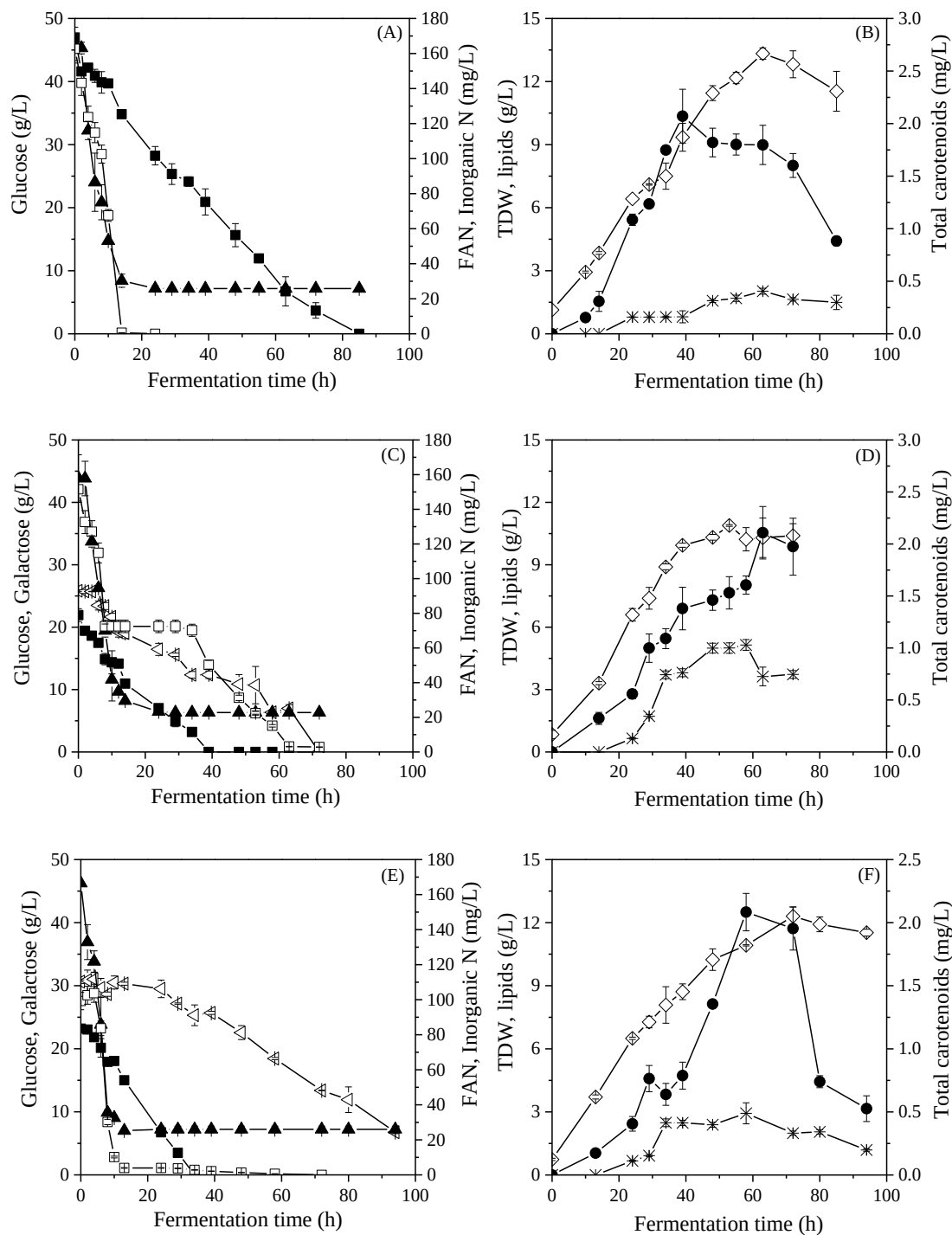


Figure 8.3 Kinetics of glucose (■), galactose (◁), free amino nitrogen (FAN) (▲) and inorganic nitrogen (◻) consumption as well as total dry weight (◇), lipids (*) and carotenoids (●) production during batch fermentations of *Rhodosporidium kratochvilovae* Y-43 using glucose (A, B) and glucose-galactose (C, D) at C/N 80, and glucose-galactose (E, F) at C/N 120. Three replicates of each experiment and analysis were taken place.

It seems that the C/N ratio can alter the metabolic pathway of *Rhodospiridium kratochvilovae* strains, promoting the production of either carotenoids or lipids. This seems to be related to the nitrogen concentration and could be explained by the fact that both biosynthetic pathways share acetyl-CoA as precursor. More specifically, acetyl-CoA carboxylase (ACC) is the key enzyme for converting acetyl-CoA into malonyl-CoA, as an intermediate step of the fatty acid biosynthetic pathway. ACC is naturally activated by the protein phosphatase 2A through dephosphorylation, while it is deactivated by phosphorylation from the AMP-activated protein kinase; it could also be allosterically triggered by citrate. At high nitrogen concentrations, the level of the produced citrate, through Krebs' cycle, is not adequate to be transferred out of the mitochondria. Thus, the available acetyl-CoA is utilized for carotenoid production, instead of fatty acids synthesis, and most likely the cytosolic ATP deactivates the ACC and enhances carotenogenesis. However, under low concentrations of nitrogen, the citrate is transported in cytosol and allosterically activates the ACC. The additional activation of ACC may reinforce the production of lipids (Fan et al., 2018; Iacobazzi and Infantino, 2014) (**Figure 8.4**).

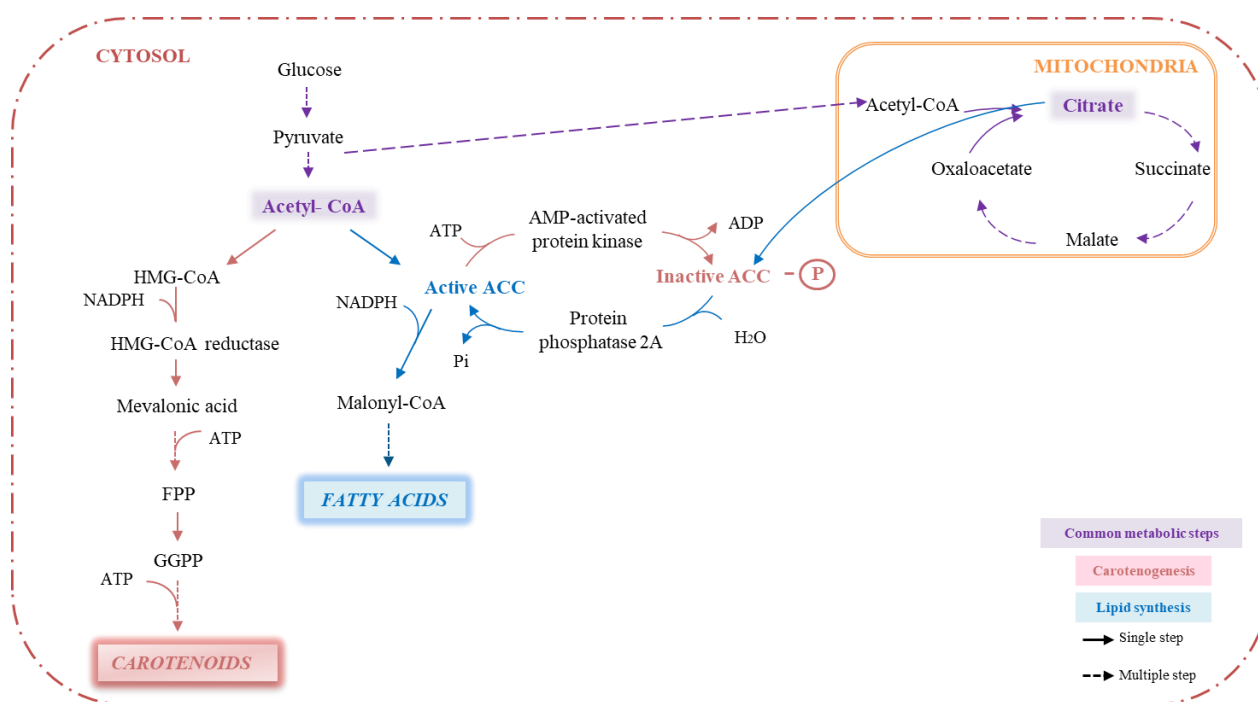


Figure 8.4 Schematic representation of carotenoid and fatty acid biosynthesis in yeast metabolism. The carotenoid and fatty acid biosynthetic pathways are dependent on nitrogen concentration. Under high C/N ratio, citrate is transferred to cytosol and allosterically activates acetyl-CoA carboxylase (ACC).

Another critical observation which -to our knowledge- has never been stated before is that in most cases the maximum concentrations (mainly for Y-42 strain) of the produced carotenoids preceded the maximum microbial oil concentrations (**Figure 8.5**). This might be related to the available nitrogen sources. As already shown in **Figures 8.2 and 8.3**, both strains rapidly assimilated both the inorganic ((NH₄)₂SO₄) and the organic (yeast extract) nitrogen. Inorganic nitrogen was depleted after 14 h of fermentation in almost every case for both strains. It has been previously reported that on cells grown at ammonium-based media the nitrogen catabolite repression genes (NCR genes) are active and as a consequence the Urea2-Gln3 regulator remains in cytosol and the metabolic pathway of fatty acids is partly inhibited (Ljungdahl and Daignan-Fornier, 2012). During fermentation, the Gln3 is dephosphorylated and transferred in the nucleus, thus in collaboration with other metabolic factors which act in parallel, fatty acid synthesis could occur.

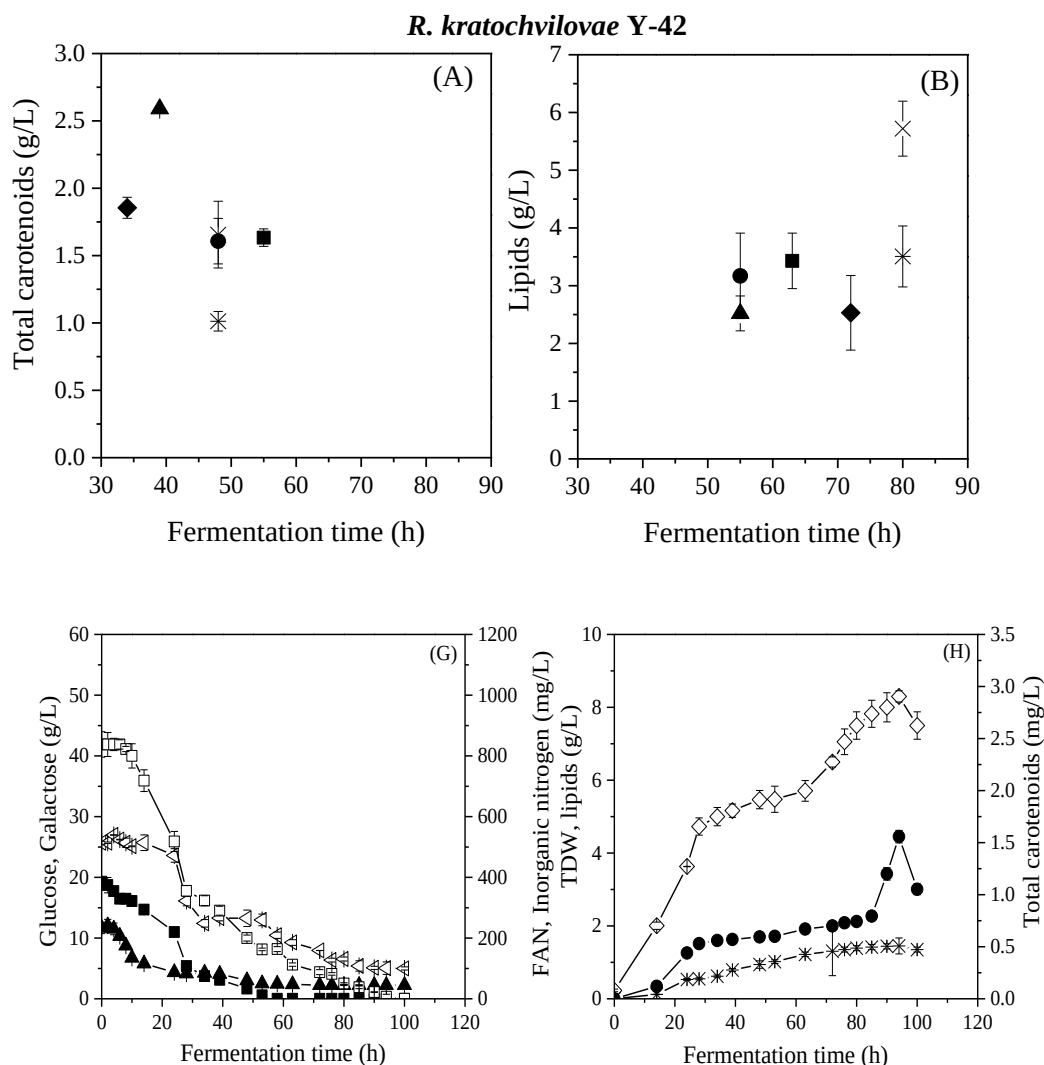


Figure 8.5 Comparison between the time frames of maximum total carotenoids production (A, C) and microbial oil accumulation (B, D) in *Rhodospiridium kratochvilovae* Y-42 and Y-43. Glucose at C/N60 (■), glucose-galactose C/N60 (●), glucose C/N80 (▲), glucose-galactose C/N80 (◆), glucose C/N120 (×), glucose-galactose C/N120 (*).

Comparing the results of this study with the literature (**Table 8.3**), it can be deduced that *Rhodospiridium kratochvilovae* Y-42 and Y-43 are two very promising strains for natural carotenoid production. Their yield is among the highest in the literature as shown in **Table 8.3**, utilizing either glucose as carbon source or in mixture with galactose. The highest total carotenoids content by an *Rhodospiridium kratochvilovae* strain achieved so far was reported by Byrtusová et al., (2021), with a value of 1.57 mg/g, using mannose and potassium nitrate as carbon and nitrogen source, respectively after 144 h of cultivation. It is also noteworthy that, in our study, both strains achieved the highest total carotenoids concentration just within 39 h of

fermentation, when glucose was the sole carbon source, whereas in most studies much more prolonged fermentation times are reported, such as 144 h (Byrtusová et al., 2021), 96 h (Tkáčová et al., 2018; Yen et al., 2019), even up to 8 days (Varzakakou et al., 2010).

Table 8.3 Total carotenoid production and biomass production from different *Rhodospiridium* strains grown on synthetic media.

Strain	Carbon source	Total carotenoids (mg/g biomass)	Biomass (g/L)	Fermentation time (h)	Reference
<i>Rhodospiridium</i> <i>kratochvilovae</i> CCY 20-2- 26	Mannose	1.57	15.0	144	Byrtusová et al. (2021)
<i>Rhodospiridium</i> <i>kratochvilovae</i> Y-42	Glucose	0.31	8.4	39	This work
	Glucose-Galactose (1:1)	0.23	7.8	34	
<i>Rhodospiridium</i> <i>kratochvilovae</i> Y-43	Glucose	0.22	9.3	39	
	Glucose-Galactose (1:1)	0.20	10.6	60	
<i>Rhodospiridium toruloides</i> NCYC 921	Glucose	0.06	5.9	72	Dias et al. (2016)
	Glucose	0.08	3.8	72	Freitas et al. (2014)
<i>Rhodospiridium diobovatum</i>	Glucose	0.32	10.7	144	Nasirian et al. (2018)
	Glycerol	0.30	10.6	144	

<i>Rhodosporidium babjevae</i>	Glucose	0.28	29.0	-	
<i>Rhodosporidium diobovatum</i>	Glucose	0.33	35.0	-	Iurkov et al. (2008)
<i>Rhodosporidium sphaerocarpum</i>	Glucose	0.49	27.0	-	
<i>Rhodosporidium paludigenum</i> DMKU3-LPK4	Glycerol	3.42 mg/L	7.6	132	Yimyoo et al. (2011)

8.3.3 Carotenoids profile

The profile of the individual carotenoids present in the extracts was firstly carried out by a spectrophotometric analysis in a scanning mode operating in a range of wavelengths. Specific peaks indicated each carotenoid; β -carotene at 450 nm (yellow), γ -carotene at 436, 462 and 492 nm (red-orange), torulene at 460, 486 and 518 nm (orange) and torularhodin at 501 and 517 nm (pink) (Britton, 2004). Carotenoids exhibited four peaks with almost the same λ_{max} value, regardless the substrate or the strain employed. More specifically, the samples presented three main peaks at 462, 486 and 517 nm and a lower peak at 435 nm. According to these results, the produced carotenoids might be a mixture of γ -carotene, torulene and torularhodin. However, chromatographic analyses were necessary in order to verify the above-mentioned findings.

Consequently, thin layer chromatography (TLC) analysis was conducted in the samples of extracted carotenoids from each experimental condition, and the retention factors (R_f) were compared with those of standard compounds. The R_f values of carotenoid standards were 0.75 for β -carotene, 0.7 for γ -carotene, 0.66 for torulene and 0.36 for torularhodin. In all examined samples, three bands were revealed after plate development with R_f values of 0.72, 0.67 and 0.35. However, it was rather unclear if each band corresponded to a single compound or a mixture. HPLC analysis was therefore essential for the individual carotenoid identification and quantification.

Identification and quantification of the individual carotenoids was finally employed through high-performance liquid chromatography analysis (HPLC), using the retention time and spectrum of carotenoid standards. All samples contained β -carotene, γ -carotene, torulene and torularhodin in both strains, regardless the C/N ratio or the carbon source. The main carotenoid was β -carotene (53-75%) in all cases, followed by γ -carotene (8.7-21.6%). The amounts of torulene and torularhodin ranged from 5 to 17% depending on the culture conditions (**Figure 8.6**). More specifically, *Rhodospiridium kratochvilovae* Y-42 presented similar carotenoid profile in both substrates, with β -carotene being the predominant compound; yet its concentration decreased at higher C/N ratios as for example at glucose-based medium β -carotene corresponded to the 73% of total carotenoids at C/N 60 g/g, whereas it dropped to

53% at C/N 120 g/g. The highest C/N ratio with glucose as substrate promoted γ -carotene synthesis, corresponding to 21.6% of total carotenoids. In case of *Rhodospiridium kratochvilovae* Y-43, β -carotene was also the main carotenoid (60-68%) in every substrate, followed by γ -carotene (13.5-18.3%). Lower amounts of torulene (8.4-10.7%) and torularhodin (6-12.2%) were also detected (**Figure 8.6B**).

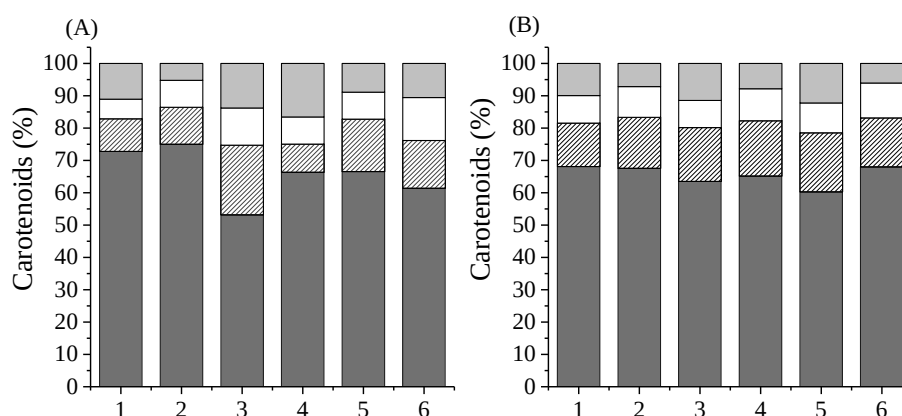


Figure 8.6 Carotenoids composition in (A) *Rhodospiridium kratochvilovae* Y-42 and (B) *Rhodospiridium kratochvilovae* Y-43 under studied culture conditions: (1) glucose at C/N60, (2) glucose at C/N80, (3) glucose at C/N120, (4) glucose-galactose C/N60, (5) glucose-galactose C/N80 and (6) glucose-galactose C/N120. β -carotene (dark grey colored bar), γ -carotene (patterned bar), torulene (white colored bar), torularhodin (light grey colored bar).

The presence of β -carotene, torulene, torularhodin and γ -carotene has been previously demonstrated in yeast strains belonging to *Rhodospiridium* genera, as well as in *Sporobolomyces* and *Sporidiobolus* (Buzzini et al., 2007). However the ratio of each carotenoid highly varies and seems to depend on both strain and the culture conditions. More specifically, in the study of Buzzini et al., (2007) torulene and torularhodin were the predominant carotenoids produced by *Rhodospiridium* strains grown on glucose and yeast extract. In another study, three *Rhodospiridium toruloides* strains cultivated on glycerol resulted in different carotenoid profiles; however torularhodin and torulene were the main carotenoids in detriment to β -carotene (Lee et al., 2014). As far as *R. kratochvilovae* strains are concerned, the studies indicating their carotenoid profile are scarce. Byrtusová et al., (2021) carried out a very comprehensive evaluation of the carotenoids' profile synthesized by

Rhodospiridium kratochvilovae CCY 20-2-26 using different carbon and nitrogen sources. This strain produced β -carotene, lycopene, torulene and torularhodin in all cases; however their ratio varied significantly among the different substrates. For example, growing the strain with glucose and ammonium sulphate (C/N equal to 70) led to similar amounts of β -carotene and torularhodin, followed by torulene and lycopene. The highest concentration of β -carotene (~60% of total carotenoids) was observed when mannose was the carbon source under low C/N (equal to 20). Unlike our study, the authors did not report the production of γ -carotene from this strain. Breierová et al., (2018) studied the same yeast using 20 g/L glucose and 4 g/L yeast extract. Under these culture conditions, the strain produced mainly β -carotene, followed by torulene and torularhodin. The production of lycopene and γ -carotene was not reported in this study. In the biosynthetic pathway, β -carotene and torulene derive from γ -carotene, which is initially formed, while torularhodin is the most oxidized form, thus the last to be produced (Cardoso et al., 2016).

Even though β -carotene production is not the highest reported in the literature, both strains were able to synthesize this valuable pigment within 39 h to 63 h of fermentation, depending on the carbon source. For example, Tkáčová et al., (2018) demonstrated the synthesis of 7.2 g/L of β -carotene using the strain *Rhodotorula glutinis* CCY-20-2-26 in glucose, but after 192 h of fermentation. Yen et al., (2019) achieved 0.4 mg/g of β -carotene after 96 h of fermentation using *Rhodotorula glutinis* BCRC 22360 grown on glycerol. In addition, and to best of our knowledge, this is the first study demonstrating the ability of *Rhodospiridium kratochvilovae* strains to produce γ -carotene. It is noteworthy that the composition of the produced carotenoids was not substantially affected by the culture medium or the strain employed. The findings of this study are promising for the production of β -carotene through a sustainable biotechnological route.

8.3.4 Shake flask fermentations using sucrose and fructose as substrates

Provided that both strains were able to grow and accumulate oil and carotenoids on various carbon sources, sucrose and fructose were subsequently evaluated. The selection of these specific carbon sources was based on the composition of other abundant renewable resources such as grape pomace and orange peel hydrolysates.

Understanding the growth pattern and carotenoid synthesis on multiple carbon sources could be the base for the development of cost-efficient and robust bioprocesses using inexpensive substrates. The selected C/N was 80 (g/g), given that this ratio resulted in the highest carotenoid concentrations for both strains. As shown in **Table 8.4**, fructose and sucrose were unfavorable carbon sources for both strains. More specifically, the highest carotenoid concentration on sucrose was only 0.8 mg/L and 0.61 mg/L on fructose, when strain *Rhodospiridium kratochvilovae* Y-42 was employed (**Table 8.4**). The carotenogenesis in *Rhodospiridium kratochvilovae* Y-43 showed a 2.5-fold decrease on sucrose, in contrast to the results obtained with glucose or the mixture of glucose-galactose (**Table 8.1**), while the reduction in total carotenoid concentration was even more evident when fructose was used, showing an approximate 4.5-fold decrease (**Table 8.4**). The profile of the produced carotenoids (**Table 8.5**) indicated that β -carotene was again the predominant carotenoid in all tested cases, ranging from 56.59% to 84.61%. The presence of γ -carotene and torulene was also observed, with their concentrations ranging from 7.65% to 15.46%, depending on the carbon source and the strain employed. Notably, torularhodin was detected only when strain Y-42 was cultivated on sucrose (16.06%), while it was completely absent in fructose for both strains (**Table 8.5**)

Comparing these results with the literature, it can be concluded that each strain could perform differently depending on the carbon source. Nagal et al., (2023) demonstrated that sucrose is not a favorable carbon source for *Phaffia rhodozyma*, neither for promoting cell growth nor for carotenogenesis. In contrast, El-Banna et al., (2012) reported that sucrose served as the optimal substrate for the yeast strain *Rhodotorula glutinis*, at a C/N ratio of 10 with ammonium sulfate as the nitrogen source, achieving a carotenoid concentration of 1.9 mg/L after 5 days of fermentation. Park et al., (2008) evaluated both sucrose and fructose as carbon sources for *Xanthophyllomyces dendrorhous* SKKU 0107. Their results indicated that sucrose was the preferred substrate, yielding 0.897 μ g of carotenoids per mg of biomass, compared to 0.763 μ g of carotenoids per mg of biomass when using fructose. Moreover, Selim et al., (2013) reported that fructose was the most favorable carbon source for carotenoid production by *Rhodotorula glutinis* NRRL Y-842, with a maximum concentration of 2.2 mg/L.

Table 8.4 Maximum carotenoid concentration and other fermentation parameters of *Rhodospiridium kratochvilovae* Y-42 and Y-43 strains using C/N ratio 80 and different synthetic media (sucrose, fructose) with total sugar concentration 50 g/L.

Carbon source	Time (h)	Total carotenoids (mg/L)	Carotenoid yield (mg/g biomass)	Sugars consumed (g/L)	FAN content (mg/L)	Inorganic nitrogen (mg/L)	Biomass (g/L)	Microbial oil (g/L)
<i>Rhodospiridium kratochvilovae</i> Y-42								
Sucrose	80	0.84±0.06 ^a	0.055±0.004	37.40±0.8	17.80±0.9	0.00	13.42±0.3	3.01±0.5 ^a
Fructose	76	0.61±0.03 ^b	0.066±0.002	42.60±0.0	24.90±1.02	0.00	12.49±0.5	2.19±0.3 ^b
<i>Rhodospiridium kratochvilovae</i> Y-43								
Sucrose	80	0.86±0.17 ^{a1}	0.107±0.021	31.21±1.3	31.50±1.3	0.00	11.03±0.6	2.31±0.2 ^{a1}
Fructose	96	0.47±0.07 ^{b1}	0.040±0.005	25.30±1.26	30.30±0.9	0.00	12.60±0.7	2.04±0.01 ^{b1}

* Different lowercase letters (a, b, a1, b1) indicate significant differences ($p<0.05$) on total carotenoids and microbial oil production between different carbon sources.

Table 8.5 Composition of carotenoids at their maximum concentrations in *Rhodospiridium kratochvilovae* Y-42 and Y-43 under the studied culture conditions.

Carbon source	C/N (g/g)	Time (h)	β -carotene (%)	γ -carotene (%)	Torulene (%)	Torularhodin (%)
<i>Rhodospiridium kratochvilovae</i> Y-42						
Sucrose	80	80	56.59	11.88	15.46	16.06
Fructose	80	76	72.80	15.11	12.09	0.00
<i>Rhodospiridium kratochvilovae</i> Y-43						
Sucrose	80	80	84.61	7.74	7.65	0.00
Fructose	80	96	76.92	9.45	13.62	0.00

8.4 Conclusions

The results of this chapter demonstrate the ability of two unexplored *Rhodospiridium kratochvilovae* strains (Y-42 and Y-43) to produce carotenoids under different culture conditions. Application of different C/N ratios and carbon sources significantly influenced the concentration of total carotenoids with the more promising results obtained when the strains were grown on glucose or on a mixture of glucose-galactose. The carotenoid profile remained unaffected by the C/N ratio; however, the choice of carbon source employed could result in differentiations. β -carotene was the predominant compound, regardless the cultivation parameters. The results are promising and reveal the significant potential of *Rhodospiridium kratochvilovae* strains as β -carotene producers under short fermentation times. This research contributes to the sustainable production of natural β -carotene that could be applied in foods and pharmaceuticals as alternative to its synthetic counterpart. Utilization of waste streams as cultivation substrates is of paramount importance to promote

circularity and enhance the sustainability of the process. Given the promising carotenoid concentrations achieved using a mixture of glucose- galactose, the next step of this research involved evaluating cheese whey, an important by-product of the dairy industry containing lactose, as a fermentation substrate.

The results presented in Chapter 8 have been published in Sustainable Chemistry and Pharmacy:

Sereti, F., Papadaki, A., Alexandri, M., Kachrimanidou, V., Kopsahelis, N. (2023). Exploring the potential of novel *R. kratochvilovae* red yeasts towards the sustainable synthesis of natural carotenoids. *Sustain Chem. Pharm.* 31, 10092

CHAPTER 9

Production of carotenoids from *Rhodospiridium kratochvilovae* Y-42 & Y-43 using cheese whey as substrate

9 Production of carotenoids from *Rhodospiridium kratochvilovae* Y-42 & Y-43 using cheese whey as substrate

9.1 Introduction

As shown in Chapter 8, different culture parameters could significantly affect the cell growth and metabolites production of the yeast strains *Rhodospiridium kratochvilovae* Y-42 and Y-43. An appropriate C/N ratio as well as the carbon source employed could enhance carotenogenesis over microbial oil accumulation. The results on commercial sugars indicated that mixtures of glucose-galactose are promising in terms of both carotenoids production and lipids accumulation, especially under a C/N ratio equal to 80 (g/g). To this end, the main objective of this chapter is to assess the feasibility of using cheese whey, as fermentation substrate as well as different nitrogen sources. Optimization of fermentation conditions was carried out, emphasizing on the effect of C/N ratio and temperature on carotenoid synthesis. Additionally, the individual carotenoids produced were identified via HPLC-DAD analysis to investigate the influence of the different conditions employed on their composition. Finally, the antioxidant activity of the biotechnologically produced carotenoid extracts was evaluated.

9.2 Shake-flask fermentations with hydrolyzed cheese whey

9.2.1 Fermentations with hydrolyzed cheese whey using commercial β -galactosidase

The initial step prior to fermentation with yeast strains *Rhodospiridium kratochvilovae* Y-42 and Y-43 using cheese whey as a substrate was lactose hydrolysis, as most *Rhodospiridium* sp. strains are unable to breakdown this sugar to its monomers (Kantry et al., 2015; Roukas et al., 2015; Poonia and Pandey, 2023). Hydrolysis was performed by adding commercial β -galactosidase to cheese whey. Results showed that the strain Y-42 produced the highest amount of carotenoids (1.87 mg/L) at 48 h, consuming 41.2 g/L of sugars (**Figure 9.1A, B**). Inorganic nitrogen was completely assimilated after 24 h, coinciding with FAN consumption, even though almost 25 mg/L of the latter remained unconsumed throughout the fermentation process. Glucose was rapidly consumed (within the first 30 h), while galactose consumption rate was boosted after glucose depletion (**Figure 9.1A**).

Carotenoid synthesis initiated at about 10 h of fermentation however their production increased rapidly after nitrogen exhaustion (Figure 9.1B).

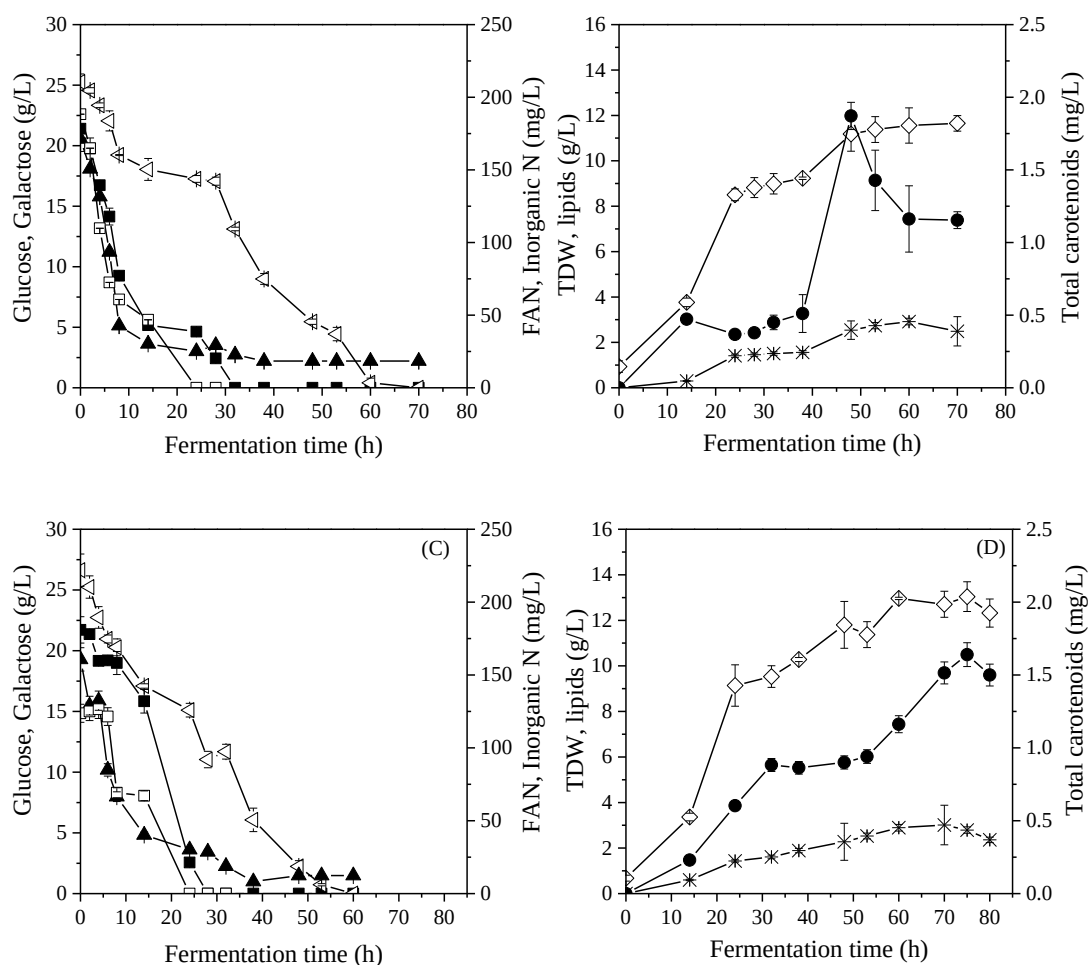


Figure 9.1 (A,C) Consumption of glucose (■), galactose (◁), free amino nitrogen (FAN) (▲) and inorganic nitrogen (□) and (B, D) evolution of total dry weight (◇), lipids (*) and carotenoids (●) during batch fermentations of *Rhodospiridium kratochvilovae* Y-42 (A, B) *Rhodospiridium kratochvilovae* Y-43 (C, D) and in hydrolyzed cheese whey.

Comparable levels of carotenoid concentration were also produced from strain Y-43 (1.64 mg/L), however at a lower production rate to strain Y-42 (Figure 9.1D). Nitrogen consumption (both FAN and inorganic) followed a similar trend to Y-42 (Figure 9.1C). Glucose and galactose were initially simultaneously assimilated; yet glucose was completely depleted after 28 h, while galactose was slowly consumed till the end of the process (60 h). Carotenoid synthesis seems to occur in two phases: the first one (14-32 h) follows growth, whereas the second phase (53-75 h) occurs at the stationary phase (Figure 9.1D). Similar kinetics were observed in the work of

Varzakakou et al., (2010), when *Blakeslea trispora* was cultivated on cheese whey for carotenoids' production. Finally, both strains showed comparable cell growth rate with 12.24 g/L total cell weight for Y-42 and 13.04 g/L for strain Y-43 (**Figures 9.1B, D**). The behavior of both strains on hydrolyzed cheese whey resembles the one obtained on commercial substrates, when mixtures of glucose and galactose were utilized (**Table 8.1**). Cheese whey has been proven an appropriate substrate for carotenoids production, even though lactose hydrolysis prior to fermentation is required. Kanzy et al., (2015) also reported high amounts of total carotenoids (3.9-5.7 mg/L) by the yeasts *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* grown on salted cheese whey. Roukas et al., (2015) achieve a significantly high carotenoids concentration with *Blakeslea trispora* (1.62 mg/L) cultivated on cheese whey, after 4 days, using a bubble column bioreactor at high aeration rates.

Despite the fact that *Rhodospiridium* sp. yeasts are considered as oleaginous, both strains (Y-42 and Y-43) accumulated low amounts of microbial oil (19.7% and 23.9% of their dry cell biomass, respectively). Therefore, consistent to our previous results, it was observed that C/N ratio 80 (g/g) significantly affected the metabolic pathway of *Rhodospiridium* strains promoting the carotenoid production over intracellular microbial oil accumulation. Kot et al., (2019) also emphasized the importance of C/N ratio on yeast metabolism. At lower C/N ratio, *Rhodotorula glutinis* LOCKR13 was able to produce 6.46 mg/L of total carotenoids after 120 h using potato wastewater with 5% glycerol as substrate, whereas at higher ratios the microbial oil accumulation was favored.

Finally, the individual constituents of carotenoid extracts were analyzed. The profile of the individual carotenoids revealed that β -carotene was the prevalent carotenoid for both strains, which is in line with the results presented in Chapter 8. Besides β -carotene, γ -carotene, torulene and torularhodin were also detected in the extracts. Fluctuations in the ratio between the different carotenoids occurred throughout the fermentation for both strains, following distinctive patterns between the two yeasts (**Figure 9.2**). For example, at the beginning of carotenogenesis, strain Y-42 produced β -carotene (89%) and torularhodin (11%) and gradually β -carotene concentration decreased to yield γ -carotene, torulene and even more torularhodin (22.8% at 38 h)

(**Figure 9.2A**). The production of torulene and torularhodin could be attributed to stress induced in the cell due to nitrogen exhaustion (Zhao and Li, 2022). When carotenoids concentration peaked (1.87 mg/L), β -carotene constituted the 80% of the extract, followed by γ -carotene (12.4%) and torulene (7.6%).

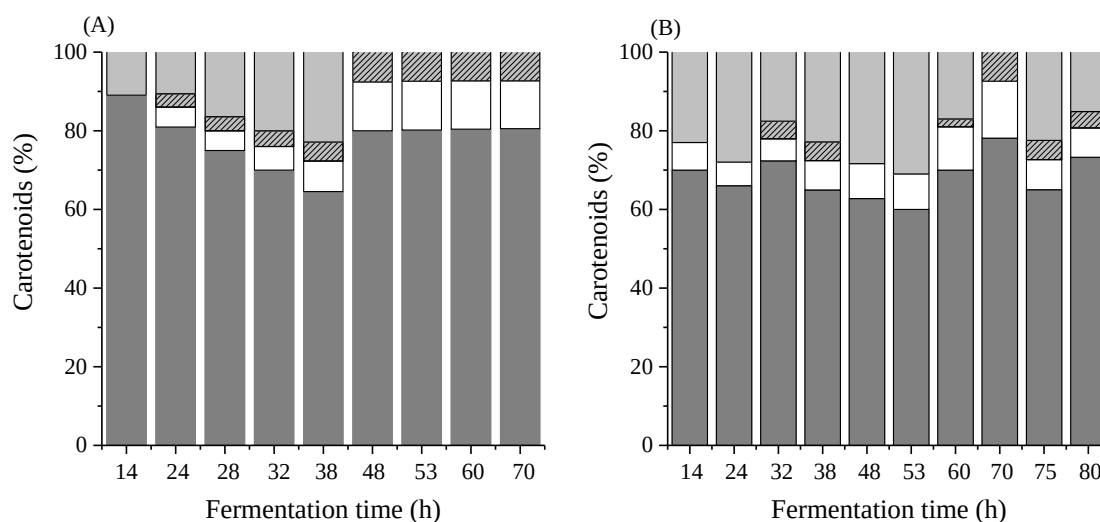


Figure 9.2 Carotenoids composition during fermentation on hydrolyzed cheese whey in (A) *Rhodosporidium kratochvilovae* Y-42 and (B) *Rhodosporidium kratochvilovae* Y-43. β -carotene (dark grey bars), γ -carotene (white color bars), torulene (patterned bars), torularhodin (light grey color bars).

Torularhodin was present almost throughout the process for strain Y-43 (**Figure 9.2B**). After 24 h of fermentation, that nitrogen was depleted, total carotenoids were composed of 66% of β -carotene, 6% of γ -carotene and 28% of torularhodin. Torulene was absent in the beginning of both phases of carotenoid synthesis, ranging generally in lower amounts (4%-7%), in comparison to strain Y-42. Finally, the composition of carotenoids at the highest concentration (1.64 mg/L) was defined by 65% of β -carotene, 7.6% of γ -carotene, 4.9% of torulene and 22.5% of torularhodin. These findings are crucial in understanding *Rhodosporidium kratochvilovae* metabolism as well as the commercial potential of these yeasts, especially if the production of the unusual carotenoids torulene and torularhodin is targeted.

9.2.2 Fermentations with hydrolyzed cheese whey using fermented wheat bran

An alternative way to hydrolyze the lactose contained in cheese whey was to utilize crude enzymes from fermented wheat bran. This process not only eliminated the need for commercial enzymes but also provided a substrate enriched with free amino nitrogen (FAN). The results showed a notable enhancement on carotenogenesis for both yeasts (**Table 9.1**). More specifically, the strain *Rhodospiridium kratochvilovae* Y-42, produced 8.24 mg/L carotenoids at 72 h of cultivation; a 4.4-fold increase compared with the results obtained using commercial β -galactosidase. Subsequently, the carotenoids production of strain *Rhodospiridium kratochvilovae* Y-43 presented a 3.7-fold improvement after 80 h (6.11 mg/L) in comparison with solely cheese whey. In both cases, microbial oil accumulation was not favored (0.85 and 1.61 g/L, respectively). Analysis of the carotenoids profile using HPLC-DAD analysis demonstrated substantial differences in comparison to the results obtained from hydrolyzed cheese whey with commercial enzymes, as lycopene was the predominant carotenoid. Lycopene accounted for more than 97% of the total carotenoids while it was observed a complete absence of β -carotene. Thus, considering the ability of both strains to synthesize high amounts of lycopene using fermented wheat bran along with cheese whey, the next set of experiments focused on optimizing culture conditions by altering critical parameters that affect carotenogenesis. The impact of different C/FAN ratio, as well as temperature, was evaluated to investigate their influence on carotenoid production and, in particular, on carotenoids composition.

Table 9.1 Maximum carotenoid production and chemical profile, biomass and microbial oil concentration during fermentation on hydrolyzed cheese whey with fermented wheat bran of *Rhodospiridium kratochvilovae* Y-42 and Y-43 under C/N ratio 80.

Yeast strain	Total carotenoids (mg/L)	Biomass (g/L)	Microbial oil (g/L)	Lycopene (%)	γ -carotene (%)	Torulene (%)	Torularhodin (%)
<i>Rhodospiridium kratochvilovae</i> Y-42	8.24 $\pm$ 0.06	13.24 $\pm$ 0.33	0.85 $\pm$ 0.21	97.85 $\pm$ 0.51	0.55 $\pm$ 0.004	0.51 $\pm$ 0.005	1.07 $\pm$ 0.007
<i>Rhodospiridium kratochvilovae</i> Y-43	6.11 $\pm$ 0.14	15.70 $\pm$ 0.14	1.61 $\pm$ 0.64	97.59 $\pm$ 0.94	1.44 $\pm$ 0.008	0.97 $\pm$ 0.003	0.00 $\pm$ 0.000

9.3 Optimization of shake flask fermentations with hydrolyzed cheese whey

9.3.1 Effect of C/FAN

Two different C/FAN ratios (40, 60 g/g) were applied in order to investigate the carotenoid production of *Rhodospiridium kratochvilovae* yeast strains. The results showed a further increase on carotenoid production on both strains (**Figure 9.3**). More specifically, when a C/FAN ratio of 40 (g/g) was employed, the strain Y-42 produced 24.86 mg/L of total carotenoids after 80 h of cultivation; an 133% increase compared with previous results obtained from hydrolyzed cheese whey with fermented bran (**Table 9.1**) and 16-fold increase in comparison to the experiments conducted with hydrolyzed cheese whey with a commercial β -galactosidase. Furthermore, 19.72 mg/L of total carotenoids were obtained from strain Y-42 after 80 h when the C/FAN ratio was set to 60 (g/g) (**Figure 9.3A**). In both cases, microbial oil accumulation was also not favored (1.68 and 0.84 g/L, respectively). Likewise, strain Y-43 showed higher levels of carotenogenesis when the C/FAN was 40 (g/g) comparing to C/FAN 60 (g/g) (14.05 and 8.61 mg/L, respectively), while low levels of microbial oil accumulation occurred (0.49 and 0.88 g/L, respectively) (**Figure 9.3B**). Overall, both *Rhodospiridium kratochvilovae* strains were able to produce higher levels of carotenoids at lower C/FAN ratios. According to Tkáčová et al., (2017), *Rhodotorula glutinis* JMT 21978 also showed improved results on carotenogenesis (188% increase) as the C/N ratio was decreased. As culture media and fermentation

parameters might further boost carotenogenesis, culture temperature was subsequently investigated.

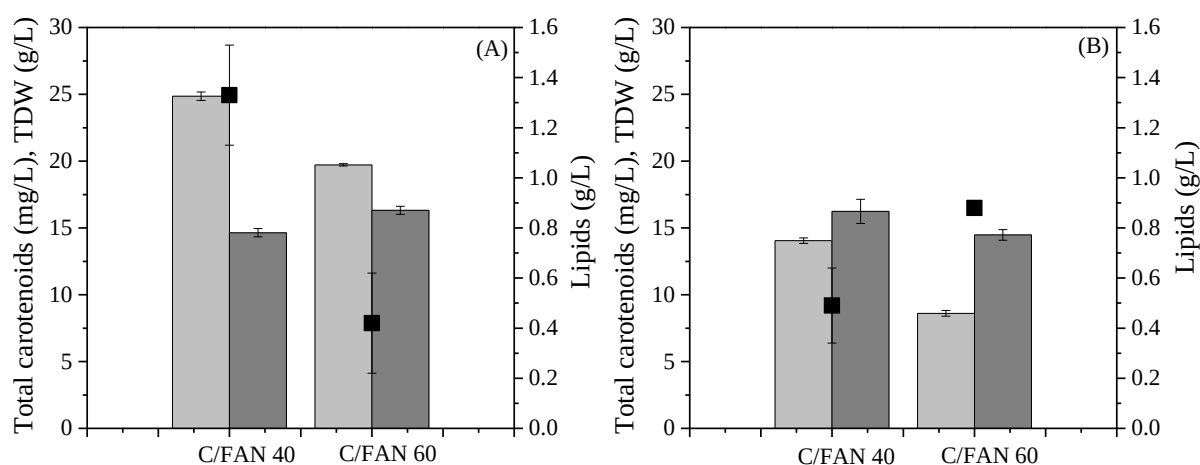


Figure 9.3 Total carotenoids (light grey bar), total dry weight (dark grey bar) and lipids (■) production during fermentation on hydrolyzed cheese whey with fermented wheat bran at different C/FAN ratios during incubation at 28 °C for (A) *Rhodosporidium kratochvilovae* Y-42 and (B) *Rhodosporidium kratochvilovae* Y-43.

9.3.2 Effect of temperature

It is widely accepted that temperature constitutes a critical fermentation factor significantly affecting microbial metabolite synthesis (Igreja et al., 2021; Hernández-Almanza et al., 2014a). Therefore, the next set of experiments addressed the evaluation of incubation temperature on the production of carotenoids on both previously examined C/FAN ratios (40 and 60 g/g). Fermentations were conducted at three incubation temperatures (23, 28 and 33 °C) in order to investigate the temperature effect on carotenogenesis. In case of C/FAN 40 (g/g), *Rhodosporidium kratochvilovae* Y-42 presented a 44% increase on the production of carotenoids at 33 °C (36.35 mg/L) compared with that at 28 °C (24.86 mg/L). Setting the temperature at 23 °C resulted in concentrations similar to those at 28 °C, with values equal to 22.52 and 24.86 mg/L, respectively (**Figure 9.4A**). Once the C/FAN ratio was 60 (g/g), the carotenoid production remained at similar levels with those at 28 °C; 20.7 mg/L at 23 °C and 17.7 mg/L when the temperature was 33 °C. Incubation temperature did not favor microbial oil accumulation regardless the C/FAN. Conclusively, the favorable conditions for carotenoids production by *Rhodosporidium kratochvilovae* Y-42 were

determined to be C/FAN 40 (g/g) and 33 °C, with the corresponding fermentation kinetics illustrated in **Figure 9.5 (A, B)**.

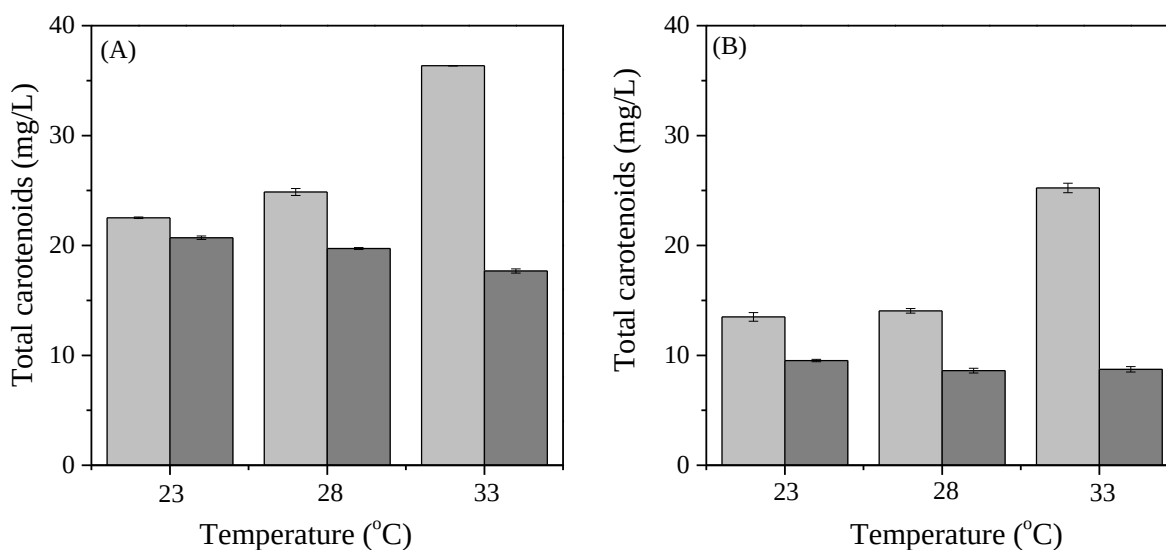


Figure 9.4 Total carotenoids production during fermentation on hydrolyzed cheese whey with fermented wheat bran at C/FAN 40 (light grey color bar) and C/FAN 60 (light grey color bar) for (A) *Rhodosporidium kratochvilovae* Y-42 and (B) *Rhodosporidium kratochvilovae* Y-43.

Increasing the temperature from 28 to 33 °C led to almost 80% increase in carotenoids concentration (from 14.07 to 25.23 mg/L), when *Rhodosporidium kratochvilovae* Y-43 was cultivated at C/FAN ratio 40 (g/g). However, carotenoid synthesis was not favored at the higher C/FAN ratio (60 g/g), regardless the temperature (**Figure 4B**). Microbial oil concentration remained at low levels under all studied conditions (**Table 1**).

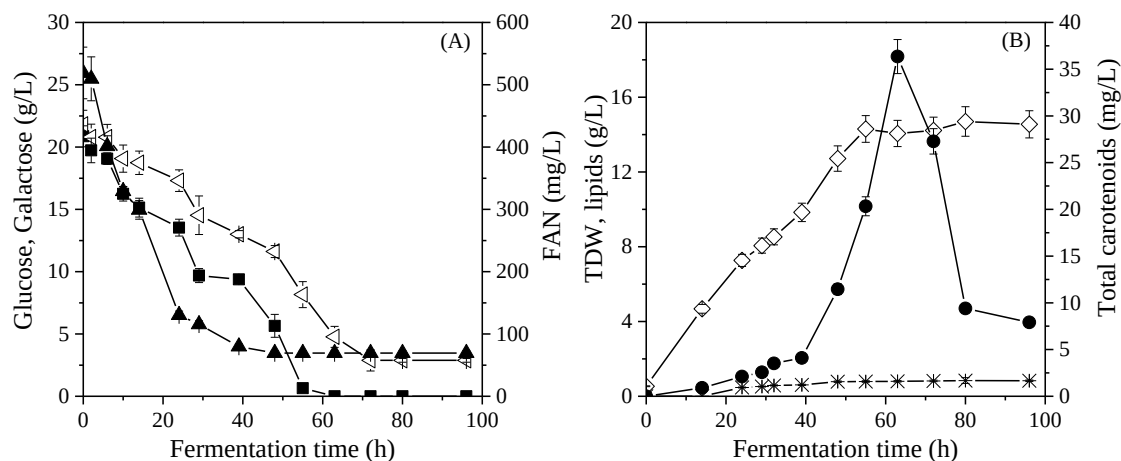


Figure 9.5 (A) Consumption of glucose (■), galactose (◻), free amino nitrogen (FAN) (▲) and inorganic nitrogen (□) and (B) evolution of total dry weight (◇), lipids (*) and carotenoids (●) during batch fermentations of *Rhodosporidium kratochvilovae* Y-42 in hydrolyzed cheese whey with fermented wheat bran with C/FAN ratio 40 (g/g) and at 33 °C incubation temperature.

Previous studies have stated that the optimum incubation temperature for carotenoid accumulation differs from that for microbial oil production. The strains *Rhodotorula glutinis* AS 2.703 and *Rhodotorula* sp. RY1801 showed higher carotenoid synthesis at 28 °C (Elfeky et al., 2020; Zhao et al., 2019), while *Rhodosporidium kratochvilovae* SY89 presented the maximum microbial oil production at 30 °C (Jiru et al., 2017). On the other hand, *Rhodotorula mucilaginosa* F-1 presented higher concentration of total carotenoids (322 µg/g) when the temperature was 25 °C (Cheng and Yang, 2016). Similar observation was made by Zhao and Li, (2022) where the effect of three different incubation temperatures (16, 25 and 32 °C) on microbial oil and carotenoid synthesis in *Rhodotorula glutinis* ZHK was investigated. Their findings implied that 32 °C were the optimum temperature for microbial oil accumulation. The absolute carotenoid concentration was not affected by the increased temperature, however the production of certain carotenoids -torulene and torularhodin- was enhanced. A possible explanation, provided by the authors, was the overproduction of reactive oxygen species (ROS) at higher temperature that forces the cell to produce specific carotenoids that are stronger scavengers to β-carotene. The results of these experiments demonstrated that carotenogenesis was boosted at 33 °C and at the lowest

C/FAN (40 g/g) in both strains, whereas microbial oil accumulation remained at low levels regardless the temperature or C/FAN ratio (**Table 9.2**).

Table 9.2 Maximum carotenoid production and chemical profile, biomass and microbial oil concentration during fermentation on hydrolyzed cheese whey with fermented wheat bran under different C/FAN ratios and temperatures of *Rhodospiridium kratochvilovae* Y-42 and Y-43.

<i>Rhodospiridium kratochvilovae</i> Y-42									
C/FAN (g/g)	Temp (°C)	Time (h)	Total carotenoids (mg/L)	Biomass (g/L)	Microbial oil (g/L)	Lycopene (%)	Torularhodin (%)	Torulene (%)	γ-carotene (%)
40	23	80	22.52±0.07 ^{a, 1}	16.79±0.01	1.92±0.29	98.50±3.20	0.65±0.02	0.44±0.01	0.41±0.02
	28	80	24.86±0.32 ^{b, 2}	14.64±0.31	1.33±0.20	98.50±1.41	0.78±0.03	0.00±0.00	0.72±0.02
	33	63	36.35±0.02 ^{c, 3}	14.55±0.50	0.84±0.15	98.85±0.53	0.31±0.01	0.47±0.01	0.38±0.01
60	23	82	20.70±0.16 ^{a*, 1*}	17.80±0.40	0.71±0.12	98.50±3.11	0.55±0.02	0.49±0.002	0.46±0.01
	28	80	19.72±0.09 ^{b*, 2*}	16.32±0.30	0.42±0.20	99.19±4.12	0.38±0.01	0.00±0.00	0.43±0.02
	33	94	17.68±0.19 ^{c*, 3*}	14.8±0.11	2.38±0.23	98.85±3.55	0.32±0.02	0.52±0.01	0.32±0.02
<i>Rhodospiridium kratochvilovae</i> Y-43									
C/FAN (g/g)	Temp (°C)	Time (h)	Total carotenoids (mg/L)	Biomass (g/L)	Microbial oil (g/L)	Lycopene (%)	Torularhodin (%)	Torulene (%)	γ-carotene (%)
40	23	80	13.50±0.39 ^{a, 1}	17.02±0.10	1.27±0.14	98.04±2.90	0.83±0.03	0.65±0.02	0.48±0.01
	28	80	14.05±0.21 ^{a, 2}	16.24±0.90	0.49±0.15	98.91±3.22	0.65±0.03	0.00±0.00	0.44±0.02
	33	96	25.23±0.43 ^{b, 3}	15.44±0.01	0.86±0.29	98.62±4.32	0.36±0.01	0.58±0.02	0.45±0.01
60	23	72	9.53±0.11 ^{a*, 1*}	17.96±0.50	0.79±0.20	97.33±1.87	0.86±0.04	0.74±0.03	1.08±0.04
	28	80	8.61±0.21 ^{b*, 2*}	14.48±0.40	0.88±0.01	98.75±4.03	0.35±0.01	0.45±0.01	0.46±0.01
	33	96	8.73±0.25 ^{b*, 3*}	17.36±0.10	1.07±0.11	98.59±1.82	0.95±0.01	0.00±0.00	0.45±0.01

* Different lowercase letters and numbers (a, b, c, a*, b*, c*, 1, 2, 3, 1*, 2*, 3*) denote significant differences ($p < 0.05$) on total carotenoids production, for each yeast strain, between the different temperatures (letters) for the same C/FAN ratio and between the two C/FAN ratios (numbers) for the same incubation temperature.

9.3.3 Carotenoids composition

Analysis of the carotenoids profile demonstrated that lycopene remained the main carotenoid produced with a range of 97.33 to 99.19% regardless the C/FAN ratio and the incubation temperature applied (**Table 9.2**). The absence of β -carotene with the simultaneous dominance of lycopene could be attributed to the metabolic pathway of carotenoids biosynthesis. The production of carotenoids is enhanced when the mevalonate pathway is triggered. More specifically, as previously mentioned, under high nitrogen concentration, acetyl-CoA carboxylase (ACC), remained inactive; thus the fatty acid biosynthetic pathway is down regulated. Consequently, the acetyl-CoA is available for the mevalonate pathway and therefore, for carotenoid production. Phytoene is the first carotenoid which occurs through the carotenoid biosynthetic pathway and several enzymes are triggered in succession in order to activate the further cascade of the produced carotenoids. One of the main enzymes in carotenogenesis is the lycopene β -cyclase, which catalyzes the β -cyclization of either both ends of lycopene or one end to generate β -carotene or γ -carotene, respectively. Then, γ -carotene acts as a precursor for the synthesis of torulene and torularhodin, with the latter being the result of hydroxylation and oxidation of torulene (Kot et al., 2018) (**Figure 9.6**). In the current study, the utilized nitrogen concentration might down regulate the fatty acid biosynthesis. At the same time, the fermentation substrate could enhance the accumulation of lycopene by deactivating the initial steps for the production of the remained, aforementioned carotenoids.

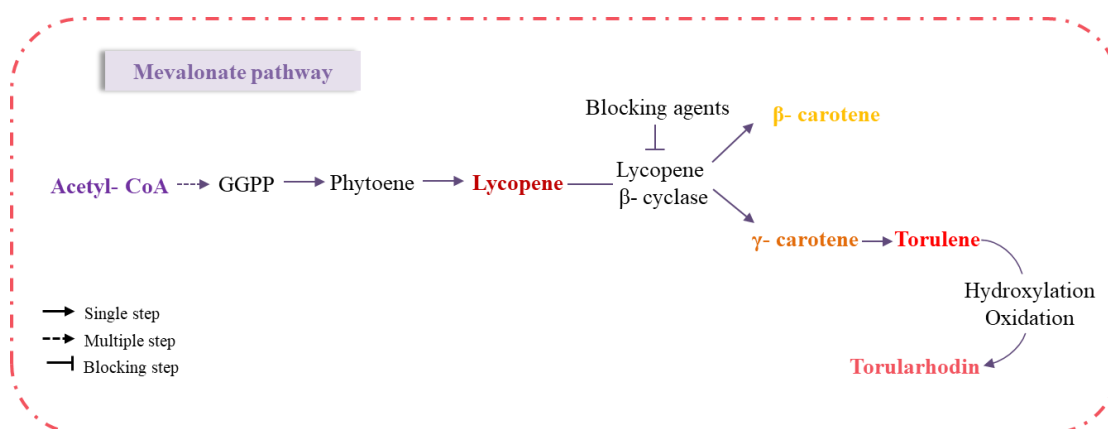


Figure 9.6 Schematic representation of the mevalonate pathway in yeasts leading to the production of different carotenoids.

Based on previous studies, the utilization of blocking agents or metabolic engineering techniques is required to suppress lycopene β -cyclase and achieve high levels of lycopene from microbial fermentations. Lycopene concentration was enhanced 50-fold by transferring genes from *Saccharomyces cerevisiae* pathway to *Yarrowia lipolytica*, constructing chimeric pathways (Liu et al., 2019). Hernández-Almanza et al., (2014b) tested the strain *Rhodotorula glutinis* in solid state fermentation on synthetic media using imidazole as a blocking agent to induce lycopene production. Under optimized conditions the strain was able to produce 340 mg/L lycopene. The European Food Safety Authority (EFSA) already allows lycopene production from *Blakeslea trispora* and its further usage in food applications. It is worth mentioning that *Blakeslea trispora* is a mass accumulator of β -carotene and requires the addition of blocking agents such as imidazole or pyridine to accomplish lycopene production (above 95%) (Mantzouridou, 2020). In view of the fact that utilization of chemical inhibitors might cause toxicological effects on the final product, several substitutes of imidazole or pyridine have been tested. Wang et al., (2014) showed that *Blakeslea trispora* produced 91% lycopene of total carotenoids at 144 h with the addition of 300 mg/L of the inhibitor 2-isopropylimidazole. The supplementation of the cultivation substrate with 50 mg/L of 2-methyl imidazole led to 94% lycopene of the total carotenoid production from *Blakeslea trispora* (Pegklidou et al., 2008). In the present study, almost solely lycopene was produced without the addition of blocking agents. However, further research is essential to elucidate the mechanism or the presence of compounds deriving from renewable resources, which might blocked the activity of lycopene β -cyclase, influencing carotenoids' composition.

9.4 Antioxidant activity of the biotechnologically produced carotenoids

It is established that carotenoids have high antioxidant activity due to the presence of conjugated double bonds on their structure (Papapostolou et al., 2023). Lycopene has eleven conjugated double bonds in its lengthy chain, thus is considered as an efficient free radical and single oxygen scavenger (Papapostolou et al., 2023; Zimmer et al., 2022). Recently, Sinha et al., (2023) demonstrated that besides exhibiting antioxidant and antimicrobial activity, carotenoids extract from *Rhodospiridium toruloides* ATCC 204091 also displayed anti-breast cancer properties. The primary role of lycopene in human's health immune system is to defend DNA against oxidative stress

for the purpose of avoiding the generation of mutations which might provoke chronic illnesses (Sen, 2019; Chen et al., 2022). Therefore, the antioxidant activity of the biotechnologically produced lycopene was next examined through ABTS• [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] analysis. Analysis of the extract derived under the optimum conditions (*Rhodospiridium kratochvilovae* Y-42 cultivated on cheese whey with fermented wheat bran, at 33 °C and at 40 g/g C/FAN) at a concentration of 1.71 mg/mL (consisting of 97% lycopene) presented 72.1% inhibition on ABTS• radical, with a corresponding IC₅₀ of 0.89 mg/mL. Comparing with tomato extract, our sample surpass its scavenging activity by 360% (IC₅₀tomatoextract = 4.095 mg/mL). These results are promising on the future applications of the derived extracts on food and pharmaceutical sector. It should be noted that toxicological tests should also be considered in order to establish the beneficial health properties of the microbially-derived carotenoid extracts (Sinha et al., 2023).

9.5 Conclusions

The results presented in this chapter indicate that hydrolyzed cheese whey constitutes an appropriate substrate for carotenoid synthesis from *Rhodospiridium kratochvilovae* Y-42 and Y-43 strains, while β-carotene was the predominant produced carotenoid. The replacement of commercial enzymes with fermented wheat bran, led to enhanced carotenoids synthesis, with high antioxidant activity, while lycopene was almost solely produced by both yeasts. This study demonstrated for the first time the feasibility to produce high amounts of lycopene, an established food additive, using *Rhodospiridium kratochvilovae* Y-42 and Y-43 grown on renewable resources. Further investigation should be carried out in order to elucidate the influence of renewable resources-based media on yeast metabolism, with ultimate goal, the sustainable production of specific carotenoids.

The results presented in Chapter 9 have been published in Food Bioscience:

Sereti F., Alexandri, M., Papadaki, A., Papapostolou, H., Kopsahelis, N. (2024). Natural lycopene and β-carotene synthesis by *Rhodospiridium kratochvilovae* yeasts: Sustainable production, chemical characterization and antioxidative properties. Food Bioscience, 57, 103425

CHAPTER 10

Production of value-added compounds by
Rhodospiridium paludigenum 2663 & 2664

10 Production of value-added compounds by *Rhodosporidium paludigenum* 2663 & 2664

10.1 Introduction

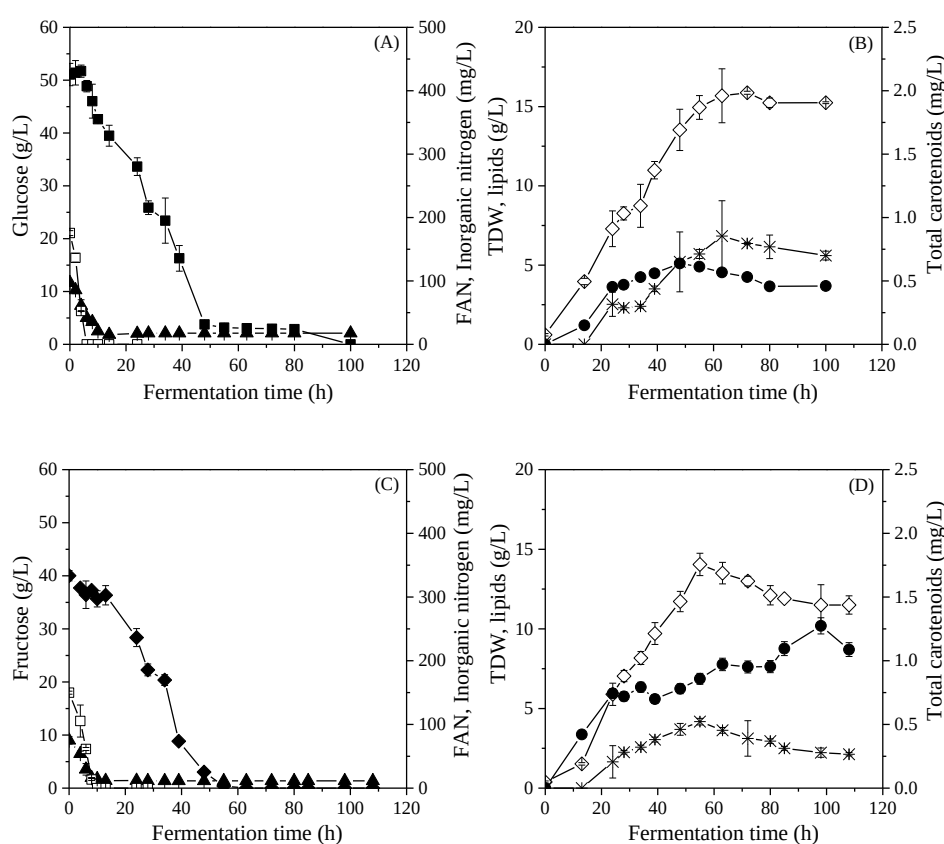
The main objective of this chapter was the investigation of two unexplored *Rhodosporidium paludigenum* strains, namely NCYC 2663 and NCYC 2664, for their ability to produce value-added compounds, such as carotenoids and microbial oil, when grown on different carbon sources. Four commercial carbon sources, namely glucose, sucrose, fructose and a mixture of glucose- galactose, were initially evaluated in order to assess the ability of these strains to accumulate oil and carotenoids intracellularly. Subsequently, bioreactor fermentations were carried out using either sucrose or molasses, aiming to elucidate the feasibility of producing multiple metabolites. Antioxidant and antimicrobial activities against common bacterial and fungal pathogens were also investigated, unraveling potential applications in the food industry.

10.2 Submerged fermentations in shaking flasks with commercial sugars

Strains *Rhodosporidium paludigenum* 2663 and 2664 were cultivated on different carbon sources aiming to investigate carotenoids and microbial oil accumulations, considering that studies dealing with metabolite synthesis from these two yeasts are scarce. In line to the experiments carried out using the *Rhodosporidium kratochvilovae* strains (Chapter 8) the selected sugars were glucose, fructose, sucrose and a mixture of glucose- galactose. Moreover, evaluation of carotenoid and microbial oil synthesis on these carbon sources would provide useful information for the potential to valorize certain renewable substrates such as cheese whey or fruit waste.

Figure 10.1 depicts the kinetics of strain *Rhodosporidium paludigenum* 2663 on the selected substrates. The strain was able to assimilate all different carbon sources and produce both carotenoids and oils at various rates. Sucrose was the most rapidly consumed sugar (**Figure 10.1E**), yielding also the highest carotenoid concentration (2.21 mg/L). Growth on glucose resulted in the highest microbial oil production (6.83 g/L) in detriment to carotenogenesis (0.64 mg/L). Strain's performance on fructose was also promising, since the sugar was completely exhausted after approximately 60

h of fermentation (**Figure 10.1C**) and accumulated 1.27 mg/L of total carotenoids (**Figure 10.1D**). Fermentation on the mixture of glucose-galactose lasted the longest time (109 h), due to the slow galactose consumption rate (**Figure 10.1E**). As it is already mentioned, nitrogen source is crucial for the synthesis of secondary metabolites. In all cases, maximum carotenoid concentration was achieved after nitrogen depletion (both FAN and inorganic nitrogen). *Rhodosporidium paludigenum* 2663 rapidly utilized ammonium sulphate and even in faster rates compared to FAN (**Figures 10.1A, C, E, G**). Many studies have confirmed that the synthesis of carotenoids in red yeasts mainly occurs during the early stationary phase (Yimyoo et al., 2011; Byrtusová et al., 2021; Fakankun et al., 2021). However, in *Rhodosporidium paludigenum* 2663, the carotenoids maxima were observed at the late stationary phase (**Figures 10.1B, D, F, H**). These findings were similar to the ones obtained by Warjoto et al., (2020), in which a *Rhodosporidium paludigenum* strain was cultivated on a substrate based on orange peels. Lee et al., (2014) suggested that during this growth phase, acetyl-CoA is mainly channeled towards carotenoid and lipid synthesis in detriment to TCA cycle.



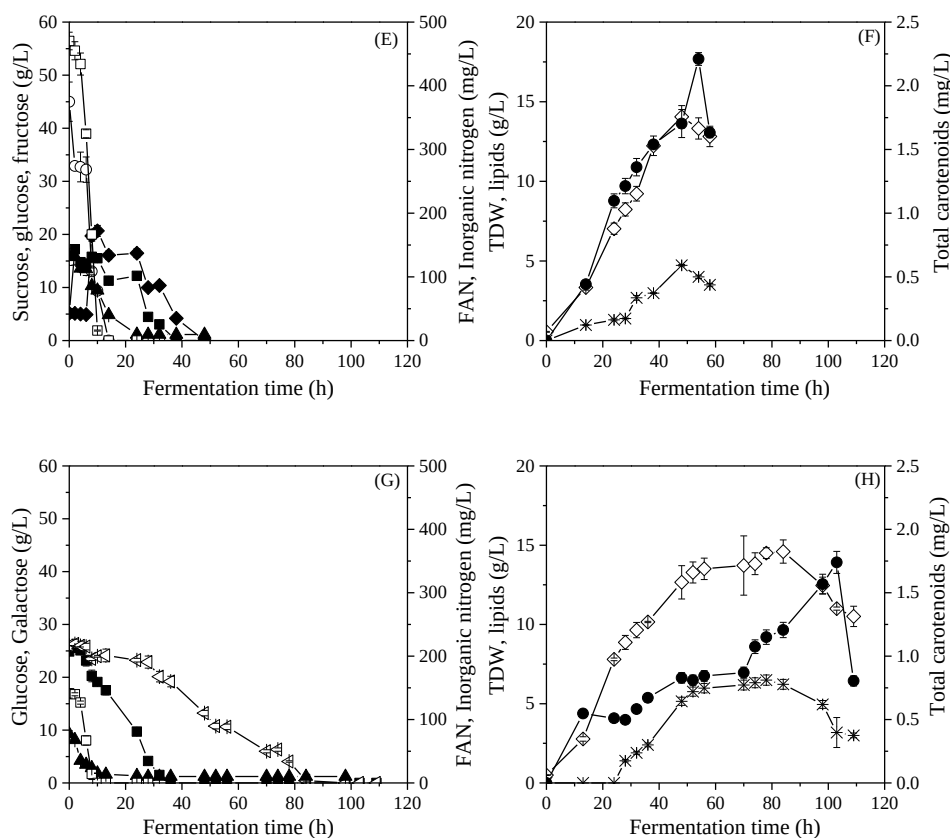


Figure 10.1 Consumption of glucose (■), fructose (◆), sucrose (○), galactose (◁), free amino nitrogen (FAN) (▲) and inorganic nitrogen (□), as well as total dry weight (◇), microbial oil (*) and carotenoids (●) production during batch fermentations of *Rhodospiridium paludigenum* 2663 using glucose (A, B), fructose (C,D), sucrose (E, F) and glucose-galactose (G, H) as carbon sources.

Microbial oil accumulation followed a different pattern than that of carotenoids, and only in sucrose the two maxima were achieved at the same time (**Figure 10.1F**). The highest microbial oil accumulation was acquired when glucose was the sole carbon source, reaching a value of 6.83 g/L (43.5% lipid content). Similar microbial oil concentration (6.49 g/L) was accumulated by the strain when a mixture of glucose-galactose was used as fermentation substrate (**Figure 10.1H**). Among the tested sugars, sucrose seemed to be the most favorable for the concomitant production of both carotenoids and microbial oil (**Table 10.1**). It is worth noting that while growing on sucrose, production of exopolysaccharides was also observed. Actually, *Rhodospiridium paludigenum* strains have been previously reported as pullulan

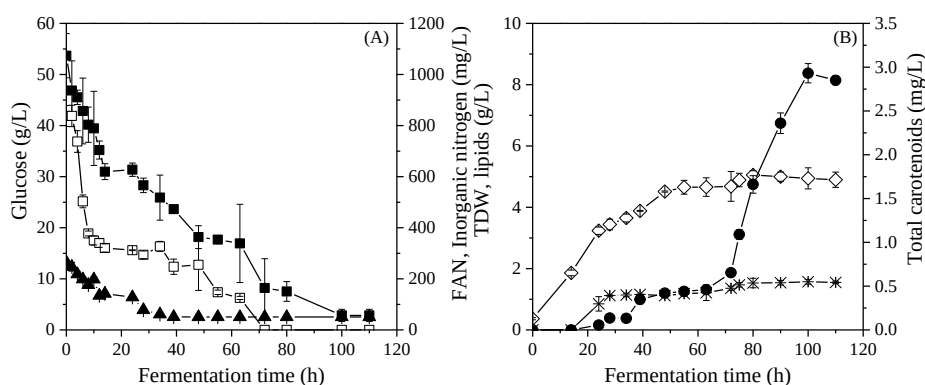
producers (Singh and Kaur, 2018). Further studies, based on sucrose as substrate, could further elucidate the potential of this strain as a multi-product cell factory.

Table 10.1 Maximum carotenoid concentration as well as total dry weight (TDW), lipid concentration and content at the same fermentation time of *Rhodospiridium paludigenum* 2663 and 2664 strains using different carbon sources.

Carbon source	Time (h)	Total carotenoids (mg/L)	Carotenoid yield (mg/g)	TDW (g/L)	Lipids (g/L)	Lipid content (%w/w)
<i>Rhodospiridium paludigenum</i> 2663						
Glucose	48	0.64 ± 0.03 ^a	0.047 ± 0.002	13.5 ± 1.3	5.20 ± 1.89 ^{a1}	38.5 ± 2.1
Fructose	98	1.27 ± 0.06 ^b	0.111 ± 0.005	11.5 ± 1.3	2.23 ± 0.29 ^{b1}	19.4 ± 0.8
Sucrose	54	2.21 ± 0.05 ^c	0.170 ± 0.007	13.3 ± 0.7	4.00 ± 0.11 ^{a1, b1}	30.0 ± 1.2
Glucose-Galactose	103	1.74 ± 0.09 ^d	0.158 ± 0.004	11.0 ± 0.1	3.18 ± 0.95 ^{a1, b1}	28.9 ± 0.5
<i>Rhodospiridium paludigenum</i> 2664						
Glucose	100	2.93 ± 0.11 ^a	0.593 ± 0.019	4.9 ± 0.3	1.57 ± 0.08 ^{a1}	31.8 ± 1.4
Fructose	80	1.94 ± 0.05 ^b	0.263 ± 0.011	7.4 ± 0.1	1.27 ± 0.01 ^{b1}	17.2 ± 0.6
Sucrose	100	1.42 ± 0.03 ^c	0.235 ± 0.008	6.1 ± 0.3	0.83 ± 0.03 ^{c1}	13.7 ± 0.5
Glucose-Galactose	94	1.56 ± 0.06 ^d	0.188 ± 0.006	8.3 ± 0.2	1.45 ± 0.22 ^{a1, b1}	17.5 ± 0.5

*Different lowercase letters (a, b, c, d, a1, b1, c1) denote significant differences (p< 0.05) on total carotenoids and lipids production, for each yeast strain, between the various carbon sources.

Strain *Rhodospiridium paludigenum* 2664 performed differently in comparison to 2663 (**Figure 10.2**). Glucose was proven as the most adequate substrate for carotenoids synthesis (2.93 mg/L), followed by fructose (1.94 mg/L). Total dry weight was in all substrates almost 2-fold lower (5-8.5 g/L) when compared to *Rhodospiridium paludigenum* 2663 (14-16 g/L). Microbial oil accumulation was also at lower levels (**Table 10.1**). The lower C/N content selected for this strain (20 g/g) could be responsible for the limited oil production; however the strain was unable to grow at C/N higher than 20 g/g (results not shown). While strain 2663 required around 10-14 h to deplete both nitrogen sources, strain 2664 fully assimilated nitrogen only when glucose and fructose were the carbon sources, and after about 72 h of fermentation (**Figure 10.2A, C**). Consequently, the maximum carotenoid concentration was accomplished -with the exception of fructose- at the end of the process (**Figures 10.2B, D, F, H**). Fructose and sucrose were entirely consumed (**Figure 10.2C, E**), whereas about 3 g/L of glucose was left unconsumed, when used as sole carbon source (**Figure 10.2A**). Glucose was firstly utilized when combined with galactose, while the consumption of the latter initiated after glucose reached a limiting concentration (~10 g/L) in the medium (**Figure 10.2G**).



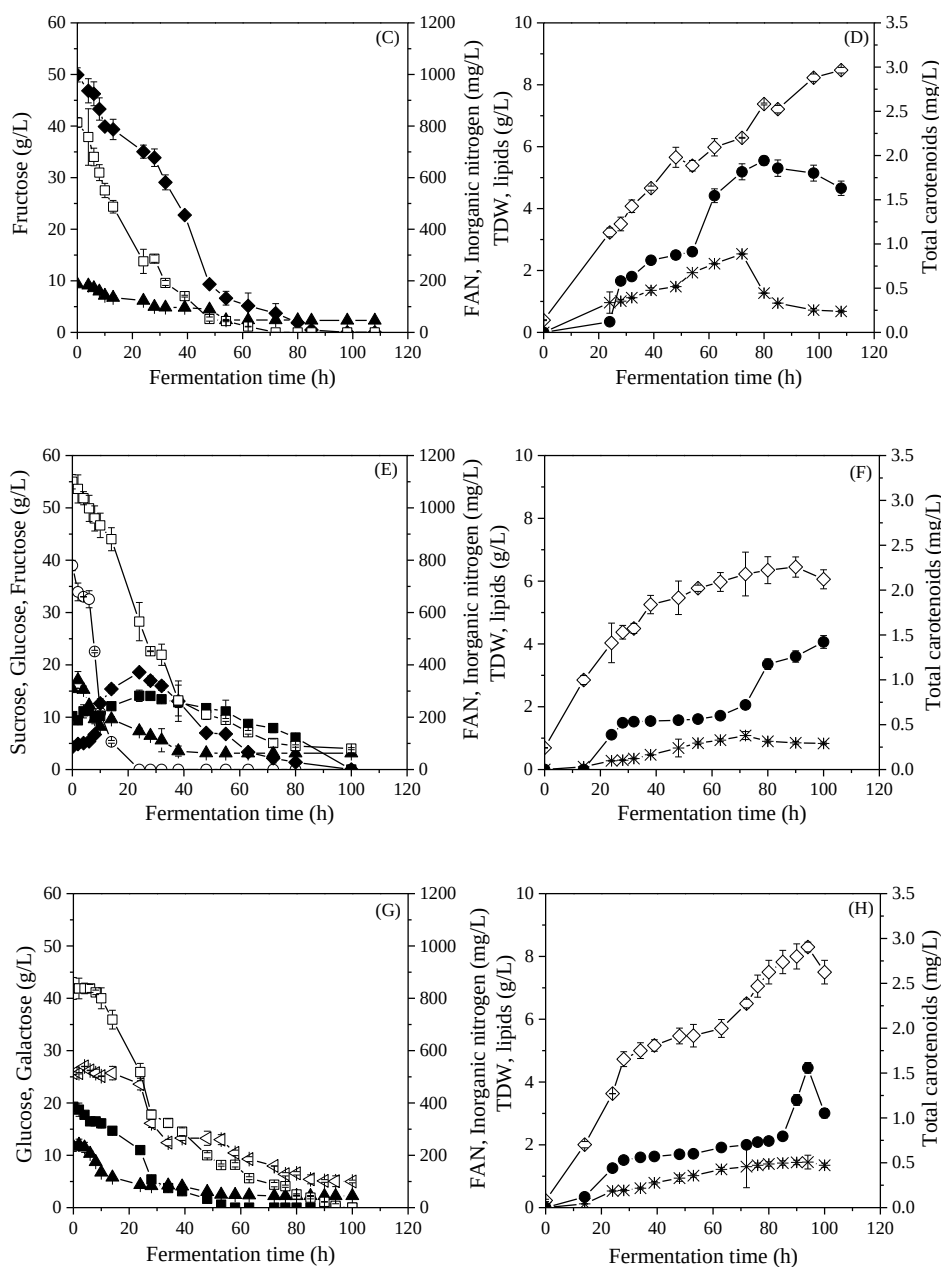


Figure 10.2 Consumption of glucose (■), fructose (◆), sucrose (○), galactose (◁), free amino nitrogen (FAN) (▲) and inorganic nitrogen (□), as well as total dry weight (◇), microbial oil (*) and carotenoids (●) production during batch fermentations of *Rhodosporidium paludigenum* 2664 using glucose (A, B), fructose (C,D), sucrose (E, F) and glucose: galactose (G, H) as carbon sources.

10.2.1 Identification of individual carotenoids composition

The chemical profile of the individual carotenoids synthesized by both strains, during cultivation on the different carbon sources, was monitored throughout the fermentation. As shown in **Figure 10.3** and **Figure 10.4**, the two *Rhodospiridium paludigenum* strains presented substantial variations. The dominant carotenoid was β -carotene for both yeasts, ranging from 58.6-94.3% (depending on the carbon source) for strain 2663 and more than 96% for strain 2664. For *Rhodospiridium paludigenum* 2663, it is evident that the carbon source affected the proportion of the individual carotenoids (**Figure 10.3**). At the beginning of carotenogenesis, β -carotene was produced in amounts corresponding to more than 85% of total carotenoids, in all cases. As fermentation was proceeding, the ratio of β -carotene to other carotenoids (γ -carotene, torulene, torularhodin) was reduced and its percentage at the maximum point differed significantly. It has been previously reported that when yeast cells operate under stress (e.g. depletion of nitrogen source, high temperatures or extreme pH values) the synthesis of torulene and torularhodin is increased, in order to regulate the production of reactive oxygen species (Mussagy et al., 2023; Zhao and Li, 2022). Torulene and torularhodin are stronger radical scavengers than β -carotene (Kot et al., 2018; Zhao and Li, 2022). Kot et al., (2019b) correlated the enhanced production of torulene using a *Rhodotorula gracilis* strain, when oxidative stress was imposed. In these experiments, the dramatic increase in these two “unusual” carotenoids was detected after nitrogen was consumed, or when the carbon source was fully depleted.

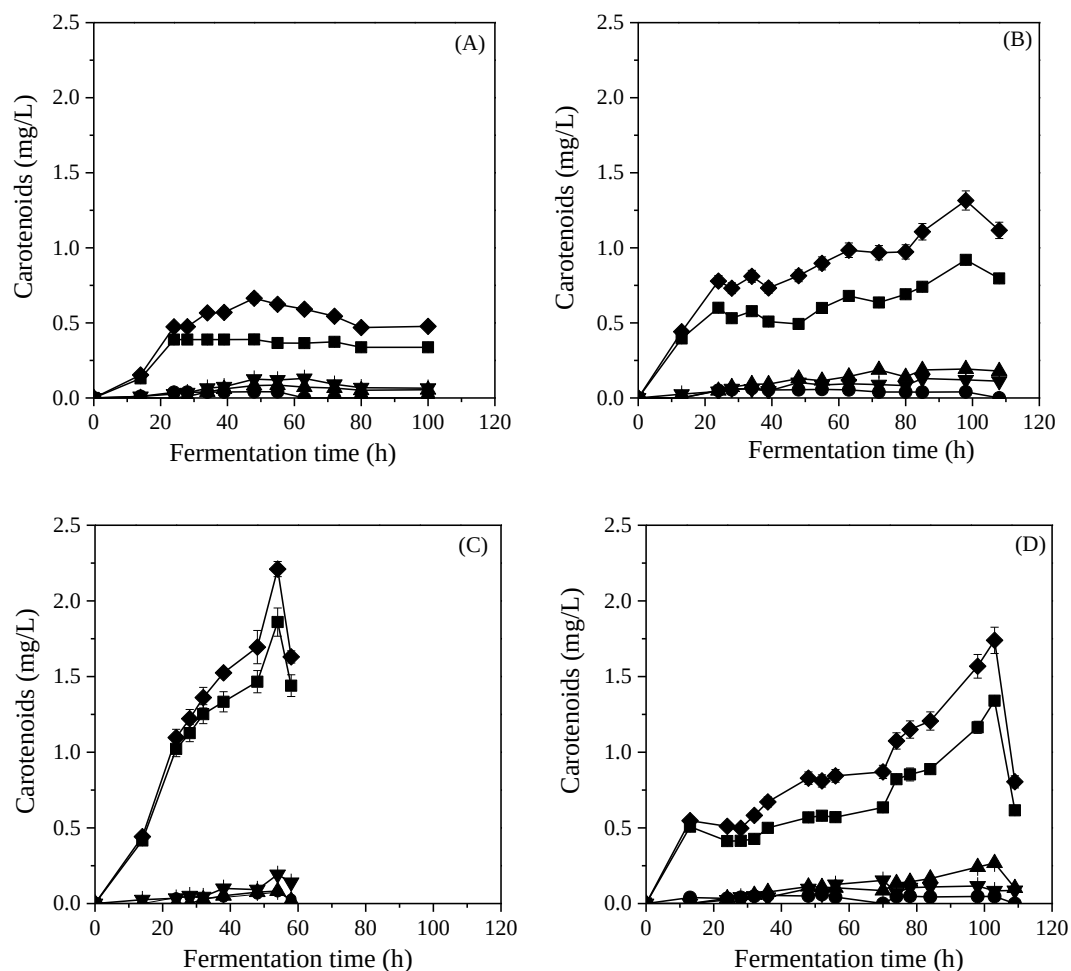


Figure 10.3 Chemical profile of the individual carotenoids produced during fermentation of strain *Rhodospiridium paludigenum* 2663 on (A) glucose, (B) fructose, (C) sucrose, (D) glucose-galactose. Total carotenoids (◆), β-carotene (■), γ-carotene (●), torulene (▲), torularhodin (▼).

Since this stressing factor was not imposed on strain 2664, β-carotene was almost the sole carotenoid detected in all substrates (**Figure 10.4**). In fructose, torulene production (2-5% of total carotenoids) coincided with FAN depletion (**Figure 10.2B**, **10.4B**). These findings could shed light into yeast metabolism on carotenogenesis and on how the adequate substrate manipulation could lead in the production of different carotenoids.

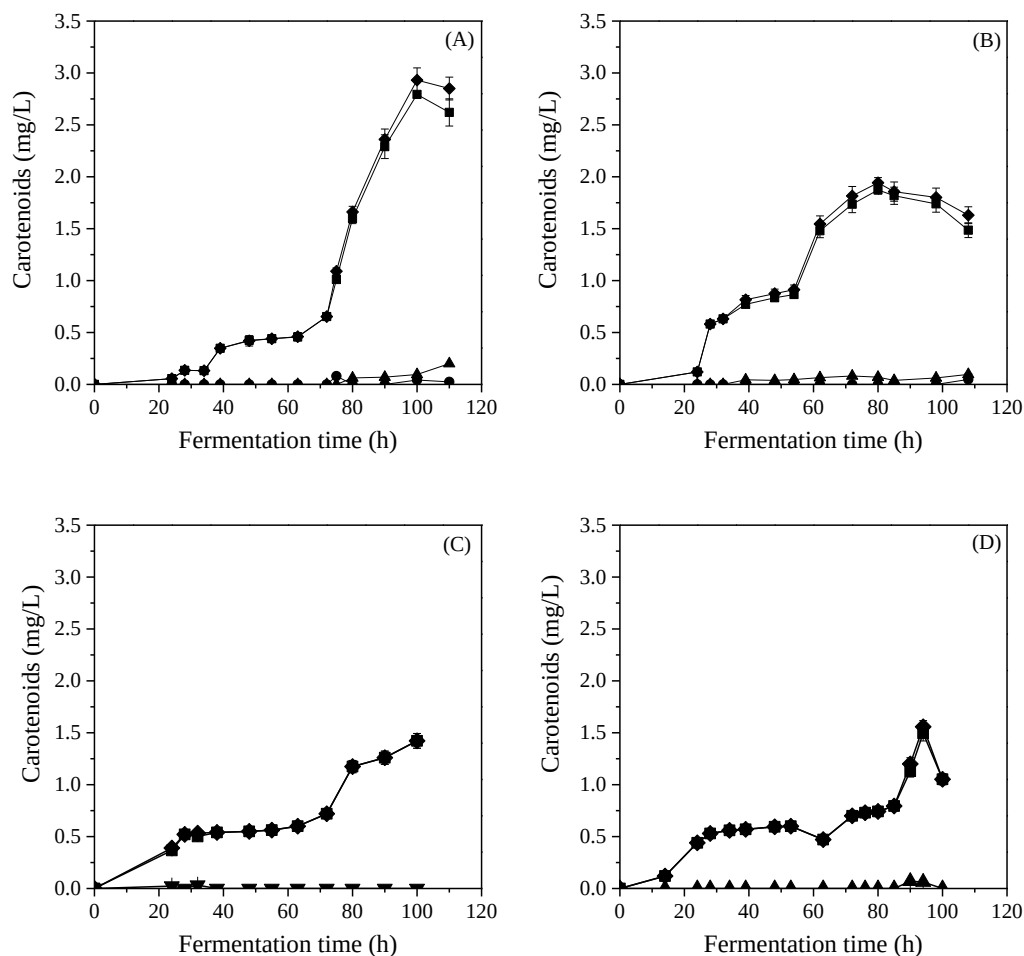


Figure 10.4 Chemical profile of the individual carotenoids produced during fermentation of strain *Rhodospiridium paludigenum* 2664 on (A) glucose, (B) fructose, (C) sucrose, (D) glucose: galactose. Total carotenoids (◆), β -carotene (■), γ -carotene (●), torulene (▲), torularhodin (▼).

10.2.2 Fatty acid profile

The composition of fatty acids is essential for the expanding potential applications of the produced microbial oil. For instance, the unsaturated fatty acids are favorable in food industry because of their ability to reinforce human's health (Coniglio et al., 2023). Thus, the profile of the produced fatty acids was next identified through GC analysis. The results of both strains indicated that the produced microbial oil was rich in unsaturated fatty acids (**Figure 10.5**). The microbial oil synthesized by *Rhodospiridium paludigenum* 2663 presented a similar fatty acid profile regardless the fermentation substrate. The dominant fatty acid was oleic acid (C18:1) with a ratio

from 48.3 to 62.6%, depending on the utilized carbon source. Palmitic acid (C16:0) also shared a high ratio ranging from 21.6 to 28.9%. Stearic acid (C18:0) and linoleic acid (C18:2) were detected in lower amounts (**Figure 10.5A**). The unsaturated fatty acids comprised the 70% of total fatty acid, while the mono-unsaturated ones (MUFAs) represented about the 90% of total unsaturated fatty acids. The microbial oil produced by the strain *Rhodospiridium paludigenum* 2664 showed a similar profile (**Figure 10.5B**). The main fatty acid was oleic acid (C18:1) and the unsaturated fatty acids were also prevalent. However, the ratio between MUFAs and poly-unsaturated (PUFAs) was different. The PUFAs consisted of around 50% of total unsaturated fatty acids, due to the increased amount of linoleic acid (C18:2) and linolenic acid (C18:3). The results of this study are in accordance with the lipoid profile reported so far regarding red yeasts. Maza et al., (2020) presented that the strain *Rhodotorula glutinis* R4 produced mainly oleic acid (~60%). Palmitic, linoleic and linolenic acid were also present in lower amounts. The strain *Rhodotorula glutinis* var. *rubescens* LOCKR13 showed that regardless the culture conditions oleic acid was the prevalent fatty acid (above 70%) (Gientka et al., 2022).

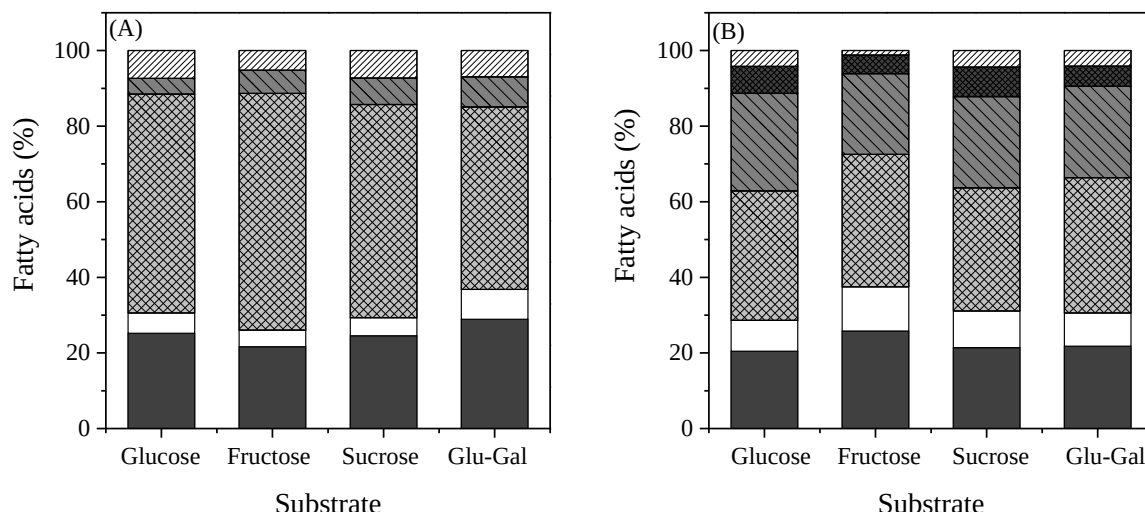


Figure 10.5 Fatty acid composition (%) of the lipids produced by (A) *Rhodospiridium paludigenum* 2663 and (B) *Rhodospiridium paludigenum* 2664. C16:0 (dark grey bar), C18:0 (white bar), C18:1 (light grey patterned bar), C18:2 (grey patterned bar), C18:3 (dark grey patterned bar), others (white patterned bar).

10.2.3 Antioxidant activity of the carotenoid-rich extracts

Natural carotenoids are known for their ability to act as strong antioxidants, scavenging oxygen and peroxy radicals (Cipolatti et al., 2019). It is also established that their antioxidant activity is strongly depended on the amount of their conjugated double bonds; torularhodin with 13 bonds is considered as the carotenoid with the highest antioxidant activity (Kot et al., 2018). Moreover, previous studies indicated that the presence of a mixture of carotenoids might act cooperatively and exhibit higher antioxidant ability than each carotenoid separately (Mandelli et al., 2012; Stahl and Sies, 2003). Considering the above, the carotenoid extract, derived from the cultivation of *R. paludigenum* 2663 on sucrose, which consisted of β -carotene, γ -carotene, torulene and torularhodin was evaluated for its antioxidant activity. A concentration of 0.43 mg/mL presented 70.2 % inhibition on ABTS• radical, with an IC_{50} equal to 0.14 mg/mL. In comparison with carrot extract composed almost solely of β -carotene (IC_{50} of 0.2 mg/mL), the microbial derived extract showed enhanced antioxidant activity (by 42.8%). A standard solution of pure β -carotene was also analyzed for its scavenging activity presenting an IC_{50} of 0.76×10^{-5} mg/mL. Hence, our results were evaluated as promising, as carotenoids extracts might find applications on different fields, such as the cosmetic, pharmaceutical and food industry.

10.2.4 Evaluation of antimicrobial properties

Natural carotenoids could have antimicrobial activity against a plethora of food-borne pathogens (Vargas-Sinisterra and Ramírez-Castrillón, 2021). Pathogenic bacterial strains such as *Listeria monocytogenes* and *Salmonella enteritidis* are considered as a common source of food contamination (Rostami et al., 2016). To this end, the carotenoid extract from *Rhodospiridium paludigenum* 2663 on sucrose was examined for its antimicrobial properties on both bacterial and fungal pathogenic strains. Preliminary experiments were initially set up in order to determine the optimal solvent to dissolve the dried carotenoids extract before testing its antimicrobial activity. Previous studies had indicated multiple solvents suitable for this purpose, like DMSO, acetone or methanol (Chen et al., 2022; Karpiński and Adamczak, 2019; Rostami et al., 2016). However, these solvents demonstrated an inhibiting effect on bacterial/fungal growth, without the addition of the carotenoid extract (data not

shown). On the contrary, 1% antifoam had a negligible impact on microbial growth and was thus selected as a proper solvent for the tests.

The results showed that the carotenoid extract was efficient against all the examined microorganisms, yet to different degrees. The growth of the pathogenic strains was completely inhibited when the carotenoid concentration ranged from 36 to 180 mg/L. The lowest MIC (minimum inhibitory concentration) was 36 mg/L against the strain *Salmonella enteritidis*. The extract was most effective on this pathogenic strain inhibiting its growth by 25 % with just 9 mg/L of carotenoids. The strain *Staphylococcus epidermitis* was also highly affected presenting a 50% inhibition with 13.5 mg/L of carotenoid extract. Despite the fact that the reduction of the strain *Pseudomonas aeruginosa* began with small amounts of carotenoids, the MIC was obtained at a concentration of 90 mg/L. The carotenoid extract was less efficient on *Staphylococcus aureus* strain presenting no inhibitory effect at concentrations up to 72 mg/L, whereas the MIC was achieved at 180 mg/L (**Table 10.2**). Karpiński and Adamczak, (2019) showed that 125 mg/L of synthetic fucoxanthin could be able to inhibit the growth of *Staphylococcus aureus*, *Staphylococcus epidermitis*, *Pseudomonas aeruginosa* and *Klebsiella oxytoca*. However, when the carotenoid extract was biotechnologically produced from microorganisms, the required concentrations in order to act as MIC were much higher in order to achieve the same inhibiting effect. According to Rostami et al., (2016), 16 g/L were the necessary amount of pigment extract from *Maoricolpus roseus* PTCC 1411 in order to completely eliminate the growth of *Salmonella enteritidis*. Moreover, 0.6 g/L of carotenoid extract derived from *Rhodotorula mucilaginosa* CCMA 0156 were the established MIC against *Listeria monocytogenes* (Moreira et al., 2018) (**Table 10.4**). Therefore, the results of the current study indicate that the produced carotenoid had high antibacterial activity against many pathogenic strains.

Table 10.2 Antibacterial activity of various carotenoid extract concentrations (1.8, 4.5, 6.0, 9.0, 13.5, 18.0, 36.0, 54.0, 72.0, 90.0, 180.0 and 360 mg/L) produced during *Rhodospiridium paludigenum* 2663 fermentation utilizing sucrose as carbon source.

Bacterial strains	Carotenoid concentration (mg/L)											
	1.8	4.5	6.0	9.0	13.5	18.0	36.0	54.0	72.0	90.0	180.0	360.0
<i>S. enteritidis</i>	1.1%	11.4%	13.2%	25.0%	26.5%	32.3%	100.0% ¹	100.0%	100.0%	100.0%	100.0%	100.0%
<i>S. epidermitis</i>	0.0%	0.0%	0.0%	28.0%	50.0%	58.0%	68.0%	100.0% ¹	100.0%	100.0%	100.0%	100.0%
<i>C. difficile</i>	0.0%	0.0%	0.0%	0.0%	11.1%	18.1%	39.2%	100.0% ¹	100.0%	100.0%	100.0%	100.0%
<i>K. oxytoca</i>	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	44.4%	100.0% ¹	100.0%	100.0%	100.0%	100.0%
<i>L. monocytogenes</i>	0.0%	0.0%	0.0%	20.6%	22.0%	33.7%	35.0%	95.4%	100.0% ¹	100.0%	100.0%	100.0%
<i>S. saprophyticus</i>	0.0%	0.0%	0.0%	0.0%	7.6%	22.4%	27.2%	36.5%	100.0% ¹	100.0%	100.0%	100.0%
<i>P. aeruginosa</i>	0.0%	15.5%	17.8%	27.7%	30.8%	31.4%	36.5%	41.9%	65.6%	100.0% ¹	100.0%	100.0%
<i>S. aureus</i>	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	97.3%	100.0% ¹	100.0%

¹MIC: minimum inhibitory concentration

Regarding the inhibitory effect of the extracts on fungal pathogenic strains, an amount of 54 mg/L inhibited their growth (**Table 10.3**). The strain *Aspergillus niger* seemed to be more sensitive to carotenoid extract, as its growth was inhibited by 51.4% in the presence of 36 mg/L of carotenoids. Chen et al., (2022) showed that a concentration of 3.25 g/L of pigment extract derived from wild berries acted as MIC against the fungus *Aspergillus niger*. Furthermore, for the complete inhibition of *Alternaria citri*, 64 g/L of pigments extracted from *Rhodotorula glutinis* PTCC 5257 were required (**Table 10.4**). Hence, compared to the literature, the carotenoid extract from *Rhodospiridium paludigenum* 2663 presented greater antifungal activity.

Table 10.3 Antifungal activity of various carotenoid extract concentrations (9.0, 18.0, 36.0, 54.0, 72.0, 90.0, 180.0 and 360 mg/L) produced during *Rhodospiridium paludigenum* 2663 fermentation utilizing sucrose as carbon source.

Fungal strains	Carotenoid concentration (mg/L)							
	9.0	18.0	36.0	54.0	72.0	90.0	180.0	360.0
<i>A. niger</i>	0.0%	3.1%	51.4%	100.0% ¹	100.0%	100.0%	100.0%	100.0% ²
<i>A. flavus</i>	0.0%	0.0%	0.0%	100.0% ¹	100.0%	100.0%	100.0%	100.0% ²

¹MIC: minimum inhibitory concentration; ²MFC: minimum fungicidal concentration

The next set of experiments was focused on the evaluation of the minimal bactericidal/fungicidal concentration (MBC/MFC). Considering that the MBC could be equal or above the corresponding MIC, the examined carotenoid concentrations were between 36 to 360 mg/L and 54 to 360 mg/L, for the bacterial and fungal strains, respectively. Testing the pathogenic bacterial strains, neither concentration exhibited bactericidal effect against them (**Table 10.2**). However, a concentration of 360 mg/L of the carotenoid extract was identified as the MFC for both *Aspergillus niger* and *Aspergillus flavus* (**Table 10.3**). According to the literature, the lowest MFC (128 g/L) was against *Alternaria citri* using pigment extract from *Rhodotorula glutinis* PTCC 5258 (**Table 10.4**) (Rostami et al., 2016). Therefore, our results corroborate on

the potential of the carotenoid extract derived from *Rhodospiridium paludigenum* as antimicrobial agent for future food applications.

Table 10.4 MIC and MBC/MFC of different pigments against pathogenic bacterial and fungal strains.

Pathogenic strains	Source	Extract	Solvent	MIC	MBC/MFC	Reference
<i>S. enteritidis</i> , <i>S. epidermitis</i> , <i>S. saprophyticus</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i> , <i>C. difficile</i> , <i>K. oxytoca</i>	<i>R. paludigenum</i> NCYC 2663	Carotenoids	1% Antifoam	36-90 mg/L	> 360 mg/L	This study
<i>A. niger</i> , <i>A. flavus</i>	<i>R. paludigenum</i> NCYC 2663	Carotenoids	1% Antifoam	54 mg/L	360 mg/L	
<i>S. aureus</i> , <i>S. enteritidis</i> , <i>L. monocytogenes</i>	<i>M. roseus</i> PTCC 1411	Pigments	Acetone	16-64 g/L	32-128 g/L	Rostami et al. (2016)
<i>A. citri</i> , <i>P. digitatum</i>	<i>M. roseus</i> PTCC 1411	Pigments	Acetone	128 g/L	<i>A. citri</i> > 256 g/L <i>P. digitatum</i> 256 g/L	
<i>S. aureus</i> , <i>S. enteritidis</i> , <i>L. monocytogenes</i>	<i>R. glutinis</i> PTCC 5257	Pigments	Acetone	16-64 g/L	32-64 g/L	
<i>A. citri</i> , <i>P. digitatum</i>	<i>R. glutinis</i> PTCC 5258	Pigments	Acetone	64-128 g/L	128-256 g/L	
<i>S. aureus</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i>	<i>R. mucilaginosa</i> CCMA 0156	Carotenoids	Methanol	0.6-1.22 g/L	0.6-1.22 g/L	Moreira et al. (2018)
<i>S. aureus</i> , <i>B. subtilis</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i> , <i>S. enteritidis</i>	wild berry (<i>Rubus coreanus</i>)	Berry	Methanol	0.39-6.25 g/L	0.78-12.5 g/L	Chen et al. (2022)
<i>A. niger</i>	wild berry (<i>Rubus coreanus</i>)	Berry	Methanol	3.25 g/L	6.5 g/L	
<i>S. aureus</i> , <i>S. epidermitis</i> , <i>P. aeruginosa</i> , <i>K. oxytoca</i>	-	Fucoxanthin	20% DMSO	125-500 mg/L	-	Karpiński and Adamczak, (2019)

10.3 Fermentations on bioreactor scale for carotenoid and microbial oil production by *Rhodospiridium paludigenum* 2663

10.3.1 Batch fermentation using commercial sucrose

Based on the previously shown results, the strain *Rhodospiridium paludigenum* 2663 was able to accumulate carotenoids, microbial oil as well as EPS when cultivated on sucrose. To this end, a bioreactor batch fermentation was carried out, coping the same conditions as in shaking flasks. As shown in **Figure 10.6A**, the carbon source assimilation rate was 0.95 g/L/h, with total available sugars being depleted after approximately 53 h of the fermentation process. Glucose was the first monosaccharide to be consumed, even though fructose assimilation was also carried out simultaneously (**Figure 10.6A**). Regarding the consumption pattern of nitrogen, IN was rapidly depleted (at about 19 h), whereas FAN consumption ceased after 24 h, leaving ~12 mg/L unconsumed (**Figure 10.6A**). These findings are consistent with our previous results obtained from shaking flask fermentations. Cell growth along with oil accumulation, carotenoids and EPS production are presented in **Figure 10.6B**. Microbial oil production was initiated at about 10 h of fermentation, reaching a maximum concentration of 6.14 g/L (36.1% w/w oil content) after 48 h. Regarding the fatty acid profile, oleic acid was the main fatty acid detected (43.4%) followed by palmitic acid (33.7%).

Carotenogenesis followed a different pattern compared to microbial oil production. Although carotenoid accumulation began within the first 20 h of fermentation, the highest concentration, equal to 1.55 mg/L, was achieved after 64 h. It is worth noting that the maximum concentration of carotenoids was observed at the end of the fermentation process (**Figures 10.6A, B**). Warjoto et al., (2020) reported that *Rhodospiridium paludigenum* InaCC Y236 achieved the maximum production of carotenoids (6.73 µg/mL) after 144 h of fermentation, while the carbon source was nearly consumed (0.83 g/L). The accumulation of carotenoids after sugar consumption could be attributed to the subsequent decrease in oil concentration (**Figure 10.6B**). A possible explanation could be that the yeast cells might assimilate the microbial oil towards carotenoids production to regulate possible oxidation stress developed in the cell following sugars depletion. Similar observation was also carried

out by Tkáčová et al., (2018) with the strain *Rhodotorula glutinis* CCY 20-2-26. The authors concluded that fatty acid degradation occurs after sugars depletion, leading to acetyl-CoA formation via β -oxidation, which is subsequently being channeled for β -carotene synthesis. The torularhodin increase at the end of the fermentation (**Figure 10.6C**) further supports this explanation, given that this carotenoid is the strongest free radical scavenger among those synthesized (Kot et al., 2019). However, the regulatory mechanisms underlying the cellular metabolism of these products remain inadequately understood, requiring further studies.

Noteworthy, EPS were also produced under these cultivation conditions. As shown in **Figure 10.6B**, their production began after 28 h, reaching a maximum concentration of 0.49 g/L at the end of the fermentation (64 h). The metabolic pathway for producing exopolysaccharides, such as pullulan, relies on the activation of cAMP-PKA signaling pathway, which regulates the fate of Msn2. Depending on whether Msn2 is located in the nucleus or the cytoplasm, the production of either EPS or microbial oil is enhanced (Yang et al., 2020). Thus, their synthesis acts antagonistically, which might explain the subsequent increase in EPS at the end of the process. EPS production from different red yeasts strains have been previously reported (Hamidi et al., 2020; Liu et al., 2021; Seveiri et al., 2020; Zhao and Li, 2022). The ability of EPS of *Rhodospiridium paludigenum* to produce exopolysaccharides has been demonstrated by Liu et al., (2021), who achieve a concentration of 4.32 g/L under optimized fermentation conditions. The authors argued that a slightly acidic pH (equal to 4) and a temperature of 30.18 °C were required for high EPS accumulation. On the other hand, Zhao and Li, (2022) observed enhanced EPS synthesis from *Rhodotorula glutinis* when a lower temperature (15 °C) was employed. It can be deduced that as in case of the other two metabolites, EPS synthesis is strain depended and more studies are required to fully elucidate the metabolic mechanisms involved. Nevertheless, Hamidi et al., (2020) reported that sucrose was the optimum carbon source for EPS production by a cold-adapted *Rhodotorula mucilaginosa* strain, which is line with our findings since EPS production was only detected in the medium containing sucrose.

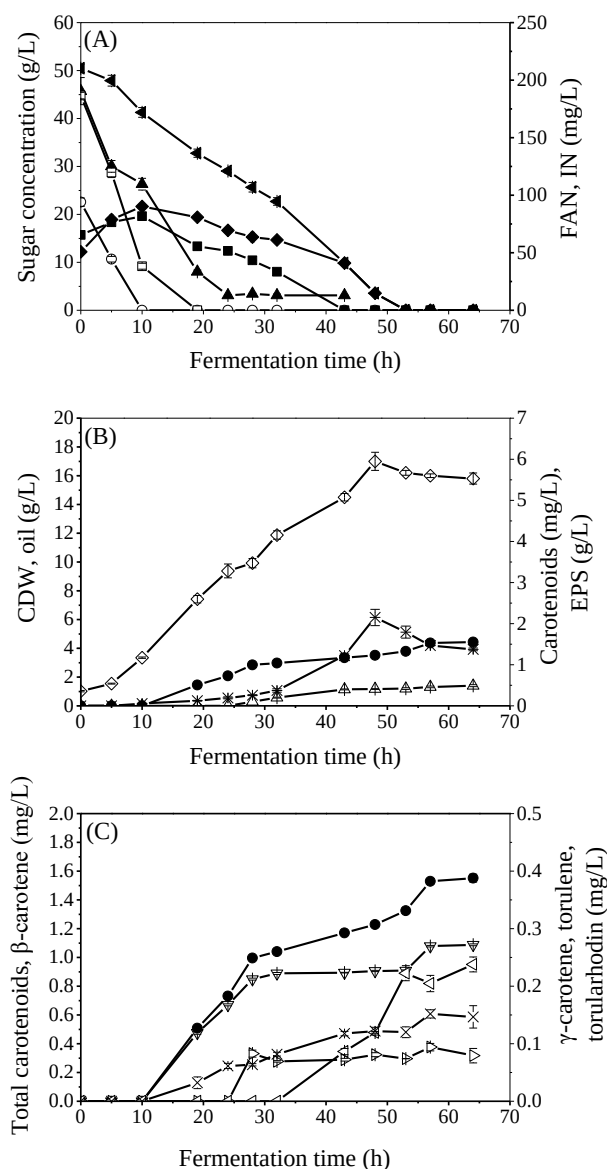


Figure 10.6 (A) Consumption of glucose (■), fructose (◆), sucrose (○), total sugars (◄), free amino nitrogen (FAN) (▲) and inorganic nitrogen (IN) (□), as well as (B) cell dry weight (◇), microbial oil (*), carotenoids (●) and EPS (△) production, and (C) individual carotenoids production where β-carotene (▽), γ-carotene (▷), torulene (◄), torularhodin (×) during batch bioreactor fermentation of *R. paludigenum* 2663.

The chemical profile of the produced carotenoids was also monitored. As depicted in **Figure 10.6C**, β-carotene was the dominant carotenoid synthesized throughout the fermentation process, comprising between 70.1 % and 93.7 % of the total carotenoids, while torulene, torularhodin and γ-carotene were also co-produced in lower

concentrations. It is noteworthy that the rapid increase of both torulene and torularhodin occurred after glucose depletion (**Figure 10.6A, C**). This observation aligns with the fact that these “unusual” carotenoids are produced under conditions of high oxidative stress (Kot et al., 2019).

10.3.2 Batch fermentation using molasses

Given the promising results obtained using sucrose, molasses were subsequently employed as an alternative carbon source. Molasses comprise a co-product of the sugar industry, finding a plethora of applications especially as fermentation substrate due to their low price and availability (Lakshmidevi et al., 2021; Ozdal and Kurbanoglu, 2019). Besides their high sucrose content, molasses are also rich in other important growth nutrients such as minerals and vitamins (Nakata et al., 2014). The kinetics of sugar consumption and metabolites accumulation are presented in **Figure 10.7**. Sugar consumption presented a similar rate (~ 1 g/L/h) to the one observed when commercial sucrose was employed. However, sugar depletion occurred a bit later in molasses (57 h) compared to sucrose (53 h). Regarding metabolites accumulation, microbial oil production started after about 23 h, when the concentration of both nitrogen sources became limiting (**Figure 10.7A, B**). The highest microbial oil concentration was 4.4 g/L, corresponding to 29.7 % content, a value $\sim 30\%$ lower than the one obtained on sucrose (6.14 g/L). When comparing to other red yeast strains, *Rhodosporidium paludigenum* 2663 performed in a rather similar manner regarding oil accumulation in molasses. Lakshmidevi et al., (2021) recorded a 4.65 g/L oil production in *Rhodotorula glutinis* and 7.55 g/L in *Rhodosporidium toruloides*, after batch cultivation in sugarcane molasses. In the work of Zheng et al., (2021) a lipid concentration of 4.35 g/L was achieved with *Rhodotorula toruloides* grown in cane molasses, while (da Costa et al., 2020) demonstrated a lipid content of only 16.5% using the strain *R. mucilaginosa* CCT3892.

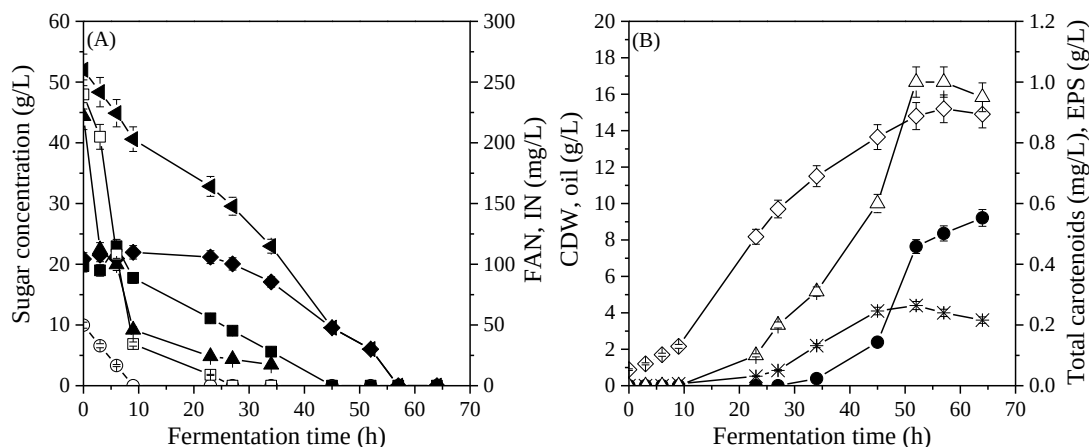


Figure 10.7 (A) Consumption of glucose (■), fructose (◆), sucrose (○), total sugars (◄), free amino nitrogen (FAN) (▲) and inorganic nitrogen (IN) (□), as well as (B) cell dry weight (◇), microbial oil (*), carotenoids (●) and EPS (△) production.

Carotenoid synthesis followed a completely different pattern compared to sucrose, as no carotenoids were detected in the extracts even after ~30 h of fermentation (**Figure 10.7B**). It is worth noting that the only carotenoid synthesized until sugar depletion was torularhodin. After this point, carotenoids were boosted reaching the highest concentration (0.55 mg/L), almost 7 hours after sugar depletion. Notably, β -carotene became the prevalent carotenoid, accounting for 87.6% of total carotenoids, while torulene and γ -carotene were not detected in extracts. Rodrigues et al., (2022) achieved the production of 1.79 mg/L carotenoids and a 43.2% lipid accumulation, when the *Rodotorula mucilaginosa* CCT 7688 was grown on sugarcane molasses supplemented with corn steep liquor, after prolonged fermentation hours (144 h). Lower carotenoid concentrations were also obtained by Lakshmidevi et al., (2021), when the strains *Rhodotorula glutinis* and *Rhodospiridium toruloides* were grown on molasses, in comparison to a glucose-based medium. This phenomenon could be attributed to the rich composition of molasses in specific minerals such as zinc and manganese (Olbrich, 2006). These metal ions could possibly negatively influence the enzymes involved in carotenogenesis, especially the ones involved in torulene and torularhodin synthesis (Mata-Gómez et al., 2014).

EPS were the only metabolite that was improved when molasses were the fermentation substrate (**Figure 10.7B**). Their production initiated after 24 h of

fermentation and continued to increase reaching 1 g/L at 52 h, a 2-fold improvement comparing to the experiment with sucrose (0.49 g/L). Molasses have been evaluated as substrate for various types of exopolysaccharides such as pullulans (Lazaridou et al., 2002) and xathan (Ozdal and Kurbanoglu, 2019). The studies on EPS production from red yeasts are scarce and are mainly focusing on stress factors such as temperature and pH (Zhao and Li, 2022). Our findings indicate that substrate plays also a crucial role and molasses seem to induce EPS production in detriment to oil and carotenoids accumulation. The different behavior of the strain on molasses led to the investigation of a fed-batch experiment, aiming to improve the final contents in both oils and carotenoids.

10.3.3 Fed-batch fermentations using molasses

The time course of substrate consumption (sugars from molasses, FAN and IN) and metabolites production in a fed-batch cultivation of strain *Rhodospiridium paludigenum* 2663 is presented in **Figure 10.8**. The bioreactor was fed with a concentrated solution of molasses at four different time intervals (**Figure 10.8A**). The fed-batch strategy significantly improved microbial oil accumulation, reaching a concentration of 10.9 g/L (0.12 g/g on consumed sugars), with a content equal to 50.7% after 86 h of fermentation. The production of microbial oil obtained in this experiment is in line with other studies, in which proper medium manipulation was employed, either with the addition of specific salts or other compounds acting as stimulants. For instance, more than 49% lipid accumulation was achieved when a mixture of molasses and cassava water was enriched with KH_2PO_4 and K_2SO_4 , in the study of Machado et al., (2022), which investigated the co-production of lipids and carotenoids with a *Rhodotorula mucilaginosa* strain. Potassium phosphate and magnesium sulfate salts were added in the medium of *Rhodotorula glutinis* CCT-2186 in order to obtain a 61.6% lipid content in the work of Mussagy et al., (2021).

Carotenoid synthesis started after 30 h of cultivation, similarly to the batch experiment, and remained at low levels (0.05 mg/L) -with torularhodin being the sole molecule- until sugar concentration became limiting (3.2 g/L). As observed also in the case of batch cultivation, low sugar concentration triggered carotenoids accumulation

(**Figure 10.8A, B**). Carotenoid synthesis continued to increase reaching a maximum at 3.3 mg/L, after complete sugar consumption (**Figure 10.8B**). This behavior was also observed when commercial sucrose was the carbon source (**Figure 10.6 A, B**). It has been previously debated that excess of glucose in the fermentation medium is continuously channeled for lipid synthesis in detriment to carotenogenesis (Tkáčová et al., 2017). When glucose is exhausted, oleic acid (with is the main fatty acid in the produced microbial oil) is metabolized via the β -oxidation pathway to form acetyl-CoA, which is subsequently used for carotenoid synthesis.

Contrary to the batch cultivation, torulene and γ -carotene were also detected to the extracts (**Figure 10.8C**). β -carotene was the main carotenoid, accounting for the 55.2% of total carotenoids in the extract, followed by torulene (25.7%), torularhodin (13.4%) and γ -carotene (5.7%). EPS production started at about 30 h and gradually increased, following a trend more similar to lipids than to carotenoids. The peak occurred at the end of the fermentation, with a value of 3 g/L, a 3-fold increment compared to the batch experiment (**Figure 10.8B**).

The concentration of carotenoids achieved on molasses (beet or sugarcane molasses) varies depending on the strain and other fermentation parameters such as temperature, pH and the addition of stimulants. The highest concentration of carotenoids achieved when the strain *Rhodotorula mucilaginosa* was grown on a mixture of molasses and cassava wastewater was equal to 3.3 mg/L (Machado et al., 2022).

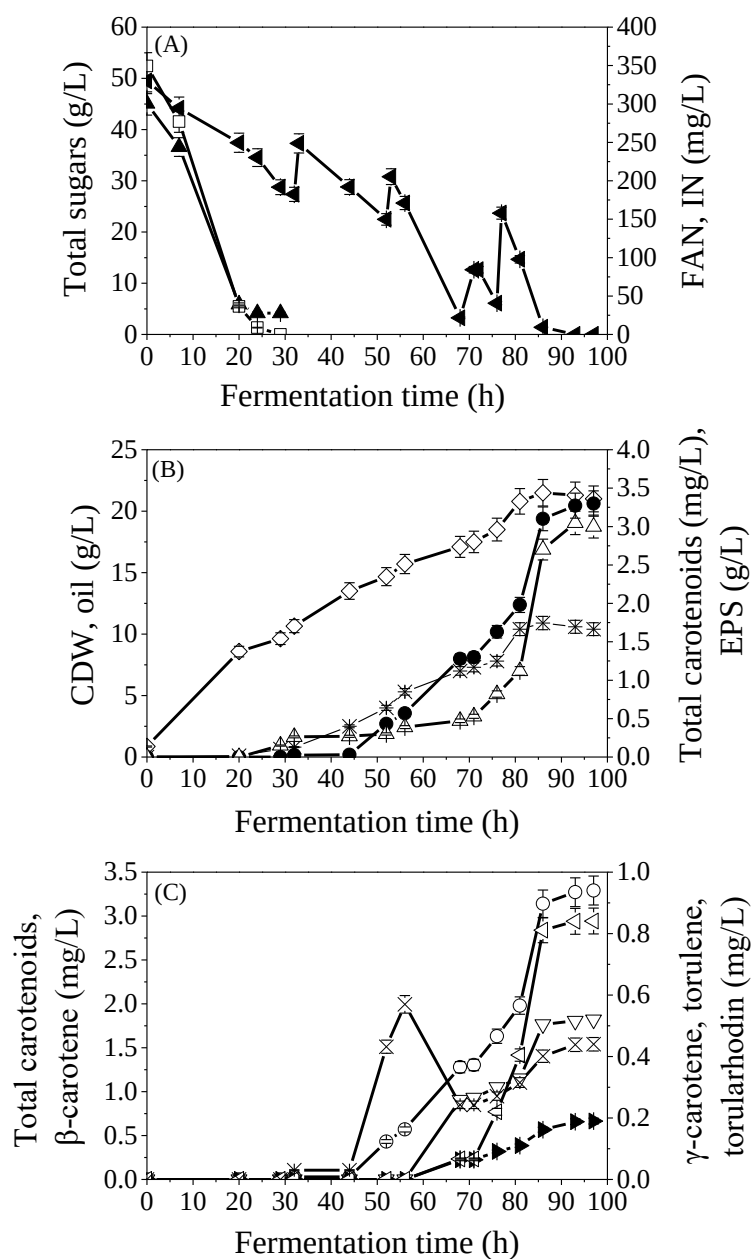


Figure 10.8 (A) Consumption of glucose (■), fructose (◆), sucrose (○), total sugars (◄), free amino nitrogen (FAN) (▲) and inorganic nitrogen (IN) (□), as well as (B) cell dry weight (◇), microbial oil (*), carotenoids (●) and EPS (△) production, and (C) individual carotenoids production where β-carotene (▽), γ-carotene (▷), torulene (◁), torularhodin (×) during batch bioreactor fermentation of *Rhodosporidium paludigenum* 2663.

The fed-batch cultivation led to higher concentrations of all metabolites produced from the strain *Rhodosporidium paludigenum* 2663, designating the potential of this strain as a multi-product cell factory. To our knowledge, this is the first study

indicating the parallel synthesis of three value-added metabolites with possible food applications.

10.4 Conclusions

The future commercialization of biotechnologically produced carotenoids depends on the identification of robust strains able to consume a variety of carbon sources and to synthesize carotenoids of market value. This is the first study to explore the fermentative behavior of two *Rhodosporidium paludigenum* yeasts (2663 and 2664) when cultivated on glucose, fructose, sucrose and a mixture of glucose-galactose. Both strains were efficient carotenoid producers, while the final product titers were strain and substrate dependent. Strain *Rhodosporidium paludigenum* 2663 was able to co-produce oil, carotenoids and EPS when grown on sucrose, leading to its evaluation in bioreactor scale both on commercial sucrose and molasses. Higher titers of all metabolites were achieved when a fed-batch strategy was followed using molasses as substrate.

The analysis of the chemical profile revealed that β -carotene was the main carotenoid in all cases while the presence of the strong antioxidants torulene and torularhodin was also unveiled. On the other hand, the lipoic fraction was rich in unsaturated fatty acids, with oleic acid found as the predominant one. Besides the complete chemical characterization of the microbial metabolites, this study also elucidated the potential bioactive properties of the carotenoids' extracts both in terms of antioxidant and antimicrobial activities, adding value towards their subsequent industrial valorization. Therefore, this study unraveled the dynamic potential of the unexplored yeast *R. paludigenum* 2663 as a robust producer of value-added ingredients targeting their incorporation in novel food formulations.

Part of the results presented in Chapter 10 have been published in Journal of Biotechnology:

Sereti, F., Alexandri, M., Papadaki, A., Papapostolou, H., Kopsahelis, N. (2024). Carotenoids production by *Rhodospiridium paludigenum* yeasts: Characterization of chemical composition, antioxidant and antimicrobial properties. *Journal of Biotechnology*, 386, 52-63

CHAPTER 11

Green recovery of carotenoids and microbial oil
using SFE-CO₂

11 Green recovery of carotenoids and microbial oil using SFE-CO₂

11.1 Introduction

The objective of this chapter was the evaluation of SFE-CO₂ as an alternative and “green” method for the concomitant recovery of oil and carotenoids produced from the yeast strain *Rhodospiridium paludigenum* NCYC 2663. Due to the lack of studies dealing with yeast biomass pretreatment methods, various cell disruption techniques (ultrasounds, beads, high speed homogenization, decompression) were also evaluated prior to SFE-CO₂ to assess the overall feasibility of the process. Moreover, a comprehensive analysis of the kinetic behavior of both carotenoids and microbial oil using SFE-CO₂ was carried out, promoting sustainable production of these valuable metabolites and suggesting a pathway for their potential use in food products.

11.2 Oils and carotenoids recovery using SFE-CO₂

11.2.1 Effect of different pretreatment methods

Cell disruption is a crucial step in recovering intracellular products, particularly carotenoids, which are highly sensitive to light, temperature, and specific solvents (Papapostolou et al., 2023). The rigid and complex cell wall of yeast cells poses a significant challenge for cell disruption (Mussagy et al., 2019). DMSO was employed, serving as the control method for the quantification analysis of carotenoids, while the modified Folch method was used as the control method for oil extraction. It should be stated that a portion of carotenoids is co-extracted along with oils during the modified Folch method, corresponding to a recovery yield of 13%. Freeze-drying was employed in all proposed schemes due to the unfavorable presence of water, given the hydrophobic nature of carotenoids (Papapostolou et al., 2023). However, it should be noted that γ -carotene was not detected after freeze-drying, indicating that even though it is a commonly employed process for thermolabile compounds, some losses could occur. Despite the significant importance of biomass pretreatment prior to SFE-CO₂, few studies have performed comprehensive evaluations and most of them are focusing on microalgae (Sarnaik et al., 2018; Viguera et al., 2016). An overview of the various examined processes is presented in **Figure 11.1**.

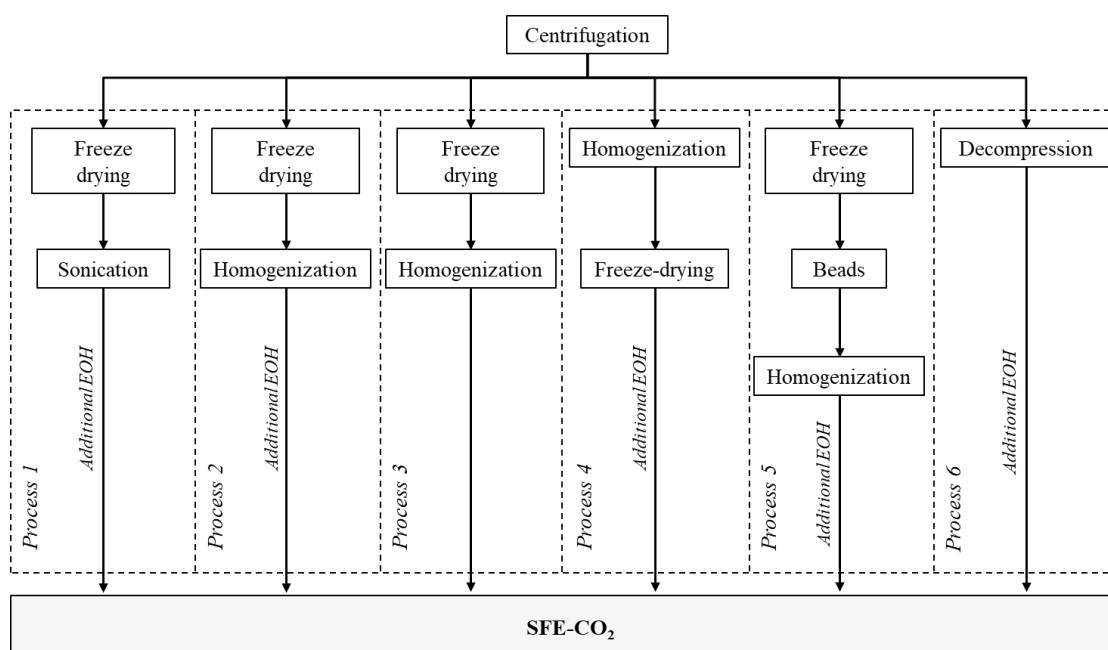


Figure 11.1 Schematic diagram of the examined pretreatment processes prior to SFE-CO₂ extraction.

As shown in **Figure 11.3A**, the different pretreatment processes significantly affect carotenoids more than oil recovery. Sonication (*Process 1*), which is commonly used for rupturing bacterial (Fidan and Zhan, 2019) and microalga biomass (Sarnaik et al., 2018; Viguera et al., 2016), proved highly unfavorable for recovering carotenoids from these red yeasts, with yields below 10%. In fact, post-ultrasound pretreatment analysis revealed carotenoid degradation during sonication and parameter stabilization at the SFE-CO₂ stage, with 51% of total carotenoids lost during sonication. In particular, β -carotene, the primary carotenoid, underwent 60.5% degradation, followed by torulene (40%) and torularhodin (8.6%). Total carotenoid content continued to decrease even after 2 h, indicating the negative effect of sonication on the overall process (**Figure 11.2**). Consequently, only 10% of carotenoids were recovered by the end of the extraction process (**Figure 11.3A**).

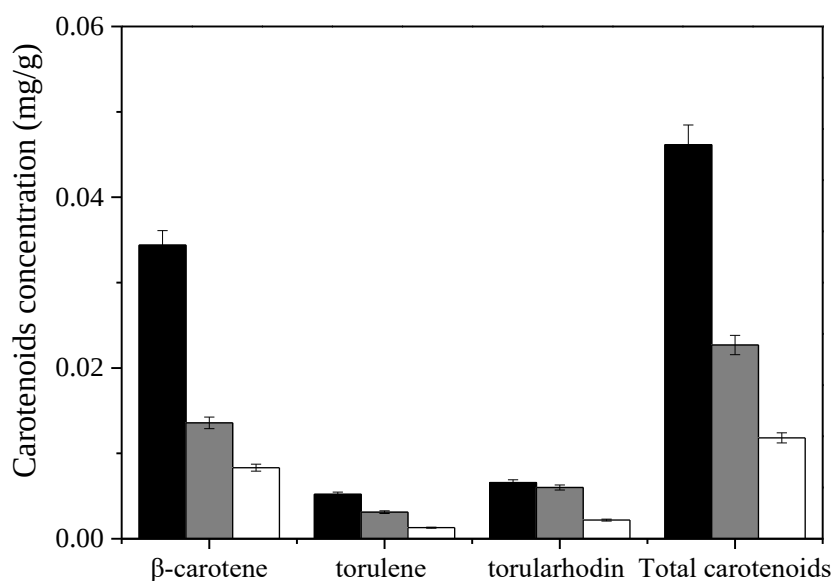
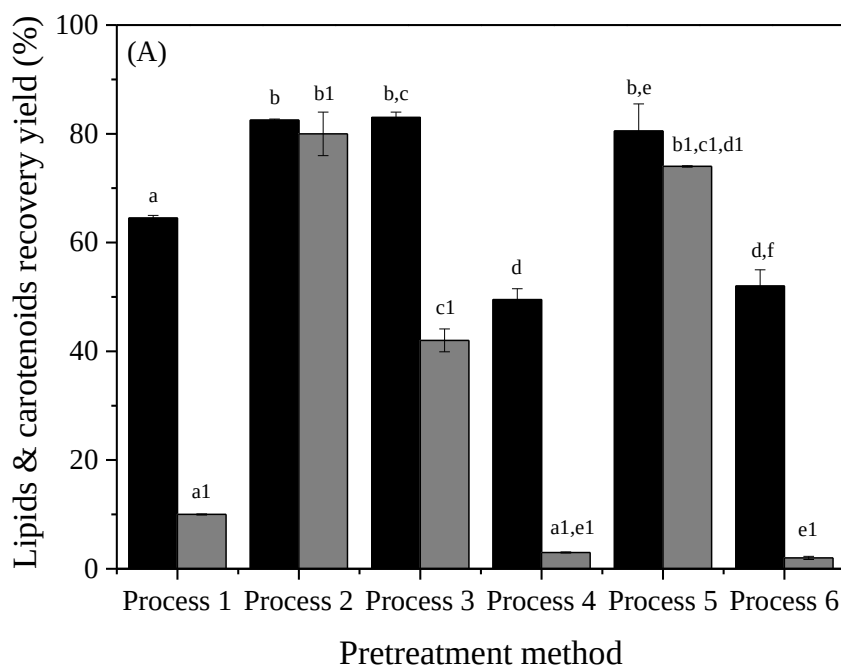


Figure 11.2 Individual and total carotenoid losses during treatment with ultrasounds. Initial concentration (black bar), concentration after sonication (grey bar), concentration 2 h after sonication (white bar).

HSP (*Process 2*) proved the most effective method for cell lysis yielding 83% and 80% of total microbial oil and carotenoids, respectively (**Figure 11.3A**). The combination with beads (*Process 5*) yielded statistically similar ($p > 0.05$) results (82.2% oil recovery and 74% of total carotenoids), indicating that extended cell lysis was induced by the HSP method. HSP is a commonly used technique in the food technology, employed for the formation of emulsion and suspensions (Hua et al., 2017). During this process, the material is subjected to high mechanical forces as well as hydrodynamic effects produced by the high rotation speed (10,000-20,000 rpm) (Hua et al., 2017; Zhou et al., 2022). Even though this method has never been evaluated for cell lysis, these results corroborate of its effectiveness.

The use of ethanol as a co-solvent in both pretreatment and throughout the SFE-CO₂ process was proven to be critical for the efficient recovery of carotenoids. More specifically, in *Process 3*, where ethanol was used only during pretreatment, carotenoid recovery was 42%, denoting a 44% decrease compared to *Process 2* (additional ethanol during SFE-CO₂). Microbial oil extraction was less affected by the

presence of ethanol in the extraction system, yielding similar recoveries (83%) to other scenarios involving homogenization with or without ethanol during SFE-CO₂ (*Processes 2 and 4*). Ethanol also played a crucial role during cell disruption, as shown in *Process 4*, where yields were significantly lower ($p < 0.05$) for both products. Torularhodin was not detected in the extract (**Figure 11.3B**), aligning with Ambrico et al., (2024), who emphasized the need for co-solvents during SFE-CO₂ due to torularhodin polar nature and tendency to form hydrogen bonds (Ambrico et al., 2024; Mussagy et al., 2023). Ludwig et al., (2021) also reported enhanced extraction yields of β -carotene from *Dunaliella salina* when ethanol was added in the system, especially at a concentration of 10%. The additional pretreatment step with glass beads (*Process 5*) was found to be unnecessary, as it showed no improvement in yields compared to homogenization alone (*Process 2*) (**Figure 11.3A**). Finally, the exposure to static compressed CO₂ with a subsequent abrupt decompression has been proposed as a technique to increase cell wall permeability (Vladić et al., 2022). However, in this study, decompression conditions were insufficient to disrupt yeast cell wall resulting in only 2% total carotenoid recovery (**Figure 11.3A**).



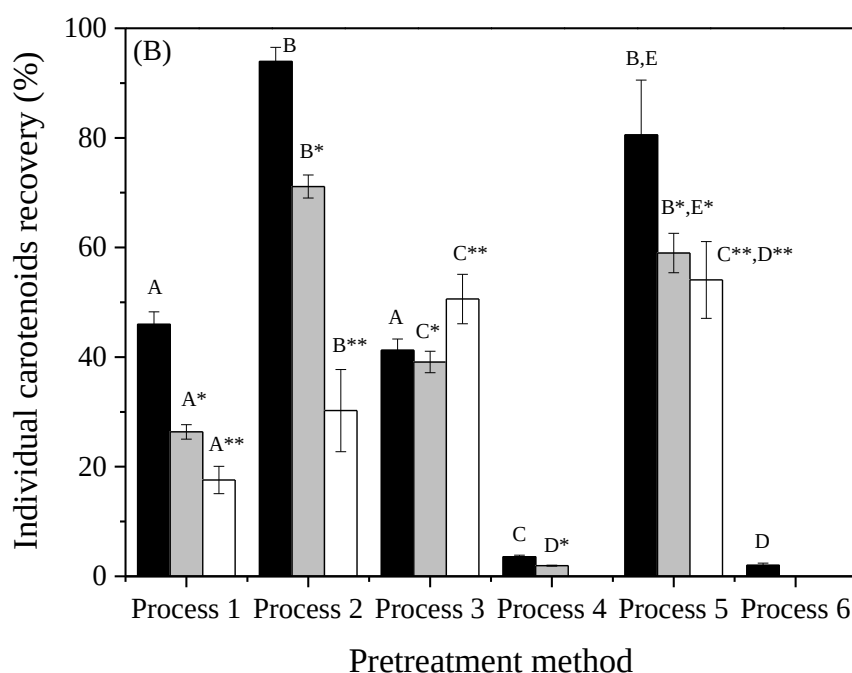


Figure 11.3 (A) Microbial oil (black bar) and total carotenoids (grey bar) recovery yields and (B) Individual carotenoids recovery yields obtained from various pretreatment processes coupled with SFE-CO₂. β-carotene (black bar), torulene (light grey bar), torularhodin (white bar). Different low case letters indicate significant differences between the different pretreatment processes regarding oils recovery (a, b, c, d, e, f), total carotenoids recovery (a1, b1, c1, d1, e1), while capital letters denote significant differences regarding the recovery yields of β-carotene (A, B, C, D, E), torulene (A*, B*, C*, D*, E*) and torularhodin (A**, B**, C**, D**).

Figure 11.3B depicts the recovery yields of individual carotenoids. β-carotene was the predominant carotenoid in the biomass (74% of total carotenoids), followed by torularhodin (14%) and torulene (11.3%). As a result, β-carotene was detected in all extracts regardless of the pretreatment method used. Significant differences ($p < 0.05$) in β-carotene recovery yields were observed when sonication was employed (*Process 1*) compared to all the other *Processes*, while no significant differences ($p > 0.05$) were obtained between the two most efficient *Processes 2* and *5* (HSH and beads/HSH combination, along with the use of ethanol throughout the extraction) with values of 93.9% and 80.6%, respectively. Torulene yields followed a similar pattern, with a percentage of 71% recovery in case of *Process 2*, while neither torulene nor

torularhodin were detected in the extracts after *Process 6* (decompression). Torularhodin recovery yields ranged between 30-54% for the most efficient processes (*Processes 2* and *5*). Analysis of residual biomass from different cases revealed an average remaining torularhodin content of 24%, likely due to its anti-oxidative nature, which makes it more prone to degradation during the process. Fatty acid composition (**Table 11.1**) was not affected by the pretreatment method, even after employing sonication (*Process 1*), which was detrimental on carotenoids. All extracts were rich in unsaturated fatty acids, which comprised more than 50% of total fatty acids, with oleic acid (C18:1) being the predominant one.

Table 11.1 Fatty acid composition (%) of microbial oil recovered by the modified Folch method, *Process 1* (pretreatment using sonication and extraction with SFE-CO₂) and *Process 2* (pretreatment with homogenization and extraction with SFE-CO₂)

Fatty acids (%)	Folch	<i>Process 1</i>	<i>Process 2</i>
C16:0	33.68	22.85	22.85
C18:0	13.01	4.48	5.20
C18:1 cis9	43.37	61.07	54.67
C18:2 cis6	3.42	5.58	4.27
Others	6.52	6.01	13.01
MUFA	45.45	64.81	58.94
PUFA	4.91	7.13	9.09
SFA	49.65	28.07	29.75

Studies on carotenoid extraction from yeasts using SFE-CO₂ are scarce, and to our knowledge, co-extraction of oils and carotenoids has not been evaluated previously. Only few reports evaluating the simultaneous extractions of oils and pigments from microalgae could be found. Mouahid et al., (2020) explored the potential of using SFE-CO₂ with and without the use of co-solvent for the recovery of valuable compounds, such as neutral oils, chlorophylls and carotenoids, from two species of microalgae. In case of the work of Molino et al., (2019), the maximum recovery of β -carotene (25.48%) occurred under different SFE-CO₂ operating conditions, compared to those used for the extraction of fatty acids from *Dunaliella salina*. Martínez et al.,

(2020) examined electroporation followed by freeze-drying before SFE-CO₂ process for carotenoid extraction from *Rhodotorula glutinis*, achieving less than 25% recovery even with ethanol addition as co-solvent. Hasan et al., (2015) reported less than 50% total carotenoid recovery from enzyme-treated *Phaffia rhodozyma* cells. The recent study of Larocca et al., (2023) reported a higher yield (73.86%) under the same operating conditions to this work, however torularhodin was the main carotenoid, and oil accumulation was not monitored in their study. Although the importance of efficient cell disruption for recovering intracellular products is undeniable, few studies have examined its impact on extraction. The comprehensive work of Viguera et al., (2016) indicated that the efficiency of oil extraction with SFE-CO₂ from *Chlorella protothecoides* was correlated with the degree of cell lysis. The authors evaluated cell rupture using microwaves, sonication, autoclaving and milling. Microwave and sonication yielded only 7%, probably because of the use of wet biomass during extraction. Milling was also insufficient, since oil recovery was only 13%. The higher yields in this study may be attributed to the concurrent extraction of carotenoids and oils, as oil addition has been shown to enhance carotenoid extraction under SFE conditions (Chalermthai et al., 2022). Carotenoids tend to form esters with oils, facilitating their co-extraction. However, in *Process 3*, despite efficient oil extraction, the lack of ethanol during SFE-CO₂ significantly ($p < 0.05$) reduced carotenoid recovery.

11.2.2 Extraction kinetics

As *Process 2* was the most efficient for co-extracting oils and carotenoids, the extraction kinetics of both products were monitored. The recovery yields of oils and total carotenoids are depicted in **Figure 11.4A**. It is evident that over 60% of carotenoids are extracted at the beginning of the process- during SFE stabilization- and after about 100 min the process is completed. Regarding individual carotenoids recovery, β -carotene and torulene exhibited similar trends, with higher extraction rates early on, whereas torularhodin was extracted at a slower rate, underscoring the importance of ethanol as a co-solvent (**Figure 11.4B**). Oil recovery followed a different pattern, with 80% extracted within 60 min and complete recovery achieved by the end of the process (**Figure 11.4A**). This trend is in line with the work of Vladić et al., (2022), who monitored the extraction yield of intracellular oil accumulated in

Tetrademus obliquus algal biomass. The authors employed decompression (1 h static SFE-CO₂ at 300 bar) while SFE-CO₂ extraction was carried out at 300 bar and 40 °C. Oil was gradually extracted, reaching an approximate yield of 65% after 2 h of operation, requiring 4 h to reach a plateau. Almost 40% of total extracted carotenoids were obtained during the first minutes of SFE-CO₂ process from the microalgae *Nannochloropsis gaditana* and *Synechococcus* sp. (Macías-Sánchez et al., 2009).

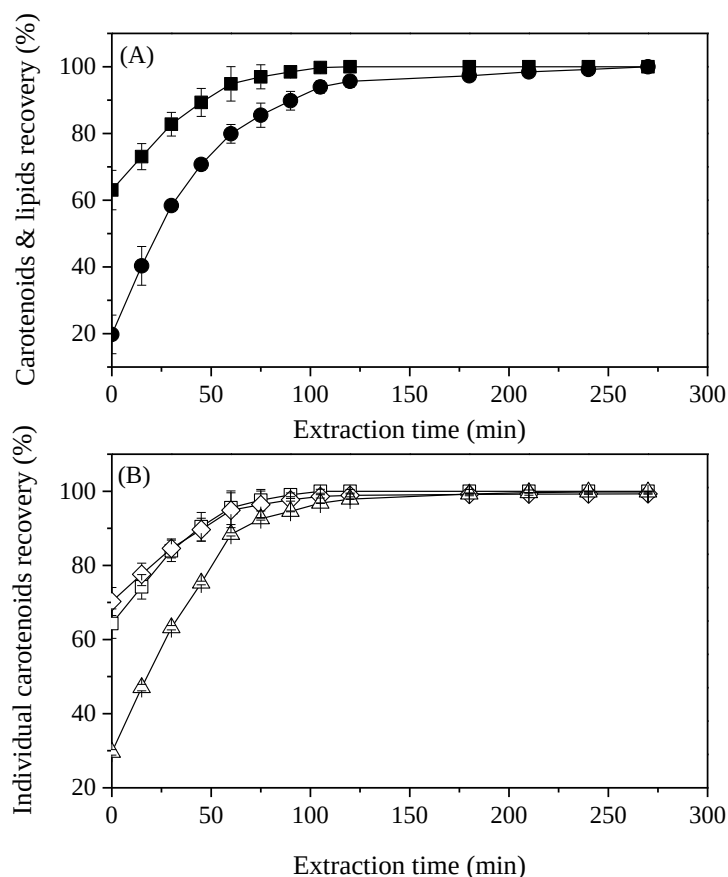


Figure 11.4 (A) Carotenoids (■) and microbial oil (●) recovery kinetics during SFE-CO₂ extraction using *Process 2*. (B) Evolution of individual carotenoid extraction under the same conditions. β-carotene (◇), torulene (□), torularhodin (Δ).

11.3 Conclusions

The results of this chapter demonstrated the feasibility of co-extracting microbial oil and carotenoids from red yeasts biomass using SFE-CO₂. The cell disruption method played a crucial role on extraction efficiency, while the use of ethanol for both cell lysis and the SFE-CO₂ process was imperative to achieve high carotenoid recovery

yields. The obtained extracts were rich in oils containing unsaturated fatty acids, that in combination with carotenoids with high antioxidant activity could potentially be used as functional ingredients in novel food systems, expanding the applications of microbial products. Consequently, the subsequent step of this research was directed toward investigating the potential applications of the carotenoid-rich microbial oil that was extracted through SFE-CO₂.

CHAPTER 12

Development of electrosprayed particles and oleogels using carotenoids-rich microbial oil

12 Development of electrosprayed particles and oleogels using carotenoids-rich microbial oil

12.1 Introduction

The main objective of this chapter was to study the development of novel food ingredients using the biotechnologically produced carotenoids-rich microbial oil. As it has been shown in previous chapters, the produced carotenoids-rich microbial oil demonstrated high antioxidant and antimicrobial activity, whereas the microbial oil composed mainly of unsaturated fatty acids. Therefore, these attributes render carotenoid-rich microbial oil highly suitable for the development of novel food ingredients with functional properties. Furthermore, the effectiveness of green extraction using SFE-CO₂, as demonstrated in the previous chapter, enhances its suitability for industrial applications, by eliminating organic solvents from the final product.

In this chapter, two applications of carotenoid-rich microbial oil were studied. Initially, electrospinning was employed for the production of capsules of carotenoid-rich microbial oil. In this case, various whey protein-based formulations were investigated as encapsulating materials targeting to the development of particles with enhanced microbial oil encapsulation efficiency. Considering that polysaccharides from plant sources have primarily been used in wall material matrices, the EPS fraction produced by *Rhodospiridium paludigenum* 2663 (Chapter 10) underwent further characterization and assessment as a potential component for incorporation into the encapsulation matrix. Therefore, this section presents both the characterization of EPS and the production of electrosprayed particles. In the second scenario, whey protein oleogels were developed using the carotenoids-rich microbial oil through an emulsion-templated approach. Structured lipids, or oleogels, represent innovative ingredients that can function both as fat replacers in food products and as protective carriers of the bioactive compounds, such as carotenoids. This chapter details the production of oleogels, along with their characterization and their capability to serve as protective carriers for microbial oil and carotenoids.

12.2 Characterization of EPS

Microbial EPS are promising for food applications due to their biodegradability, unlike their synthetic counterparts (Hamidi et al., 2020). While yeasts are recognized as potential EPS producers, the characterization and the potential uses of yeast-derived EPS have yet to be evaluated. To address this gap, the first step was to characterize the crude EPS fraction after ice-cold ethanol precipitation and analyze some key properties which could reveal their possible applications.

12.2.1 FTIR analysis

FTIR analysis was initially employed in order to characterize the crude EPS fraction. The presence of characteristic bands in the IR spectrum would confirm its structure as a polysaccharide. As shown in **Figure 12.1**, a broad band at 3276 cm^{-1} was observed in the sample. This broad band in the range of $3600\text{--}3000\text{ cm}^{-1}$ is due to the stretching vibrations of abundant OH groups, which are typical of polysaccharides (Hong et al., 2021). Additionally, the bands present at 2922 and 2851 cm^{-1} can be mainly assigned to the antisymmetric and symmetric stretching vibrations of CH_2 groups and stretching vibrations of CH groups in the polysaccharide structure (Wang et al., 2022). Finally, the region between 1200 and 800 cm^{-1} , commonly referred to as the "fingerprint" region of polysaccharides, showed varying bands between 1174 and 1140 cm^{-1} (Pasandide et al., 2017). These bands are characteristic of different glycosidic linkages found in various polysaccharides. Hamidi et al., (2020) reported that the glycosyl linkages identified after GC-EI-MS analysis were 1,2-, 1,3-, and 1,6-, indicating, that the analyzed EPS were a highly branched β -D-glucan polymer. The examined sample presented an intense band at 1021 cm^{-1} that can be assigned to the C-O stretching vibration of ether bond, indicating that the yeast strain *Rhodospiridium paludigenum* 2663 successfully produced exopolysaccharides.

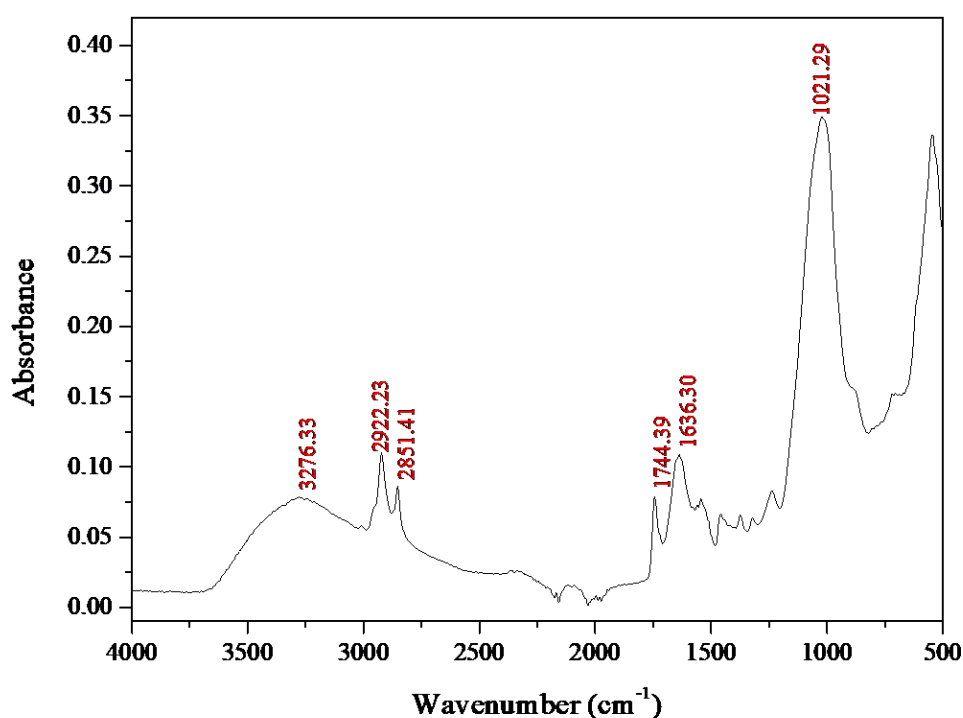


Figure 12.1 FTIR spectrum of crude EPS fraction obtained by *Rhodosporidium paludigenum* 2663.

12.2.2 Determination of EPS monosaccharide composition

The identification of the individual monosaccharides comprising the crude EPS fraction was conducted using both HPLC and TLC analysis. Prior to analysis the fraction underwent acid hydrolysis in order to break down the polymer into its components. HPLC indicated that the crude EPS fraction was mainly composed of glucose. Fungi (including yeasts) are able to synthesize various types of EPS such as glucan-type, mannan, and glucomannan (De Baets et al., 2002). Liu et al., (2021) reported that the EPS produced under the optimal pH and temperature conditions by an *Rhodosporidium paludigenum* strain consisted of glucose and mannose. Glucose represented the main monosaccharide in the EPS synthesized by a cold-adapted *Rhodotorula* strain (Hamidi et al., 2020).

TLC analysis was also carried out on both the non-hydrolyzed (labeled as bar 1) and the hydrolyzed sample (labeled as bar 2). **Figure 12.2** presents the results of TLC analysis using a mobile phase consisting of chloroform: methanol: acetic acid: dH₂O

in a ratio of 25:15:4:1. A mixture of 20% (v/v) H₂SO₄ and 5% (v/v) phenol solution were employed as visualization reagent. Four different sugar monosaccharide standards -glucose, sucrose, fructose, and galactose- were also analyzed. The with respective R_f values of the standard compounds were 0.61, 0.63, 0.62, and 0.56. As expected, no bands were observed in the non-hydrolyzed sample, while five distinct bands appeared in the hydrolyzed sample. The R_f values, beginning from the lowest band on the TLC plate, were 0.45, 0.5, 0.57, 0.61, and 0.69 (**Figure 12.2**). Consequently, glucose was identified in the sample based on the observed R_f values. The presence of galactose could also be deduced by the TLC bands of the standard (R_f =0.56) and the hydrolysed sample (R_f =0.57). The presence of the other bands indicated the presence of other carbohydrates in the crude EPS fraction. Mannose has been identified in yeast-derived EPS fractions, in different molar ratios with glucose (Hamidi et al., 2020; Liu et al., 2021; Seveiri et al., 2019). Red yeast EPS composed of galactose, arabinose and fucose have been previously demonstrated (Vazquez-Rodriguez et al., 2018; Yousefbeyk et al., 2018). It is obvious that further studies along with the utilization of more sophisticated analytic techniques (e.g GC-MS analysis) are required to fully elucidate the structure of *Rhodospiridium paludigenum* 2663 EPS.

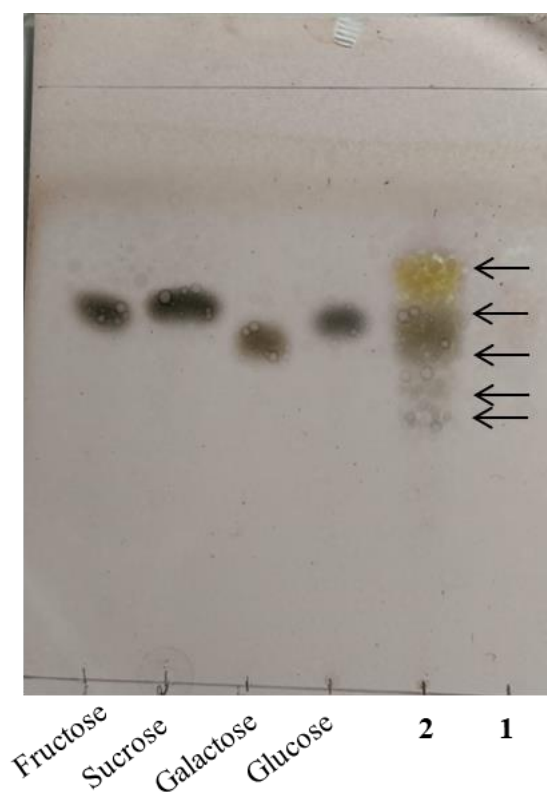


Figure 12.2 TLC analysis of sugars presented in the crude EPS fraction produced by *Rhodospiridium paludigenum* 2663.

TLC was further utilized to detect the presence of impurities in the crude EPS fraction such as proteins and lipids. As shown in **Figures 12.3A** and **12.3B** the obtained EPS fraction was contaminated with both proteins and lipids. The analyzed samples were before (labeled as bar 1) and after acid hydrolysis (labeled as bar 2). TLC plates were developed with isopropanol: dH₂O in a ratio of 7:3 and the spray reagent was a ninhydrin solution. Lipid identification was carried out using a mixture of hexane, diethyl ether, and acetic acid in a ratio of 42.5:7.5:0.5, with iodine vapors used as the visualization reagent. Five bands were observed in **Figure 12.2A** and three distinct bands in **Figure 12.2B**, indicating the presence of both proteins and lipids in the crude EPS extract.

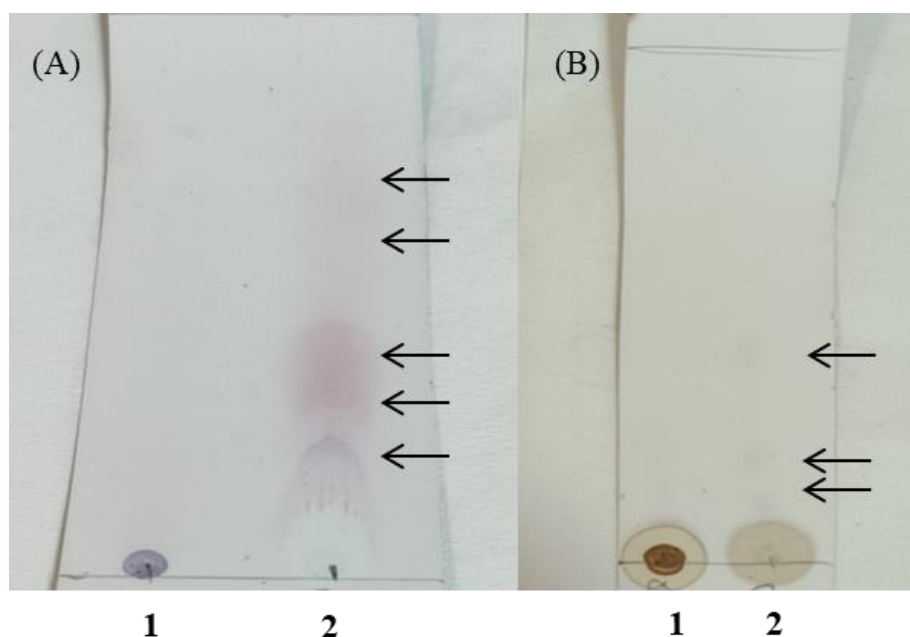


Figure 12.3 TLC analysis of EPS for proteins (A) and lipids (B) detection. Lane 1: non-hydrolyzed EPS, Lane 2: hydrolyzed EPS.

12.2.3 Oil holding capacity, surface tension and emulsification properties

The characterization of the physical properties of EPS focused on their oil holding capacity, surface tension, and emulsification index. Since the TLC analysis indicated the presence of impurities, such as lipids and proteins, in the extracted EPS, further purification was performed using three different approaches. Eventually, the physical properties were evaluated in three distinct fractions: crude EPS (fraction 1), crude EPS treated with dialysis membranes and filtration (fraction 2), crude EPS treated with TCA (prior to precipitation with ice-cold ethanol), with dialysis membranes and filtration (fraction 3) and the results are presented in **Table 12.1**.

OHC is an important property that determines the hydrophobicity index of compounds and their interactions with other food constituents. OHC reflects compound ability to absorb and retain lipids in food formulations (Shen et al., 2022). Regarding the OHC, fraction 2 exhibited the highest capacity of 4.28 g/g. Kavitate et al., (2024) reported an OHC of 3.79 g/g for EPS derived from the *Enterococcus hirae* strain OL616073, while Lobo et al., (2019) demonstrated a 3-fold higher OHC for crude EPS fraction from *Streptococcus thermophilus* CRL1190 (fraction which was equivalent to the fraction 1 of this study).

Moreover, fraction 2 presented the lowest surface tension (**Table 12.1**). Surface tension is a key property that reflects the surface activity of substances. Compounds able to reduce the surface tension of a liquid are referred to as surface-active materials, which are commonly used in colloidal food systems, such as emulsions. These materials possess both hydrophilic and lipophilic groups, enabling them to orient at the interface between the two phases of a biphasic system, thus facilitating the stabilization of the emulsion (Sahin and Sumnu, 2007). The EPS fractions 1 and 3, did not exhibit considerable surface tension reduction, considering that the surface tension of ddH₂O is approximately 70 mN/m. On the other hand, fraction 2 showed the highest reduction in surface tension (**Table 12.1**). Likewise, Tansel and Tansel, (2013) used a modified protocol involving dialysis membranes to recover EPS from bacteria strains, obtaining surface tension values ranging from 49.34 to 53.48 mN/m.

Finally, the emulsification index of EPS was also evaluated. The obtained results (**Table 12.1**) showed that emulsification index ranged from 47% for the fraction 1 to 53% for fractions 2 and 3. Mohammed et al., (2021) demonstrated that a crude EPS extract (equivalent to the fraction 1 of this study) derived from *Rhodotorula mucilaginosa* YMM19 resulted in an emulsification index of 60%. Similarly, Tarannum et al., (2023) obtained comparable results for crude EPS extract produced from lactic acid bacteria.

Table 12.1 Evaluation of the effect of three different precipitation methods on the OHC, surface tension and emulsification index of the extracted EPS.

Physical properties	Fraction 1	Fraction 2	Fraction 3
OHC (g/g)	1.14 ± 0.33 ^a	4.28 ± 0.72 ^b	3.14 ± 0.70 ^b
Surface tension (mN/m)	63.00 ± 0.43 ^a	45.90 ± 0.44 ^b	64.10 ± 0.43 ^a
Emulsification index (%)	47.00 ± 1.21 ^a	53.00 ± 3.27 ^b	53.00 ± 4.24 ^b

* Different lowercase letters (a, b) indicate significant differences ($p < 0.05$) between different fractions within each physical property.

Conclusively, the results of this study suggested that EPS fraction 2 holds potential as a colloidal ingredient in biphasic systems, as it demonstrated the highest OHC, the lowest surface tension, and a strong emulsification index. These attributes would typically indicate that EPS fraction 2 could effectively stabilize emulsions and interface well in biphasic systems. Therefore, the EPS fraction 2 was selected for incorporation into the whey protein/ carotenoid-rich microbial oil emulsions prior to the electrospinning process, as described in the following section.

12.2 Development of electrosprayed particles

12.2.1 Evaluation of encapsulation efficiency of microbial oil

The initial steps in the formation of particles or capsules containing carotenoid-rich microbial oil involved the assessment of various whey protein-based formulations for the development of an effective encapsulation matrix. As previously mentioned, the combination of encapsulation techniques could lead to enhanced encapsulation efficiency of bioactive compounds, with the combination of microemulsion and electrospinning standing out among these methods (Chen et al., 2023; Drosou et al., 2022; İnan-Çınkır et al., 2023). The effectiveness of an emulsion primarily depends on the incorporation of surfactants to prevent gravitational separation of the biphasic system (McClements, 2015). To this end, the surfactants Tween-80 and Triton X-100 were utilized in the preparation of the whey protein/microbial oil emulsion and assessed for their impact on the encapsulation efficiency (EE) of microbial oil after the electrospinning process.

As shown in **Table 12.2**, the concentration of Tween-80 appeared to have a notable effect on the EE of microbial oil. At a concentration of 0.05%, no encapsulation of microbial oil occurred. In contrast, when the concentration was increased to 0.5%, 45% of the microbial oil was successfully encapsulated within the matrix. On the contrary, the emulsion prepared with Triton X-100 as the emulsifier (0.5%) resulted in particles with lower EE than that achieved with Tween-80. This could be partially explained by the higher HLB (hydrophilic-hydrophobic balance) of Tween-80 (HLB 15.0) than Triton X-100 (HLB 13.6) (Kruglyakov et al., 2002). It should be stressed that higher HLB value is assigned to a more hydrophilic character of the emulsifier (Griffin, 1954). Although, both Tween-80 and Triton X-100 fall within the 8-18 HLB

range, making them suitable for oil-in-water (o/w) emulsions like the whey protein/microbial oil emulsion, their slight HLB difference may led to the different EE of the microbial oil. Furthermore, as it has been reported, that the effectiveness of an emulsifier is determined by an optimal HLB value along with the presence of the correct chemical groups (Griffin, 1954). Therefore, both the concentration and type of emulsifier could influence the final EE of the microbial oil.

Table 12.2 Encapsulation efficiency (%) of microbial oil using various whey protein-based formulations as the encapsulation matrices.

Wall Material		Microbial oil (%, w/w)	Encapsulation efficiency (%)
Protein (%, w/w)	Emulsifier (%, w/w)		
10% WPC, pH 8	0.05% Tween-80	3%	0%
	0.5% Tween-80		43.30%
	0.5% Triton X-100		33.00%
	0.05% EPS		28.00%
	1% EPS		71.15%

As mentioned in previous chapter, the yeast *Rhodospiridium paludigenum* 2663 was capable of producing EPS and specifically the fraction 2 was highlighted to be a potential emulsifier (Section 12.2.3). As presented in **Table 12.2**, the use of EPS (1%) positively influenced the EE of microbial oil, resulting in particles with 71.15% EE of the microbial oil. It is worth noting that the composition of the microbial oil remained consistent before and after the electrospinning process, with C18:1 comprising more than 50% of the total fatty acids.

12.2.2 Evaluation of encapsulation efficiency of carotenoids

The carotenoids present in microbial oil were also evaluated for their encapsulation efficiency. Their EE was examined under the optimal encapsulation conditions identified in the previous section. **Figure 12.4A** depicts the emulsion produced prior

to the electrospinning process, while **Figure 12.4B** presents the final electrospayed particles.

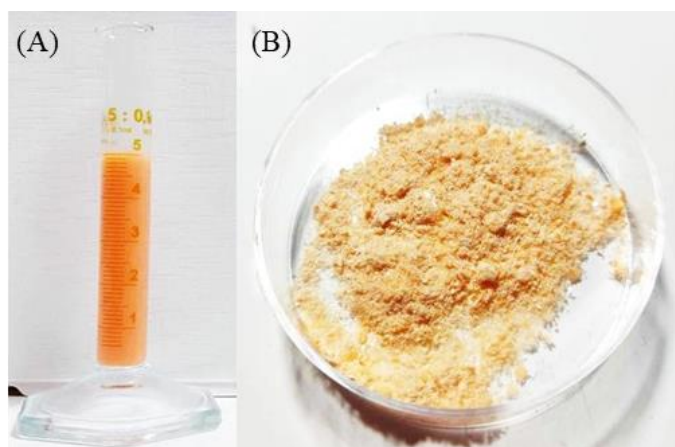


Figure 12.4 The produced whey protein/microbial oil emulsion (A) and the resulted particles after electrospinning process (B).

The produced electrospayed particles were subsequently examined to obtain the electrospinning yield and EE of carotenoids. It should be stressed that the electrospinning yield of the carotenoids differs from the EE. Specifically, electrospinning yield refers to the proportion of carotenoids present in the final electrospayed particles compared to the initial concentration carotenoids before electrospinning process. On the other hand, the encapsulation efficiency (EE) of carotenoids represents the proportion that was successfully incorporated into the particles.

HPLC-DAD analysis revealed that 84.3% of the total carotenoids were retained in the final product (electrospinning yield). Specifically, 89% of the initial β -carotene concentration was present in the particles. Likewise, the concentration of torulene remained at 90.9% of its initial concentration in the microbial oil prior to encapsulation. However, the concentration of torularhodin was reduced by approximately 50% in the final product, indicating its increased sensitivity to oxidation. The obtained results are comparable to those from previous studies that employed microemulsion electrospinning as the encapsulation technique (**Table 12.3**). İnan-Çinkır et al., (2023) reported an electrospinning yield of 79.74% for carotenoid extract from watermelon pulp using this technique. Moreover, the encapsulation of torularhodin extracted from *Sporidiobolus pararoseus* JD-2 resulted

in 82.47% electrospinning yield of carotenoids (Liu et al., 2023). In contrast, Drosou et al., (2022) used pullulan in the encapsulating matrix, achieving greater results with a β -carotene electrospinning yield of 95.77%.

Moreover, the EE of carotenoids reached 42.2%, indicating that more than half of the carotenoids were located on the surface of the particles. Further analysis of the composition revealed that EE varied by carotenoid type. Specifically, torulene and torularhodin were fully encapsulated in the final product, whereas the majority of β -carotene (68%) was found on the surface. Niu et al., (2020) showed up to 39% EE of synthetic β -carotene, which aligns closely with our findings. On the contrary, Chen et al., (2023) reported a significantly enhanced EE of lycopene, achieving 98.75% following optimization of the encapsulation process.

In conclusion, this study demonstrated the potential of using *Rhodospiridium paludigenum* as a single-cell factory for the simultaneous production of carotenoids, microbial oil, and EPS, which can be utilized for the development of novel electrosprayed particles with carotenoid EE comparable to those reported in the literature. Further research focusing on the electrospinning process parameters, such as applied voltage, distance from the collector etc. as well as the ingredients of the encapsulation matrix could lead to higher EE of carotenoids.

Table 12.3 Summary of electrospinning yield and encapsulation efficiency of carotenoids encapsulated in different matrices using microemulsion electrospinning process.

Carotenoid	Source	Encapsulating matrix	Core material	Electrospinning yield (%)	Encapsulation efficiency (%)	References
β -carotene	Synthetic	20% (w/v) Pullulan	0.25mg/mL β -carotene in corn oil	95.77		Drosou et al., (2022)
β -carotene	Synthetic	2.3% (w/w) Alkali lignin, 16% (w/w) PVA	2% (w/w) soybean oil, β -carotene	90.20		Xiang et al., (2022)
β -carotene	Synthetic	40% (w/w) Gum Arabic, 25% (w/w) WPI, 0.02% (w/w) Sodium azide	5% Oil phase: 10% (w/w) β -carotene in corn oil	93.32		Falsafi et al., (2022)
Carotenoid extract	Watermelon pulp (<i>Crimson tide</i>)	3.01g Span 20, 25% (w/v) Zein powder, Gelatin, Pectin	0.5g of watermelon pulp in 4.4g glycerol	79.74		İnan-Çinkır et al., (2023)
Torularhodin	<i>Sporidiobolus pararoseus</i> JD-2	1 mg/mL Sodium alginate, 1 mg/mL Chitosan	300 mg torularhodin in 5g corn oil	82.47		Liu et al., (2023)
β -carotene	Synthetic	1% (w/w) Whey protein isolate, 8% (w/w) Pullulan	5% (w/w) β -carotene, Medium-chain triacylglycerols		39.00	Niu et al., (2020)
Lycopene	Synthetic	1% (w/v) Whey protein isolate, 1% (w/v) Tricholoma lobayense Heim-3	0.2% (w/v) lycopene in corn oil		98.75	Chen et al., (2023)
Carotenoid extract	<i>Rhodospiridium paludigenum</i> 2663	10% Whey protein concentrate, 1% exopolysaccharide extract from <i>R. paludigenum</i> 2663	Carotenoids-rich microbial oil containing β -carotene, torulene and torularhodin	84.3	42.2	This thesis

12.2.3 FTIR analysis

Further analysis of the chemical profile of the produced electrosprayed particles was conducted through FTIR. **Figure 12.5A** illustrates the FTIR spectrum of the produced electrosprayed particles. To determine the presence of EPS, microbial oil and whey protein the individual spectra of each component were also analyzed, as shown in **Figures 12.5B, 12.5C, and 12.5D**, respectively. Regarding the spectrum of whey protein, the presence of three characteristic bands indicates protein identification. Specifically, a band in the $3300\text{--}3070\text{ cm}^{-1}$ region corresponds to the stretching vibration of the -NH bond in proteins (Amide A band) (Barth, 2007), with the whey protein sample exhibiting a corresponding band at 3276 cm^{-1} (**Figure 12.5D**). Additionally, sharp peaks near 1650 cm^{-1} and 1550 cm^{-1} are attributed to Amide I and Amide II bands, respectively. The Amide I band primarily arises from the stretching vibration of the C=O bond, while the Amide II band results from a combination of the in-plane bending of the NH bond and the stretching vibration of the CN bond (Barth, 2007). As shown in **Figure 12.5D**, the whey protein also displayed both of these characteristic bands at 1629 cm^{-1} and 1510 cm^{-1} respectively. The less intense bands in the $3000\text{--}2800\text{ cm}^{-1}$ region (2922 and 2855 cm^{-1}) indicated the presence of CH, CH₂, and CH₃ groups (Wang et al., 2022), whereas the peak at 3060 cm^{-1} can be assigned to the Amide B band (Barth, 2007).

In the spectrum of microbial oil (**Figure 12.5C**), the presence of bands in two regions facilitated its identification. Particularly, sharp bands in the $3000\text{--}2800\text{ cm}^{-1}$ region are expected due to the abundance of CH, CH₂, and CH₃ bonds in oil. Indeed, microbial oil exhibited bands in this region, at 2922 and 2851 cm^{-1} . Furthermore, the presence of an intense band at 1744 cm^{-1} corresponds to the stretching vibration of the ester carbonyl functional groups in triglycerides (Lucarini et al., 2018), which was also observed in the microbial oil spectrum. With respect to the spectrum of EPS already analyzed in Section 12.2.1, the spectrum obtained from the produced electrosprayed particles exhibited all the characteristic bands of each component used.

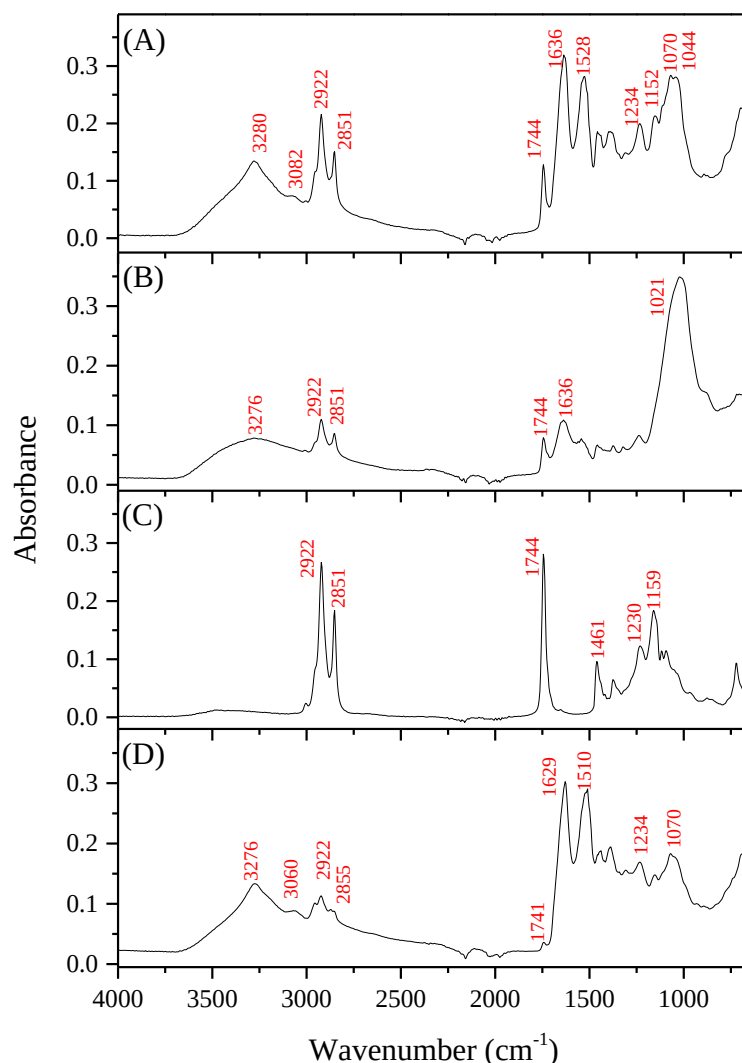


Figure 12.5 FTIR spectrum of electro sprayed particles (A), EPS (B), microbial oil (C) and whey protein (D).

12.3 Development of whey protein oleogel

Oleogels are considered an effective carrier for protecting bioactive compounds, while they can also serve as healthier fat substitutes in several food systems due to their solid-like structure (Martins et al., 2020; Perța-Crișan et al., 2023). To this end, the next set of experiments focused on the formation of an oleogel containing microbial oil rich in carotenoids derived from yeast strain *Rhodospiridium paludigenum* 2663 and the evaluation of its stability during the oleogelation process and upon thermal treatment. Additionally, the assessment of its physical properties was included as part of the characterization, providing insights into its potential use in food applications..

12.3.1 Preparation and visual appearance of the oleogel

Oleogelation involved three preparation steps; development of the whey protein/microbial oil emulsion through homogenization, the freezing of emulsion at -80 °C and finally the freeze drying of the emulsion to obtain the oleogel. As depicted in **Figure 12.6A** homogenization produced an orange-colored emulsion due to the presence of carotenoids. The subsequent preparation steps produced a macroscopic solid structure with an evenly distributed orange color, indicating a homogenous oleogel. The resulting oleogel exhibited a macroscopically cohesive structure with no observable oil leakage (**Figure 12.6B**). Then, the oleogel was manually mixed with a spatula until a homogeneous sample was obtained (**Figure 12.6C**), which was further characterized.

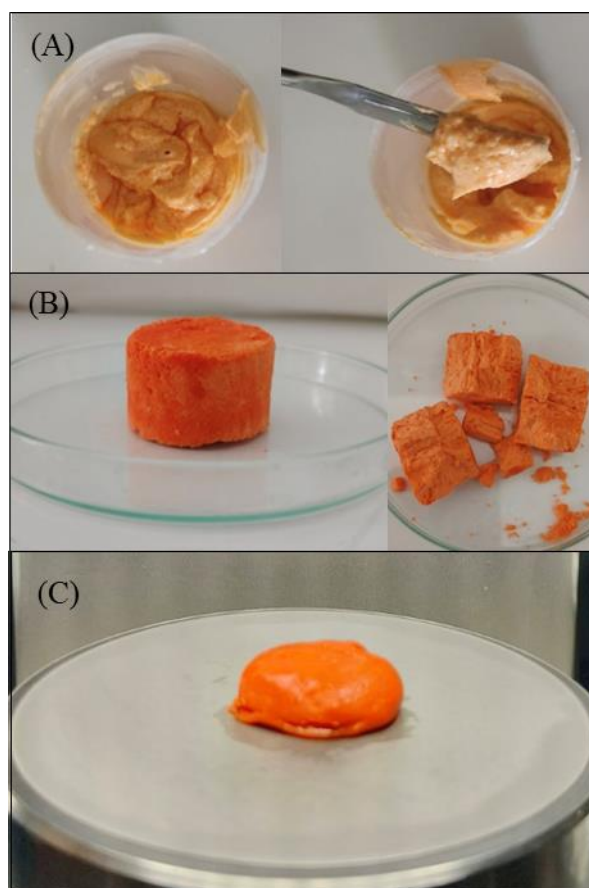


Figure 12.6 Whey protein/carotenoids-rich microbial oil formulations during oleogelation process: (A) emulsion, (B) oleogel after freeze-drying and (C) oleogel after shearing.

12.3.2 Characterization of oleogel

12.3.2.1 FTIR analysis

Figure 12.7 illustrates the FTIR spectrum of the oleogel. The presence of whey protein was confirmed by three characteristic bands, as previously mentioned; Amide A at 3276 cm^{-1} , Amide I at 1629 cm^{-1} and Amide II at 1524 cm^{-1} . Additionally, it was confirmed that the oleogel contained microbial oil, as all of its characteristic bands were observed in the sample. Specifically, the peaks at 2922 and 2855 cm^{-1} can be assigned to the antisymmetric and symmetric CH_2 stretching vibration (Lin-Vien et al., 1991) characteristic group of fatty acid structure. Also, as aforementioned, the band at 1744 cm^{-1} is associated with the vibration of ester carbonyl groups, a characteristic feature in lipid structures (Lucarini et al., 2018).

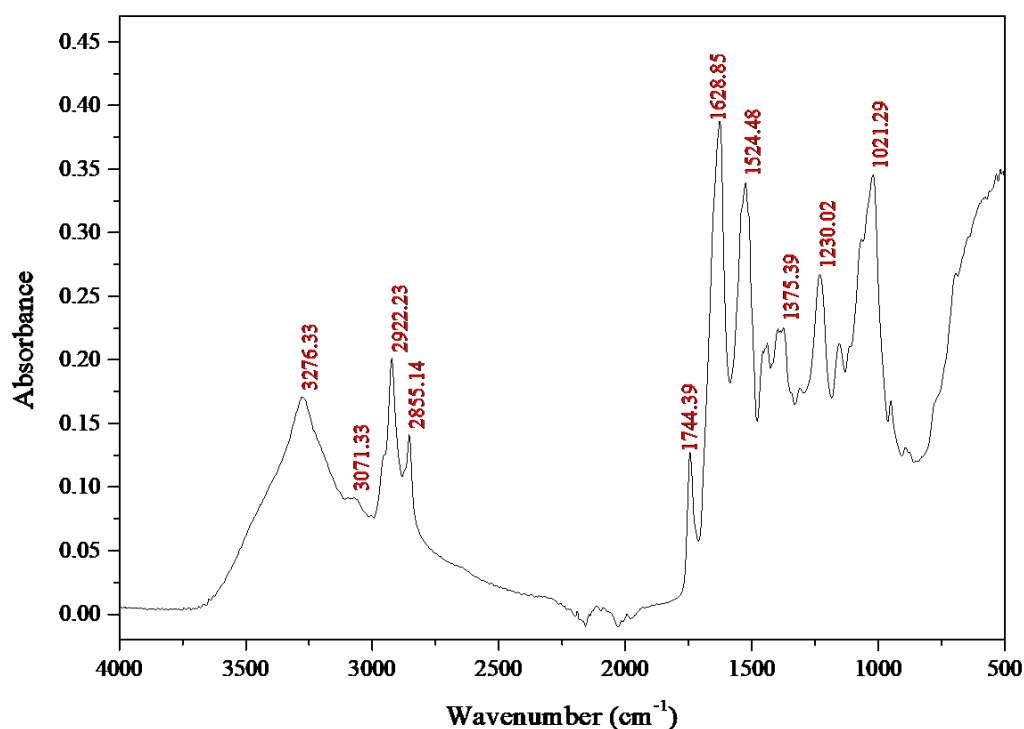


Figure 12.7 FTIR spectrum of the produced oleogel.

12.3.2.2 Oil bind capacity and textural attributes

Next step was the evaluation of the oil binding capacity of the produced oleogel as well as its textural properties. The OBC demonstrated no leakage of microbial oil

from the oleogel, resulting in complete binding (100%). Ropciuc et al., (2023) reported that, depending on the oleogelators used, the OBC could range from 64.34 to 99.73%. Indeed, a recent study showed that using different lipophilic oleogelators influenced the OBC of astaxanthin-loaded oleogels and the highest OBC, approximately 95%, was achieved with a mixture of γ -oryzanol and β -sitosterol (Shuai et al., 2024).

According to the textural analysis, the measured firmness was 4.3 N. Papadaki et al., (2019b) demonstrated that a wax-based oleogel containing microbial oil exhibited a firmness of 14.5 N, while the combination of microbial and soybean oil resulted in a wax-based oleogel with lowest firmness (1.9 N). Obviously, firmness of the oleogels is strongly dependent on the oil type as well as the oleogelator.

12.3.2.3 Rheological analysis

In this section, the rheological behavior of the produced oleogel is presented. Initially, the flow behavior of the oleogel was evaluated through a viscosity test as a function of shear rate (**Figure 12.8**). Viscosity properties are important to be measured since they are related to the minimum stress that food products need to maintain their continuous flow. The results showed viscosity decreased as the shear rate was increasing. This confirmed that the oleogel is a non-Newtonian fluid, presenting a typical shear thinning behavior. Similar findings have been demonstrated for emulsion-oleogels containing fucoxanthin (Shang et al., 2023).

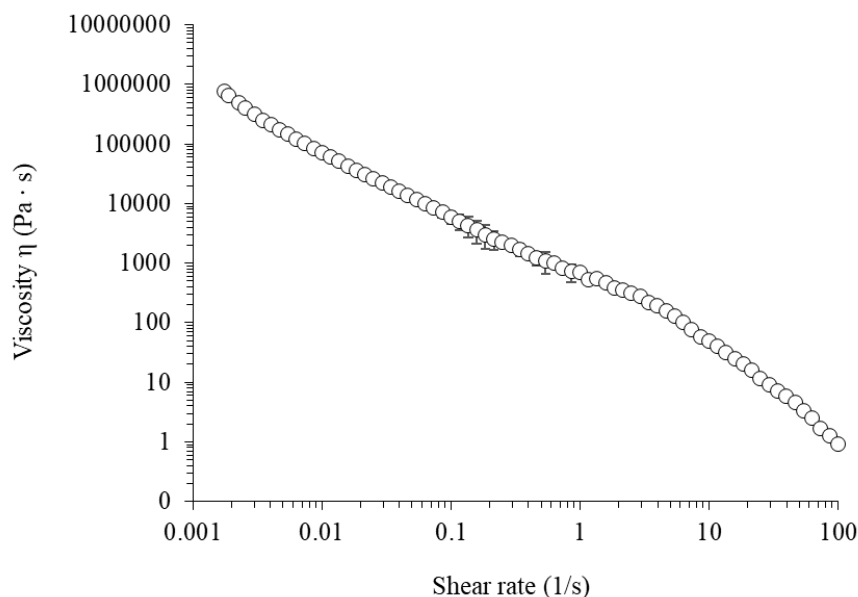


Figure 12.8 Viscosity of the oleogel

Subsequently, the viscoelastic properties of the oleogel were evaluated through strain and frequency sweep experiments. The results of strain sweep test are presented in **Figure 12.9A**, in which linear (LVR) and non-linear regions were observed. In the LVR, the oleogels demonstrated a solid-like structure as the G' was higher than G'' . The existence of a firm gel-like network structure was also identified by the $G'_{\max}$ which was 1.5 MPa or 1.5×10^6 Pa. Until now, there exists only one study investigating the development of whey protein isolate–carboxymethyl chitosan aerogel–astaxanthin oleogel, in which rheological analyses have not been performed (Zhao et al., 2023). Nevertheless, another study showed that the astaxanthin macadamia oil oleogels, structured with lipophilic oleogelators (mixture of γ -oryzanol and β -sitosterol), presented lower gel strength ($G'_{\max}$ around 2×10^4 Pa) than the oleogel of this thesis (Shuai et al., 2024). Furthermore, a 5% decrease of G' indicates the initiation of the non-linear region, in which G' and G'' values are continuously decreasing until the crossover point. The critical strain, which is the strain values at the end of the LVR, was 0.06%, demonstrating that above this critical strain the oleogel becomes more fluid-like. The critical strain could be considered as low, thus showing a low resistance to structure breakdown. This is commonly found in whey protein oleogels and it is strongly affected by the preparation method. For instance, supercritical-dried whey protein aerogels resulted in oleogels with significantly

greater LVR and higher critical strain values, than freeze-dried particles (Plazzotta et al., 2020). Notably, the G''/G' or the $\tan\delta$ (loss tangent) value in the LVR was about 0.1, confirming not only the predominance of the gel-like structure, but also the strong gel properties (Shuai et al., 2024). In particular, the closer the loss tangent value is to 1, the weaker the strength of the oleogel and vice versa (Wang et al., 2023). Consequently, the viscoelastic properties showed that oleogel presented a stable three-dimensional network structure. The crossover point ($G'=G''$), also known as the flow point, determines the transition to a viscous state. The cross over point was detected at 4.07% strain which indicated. that a disruption of the oleogel structure is occurred and after that strain the oleogel present a liquid-like behavior (Tirgarian et al., 2023).

Frequency sweep experiment was conducted at a strain value of 0.01%, which was within the LVR. The solid-like behavior ($G'>G''$) of the oleogels was also evident in the frequency sweep tests (**Figure 12.9B**). The G' and G'' curves were linear without presenting a crossover point. This behavior is typical of strong gels confirming their good tolerance against deformation (Espert et al., 2022). Moreover, a slight increase in G'' with increasing frequency values was negligible, indicating that all oleogels exhibited non-frequency-dependent behavior.

Conclusively, the rheological analysis confirmed the production of an oleogel with solid-like character and high mechanical strength. The low critical strain suggested that more studies should be performed concerning the oleogel preparation and composition to increase its tolerance against deformation. On the other hand, the low viscosity at high shear rates indicated the high spreadability capacity of the oleogels. Typically, the shear rate of spreading creamy food products on the surface of a bread is usually 10-100 1/s. Therefore, this oleogel could be utilized as fat substitute in spreadable food system, to control their structure and spreadability.

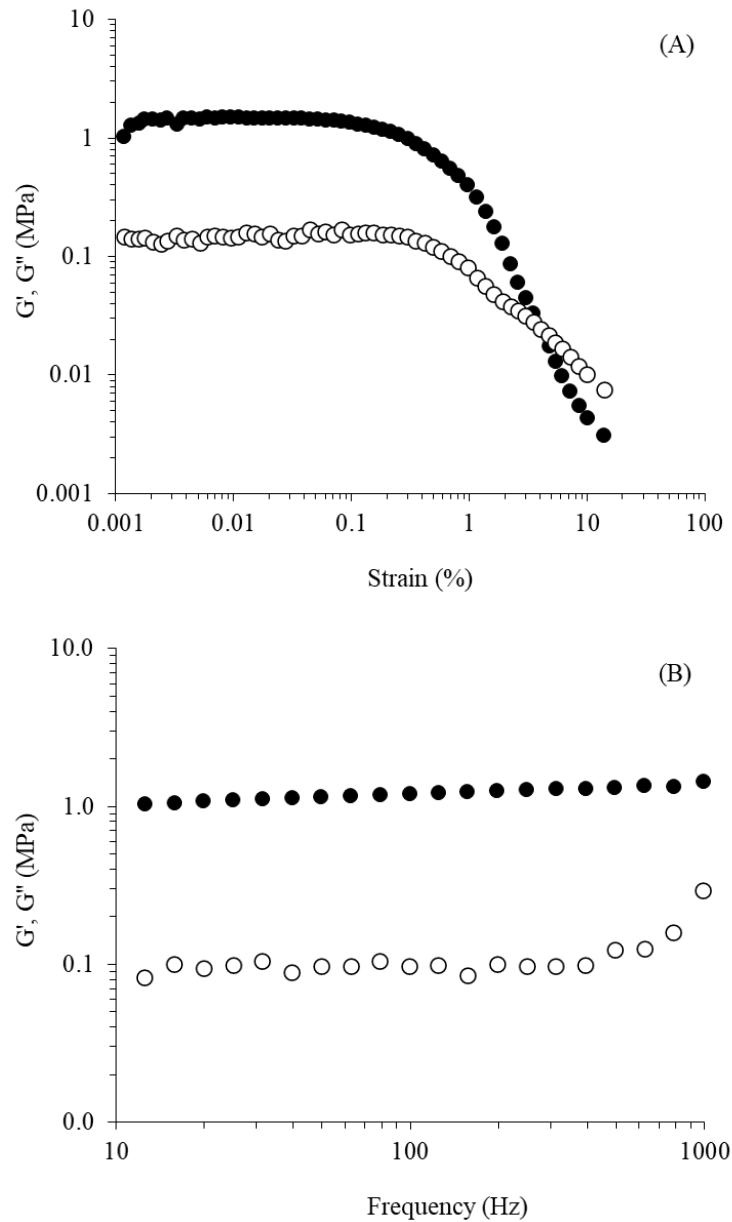


Figure 12.9 Storage modulus (G') and loss modulus (G'') during strain sweep tests (A) and frequency sweep tests (B) of the oleogel.

12.3.3 Carotenoids stability during oleogelation process

The stability of the carotenoids during the oleogelation process was evaluated by measuring the oleogelation yields of carotenoids at each preparation step, as well as assessing the oxidation of microbial oil before and after oleogelation.

The microbial oil remained protected throughout the oleogelation steps, with its oxidation level consistently maintained at its initial value of 7.68 meq/kg. However, a

gradual carotenoid degradation was observed during the oleogelation process, resulting in a final oleogelation yield of approximately 45% (**Figure 12.10**). More specifically, each preparation step had a significant impact on carotenoids concentration ($p < 0.05$). Previous studies on encapsulation techniques have reported that the choice of encapsulation matrix significantly impacts the encapsulation efficiency of carotenoids (Saifullah et al., 2019). Additionally, the use of a single oleogelator might strongly influence the physicochemical properties of the resulting oleogel (Hu et al., 2022). Consequently, further investigation into the encapsulation matrix for oleogelation might improve the final carotenoid yield.

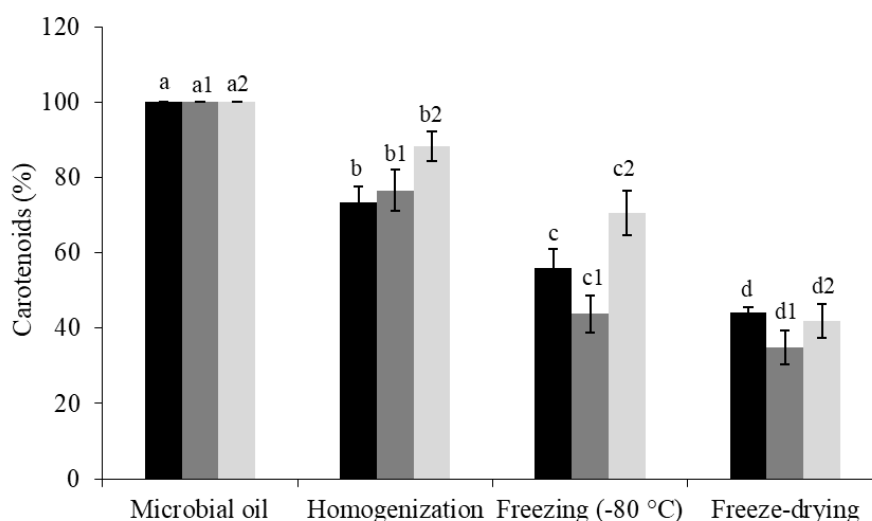


Figure 12.10 Carotenoids (%) during preparation steps of the oleogelation process: β -carotene (black bars), torulene (dark grey bars) and torularhodin (light grey bars). Different letters on the bars indicate statistically significant differences between preparation steps for each carotenoid (^{a, b, c} β -carotene; ^{a1, b1, c1} torulene; ^{a2, b2, c2} torularhodin) ($p < 0.05$).

The carotenoids degradation data during oleogelation fitted well ($R^2 > 0.96$) with the zero-order kinetic model, which revealed different constant rates (k) for each carotenoid. As shown in **Table 12.4**, β -carotene degradation exhibited the lowest k among the carotenoids, followed by torularhodin and torulene. Torulene demonstrated the highest reduction rate (k 22.83). The R^2 values indicated that the reduction of each carotenoid exhibited a linear relationship across each preparation step, which could be efficiently expressed through this kinetic model.

Table 12.4 Zero order kinetic models for the degradation of each carotenoid during oleogelation process.

Carotenoid	Zero order fitting	R ²
β-carotene	y= -18.475x+114.59	0.9683
Torulene	y= -22.829x+120.89	0.9601
Torularhodin	y= -19.205x+123.24	0.9613

12.3.4 Microbial oil and carotenoids stability during thermal treatment of oleogels

The next step of this study involved the evaluation of thermal stability of the carotenoid-rich microbial oil of the oleogel. For this purpose, three different temperatures were selected; 50, 70 and 90 °C. The chosen temperatures represent different common heating levels of food formulations, with 50 °C considered a mild heating e.g. to facilitate mixing of food ingredients, while 70 °C and 90 °C corresponding to high-temperature heating processes e.g. resembling pasteurization process. The thermal stability of microbial oil and carotenoids was investigated at various time intervals during heating to evaluate both the oxidative stability and the carotenoids degradation. Microbial oil in a non-encapsulated form was used as the control of this experimental set-up.

12.3.4.1 Oxidative stability

The oxidation of microbial oil during thermal processing was expressed as peroxide value (PV). As shown in **Figure 12.11** the oxidation of non-encapsulated microbial oil was higher, than oleogel-encapsulated microbial oil, as the temperature and time were increased. Particularly, under mild heating conditions (50 °C), the non-encapsulated microbial oil exhibited an oxidation increase of 32.26% after 90 min, compared to the sample at 0 min. At the same temperature, the increase in oxidation for the oleogel-encapsulated microbial oil was 14.56%, which was significantly lower than that of the non-encapsulated ($p<0.05$). Additionally, heating at 70 °C had a

significant impact on the oxidation of non-encapsulated microbial oil, resulting in an approximate 114% increase, whereas the oxidation of the oleogel was about 37.22% at 90 min. High-temperature heating conditions (90 °C) caused the greatest oxidation in both non-encapsulated (132.26%) and oleogel-encapsulated (44.98%) microbial oil, as expected due to the increased oxidation rates that are usually observed at elevated temperatures. However, oleogelation was able to maintain oxidation levels approximately 3 times lower at both 70 and 90 °C, demonstrating the effectiveness of oleogels in protecting thermo-sensitive compounds. Similarly findings to these results have been demonstrated for astaxanthin-loaded whey protein isolate oleogels prepared via the aerogel-templated method. In particular, researchers reported the PV values increased in a slower rate trend in the oleogel than that in the bulk oil (Zhao et al., 2023). Overall, the microbial oil within the oleogel remained significantly protected compared to the non-encapsulated oil after 90 min of thermal processing at the temperature range of 50-90 °C. This phenomenon suggested that the gel structure played a critical role in retarding oxidation. Conclusively, the enhanced oxidative stability of oleogels at various temperatures, suggested their potential use in the development of functional food products which must be thermally processed.

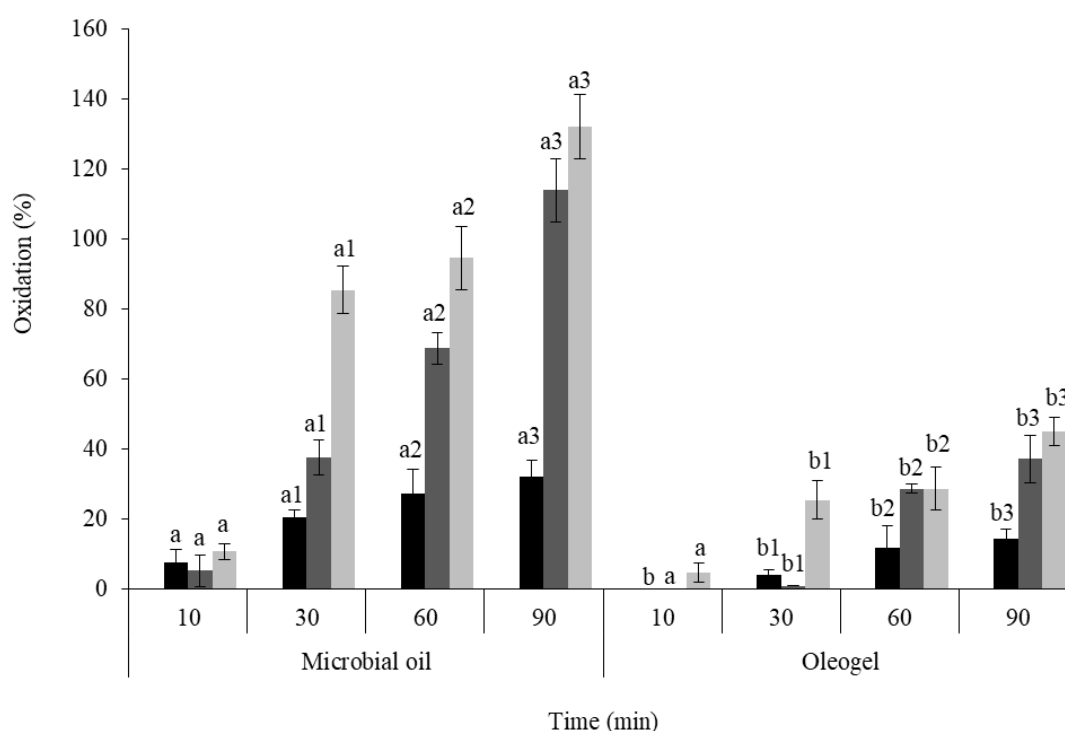


Figure 12.11 Oxidation (%) of the non-encapsulated microbial oil and oleogel-encapsulated microbial oil at different time intervals at 50 °C (black bars), 70 °C (dark grey bars) and 90 °C (light grey bars). Different letters on the bars indicate statistically significant differences between non-encapsulated microbial oil and oleogel-encapsulated microbial oil for each time interval at each temperature (^{a, b} 10 min; ^{a1, b1} 30min; ^{a2, b2} 60 min; ^{a3, b3} 90 min) ($p < 0.05$).

As illustrated in **Figures 12.12A** and **12.12B**, a first-order kinetic model was applied to determine the oxidation rate constant (k) at each temperature for both non-encapsulated microbial oil and oleogel-encapsulated microbial oil. **Table 12.5** demonstrated that k rate constants increased as the temperature increased, with the oleogel formulation providing higher protection against oxidation to the microbial oil, due to the lower k values. The non-encapsulated microbial oil was more susceptible to oxidation, as k was approximately 2-fold higher than this of oleogel-encapsulated microbial oil.

Among temperatures, k was the lowest at 50 °C for both formulations, suggesting that mild heating conditions might not strongly influence microbial oil oxidation. In contrast, at 90 °C, the oxidation rate appeared to increase, with k approximately doubling in both cases. Furthermore, as shown in Table 12, the R^2 values (> 0.93)

indicated that the first-order kinetic model was well fitted to the oxidation data at any temperature.

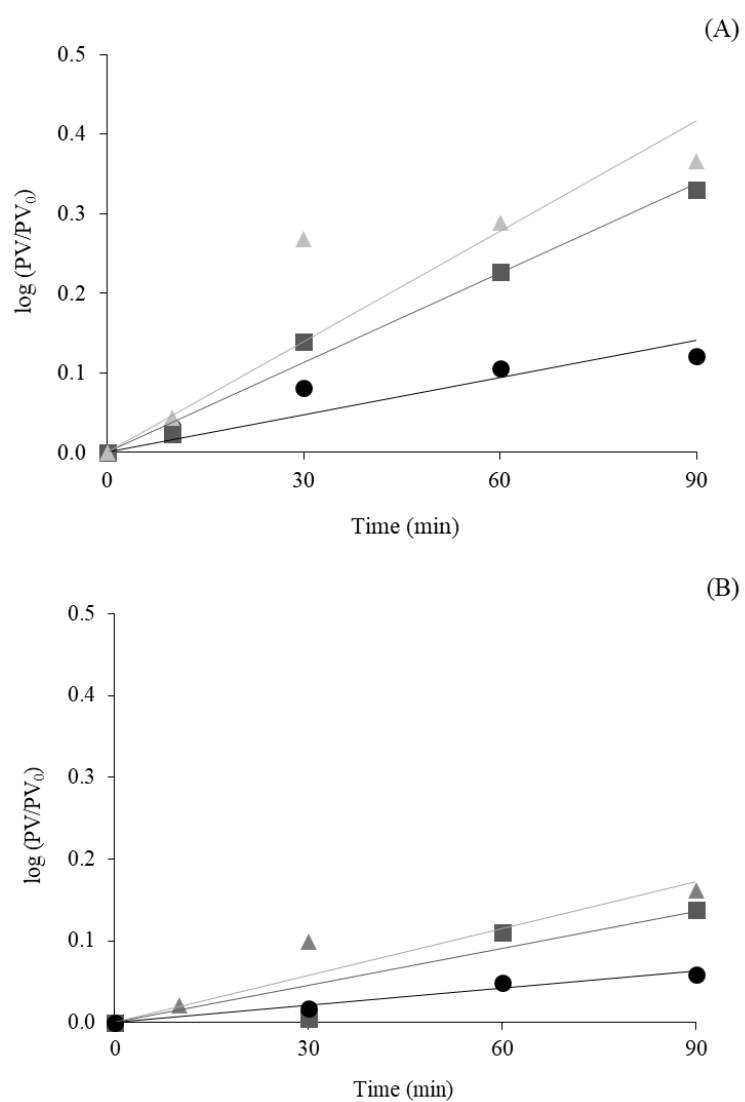


Figure 12.12 Fitting of oxidation data with the first-order kinetic model: non-encapsulated microbial oil (A) and oleogel-encapsulated microbial oil (B) during thermal treatment at 50 °C (●), 70 °C (■) and 90 °C (▲).

Table 12.5 First-order kinetic parameters: Oxidation rate constant values (k) and R^2 values of non-encapsulated microbial oil and oleogel-encapsulated microbial oil during thermal treatment at 50, 70 and 90 °C.

Temperature (°C)	Microbial oil		Oleogel	
	k	R^2	k	R^2
50	0.0016	0.9430	0.0007	0.9878
70	0.0038	0.9947	0.0015	0.9334
90	0.0046	0.9333	0.0019	0.9614

Fitting the oxidation rate constants data to the Arrhenius model, the activation energies E_a were calculated, giving insights of the impact of temperature changes on the oxidative stability of non-encapsulated and oleogel-encapsulated microbial oil. As shown in **Figure 12.13** the Arrhenius model were fitted well ($R^2 > 0.90$) for both non-encapsulated and oleogel-encapsulated microbial oil. The obtained E_a for non-encapsulated microbial oil was 26.05 kJ/mol, while the E_a for oleogel was 24.58 kJ/mol. A lower E_a indicates a reduced sensitivity of the material to thermal processes. This means that oleogel formulation had lower thermal sensitivity (lower E_a) compared to the non-encapsulated microbial oil form. Therefore, the oleogel provided enhanced protection to the microbial oil, which indicated that oleogels can serve as an effective protective carrier for microbial oil during thermal processing.

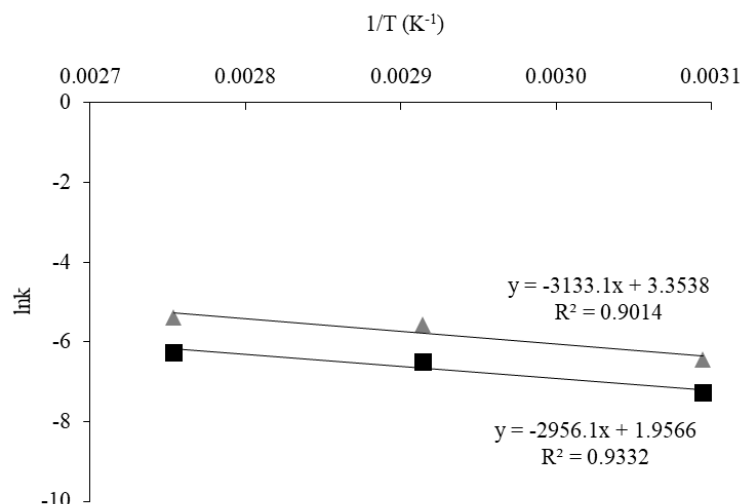


Figure 12.13 Fitting of oil oxidation data with the Arrhenius model for non-encapsulated microbial oil (▲) and oleogel-encapsulated microbial oil (■).

12.3.4.2 Carotenoids degradation

In addition, the degradation of carotenoids during thermal processing was investigated to evaluate their stability. The reduction of carotenoids concentration in non-encapsulated microbial oil was compared to that in the oleogel-encapsulated at 50, 70, and 90 °C, across different time intervals. According to **Figure 12.14**, temperature affected the degradation of carotenoids, with higher temperatures leading to a greater reduction in both conditions. Specifically, at 50 °C, the degradation of total carotenoids reached 84.27% by the end of the process (90 min), which was significantly higher compared to the reduction observed in the oleogel ($p < 0.05$). The oleogel formulation seemed to efficiently protect the carotenoids from degradation, showing only a 6% reduction in their concentration after 90 min. Moreover, at 70 °C, the difference in carotenoid degradation between the two formulations was even more pronounced, with the oleogel form resulting in a significantly higher carotenoid stability (78.8%) after 90 min of thermal processing. Finally, the high-temperature treatment (90 °C), appeared detrimental to the carotenoids in the non-encapsulated microbial oil, as they were completely degraded by the end of the process. In contrast, the carotenoid yield in oleogel was 52.30%, highlighting the protective effect of oleogels on carotenoids even at high temperatures.

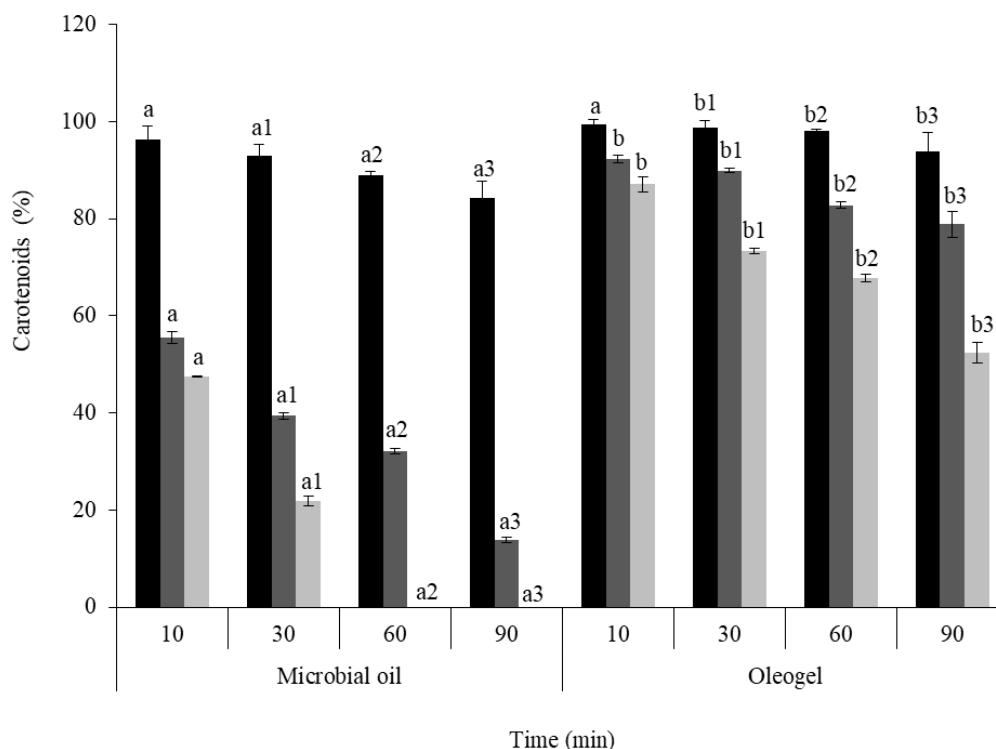


Figure 12.14 Carotenoids (%) of the non-encapsulated microbial oil and oleogel-encapsulated microbial oil at different time intervals at 50 °C (black bars), 70 °C (dark grey bars) and 90 °C (light grey bars). Different letters on the bars indicate statistically significant differences between non-encapsulated microbial oil and oleogel-encapsulated microbial oil for each time interval at each temperature (^{a, b} 10 min; ^{a1, b1} 30min; ^{a2, b2} 60 min; ^{a3, b3} 90 min) ($p < 0.05$).

Thermal degradation kinetics of carotenoids were evaluated during thermal treatment. Specifically, a first-order kinetic model was applied, as shown in **Figures 12.15A** and **12.15B**, to determine the (k) rate constants for each case. The slope of the lines increased as the temperature increased, with the carotenoid degradation rate to be consistently higher in non-encapsulated microbial oil than in the oleogel at each temperature. **Table 12.6** presents the k values derived from the kinetic model indicating that carotenoids protected within the oleogel exhibited a lower degradation rate at every temperature. At 50 °C, the degradation rate was the lowest for carotenoids in both formulations.. At higher temperatures, the degradation rate was greater compared to that at 50 °C. The k value for carotenoids in the non-encapsulated microbial oil at 90 °C was approximately 25 times higher than the k value at 50 °C. In contrast, carotenoids encapsulated within the oleogel exhibited lower k values at both 70 and 90 °C, demonstrating that oleogels provide a high carotenoid stability during

thermal treatments. Overall, the degradation of carotenoids was described well by the first-order kinetic model ($R^2 > 0.92$) in the temperature range of 50–90 °C, which is in agreement with previous results for thermal degradation kinetics of lutein, zeaxanthin, β -cryptoxanthin and β -carotene at 25, 35, and 45 °C (Xiao et al., 2018).

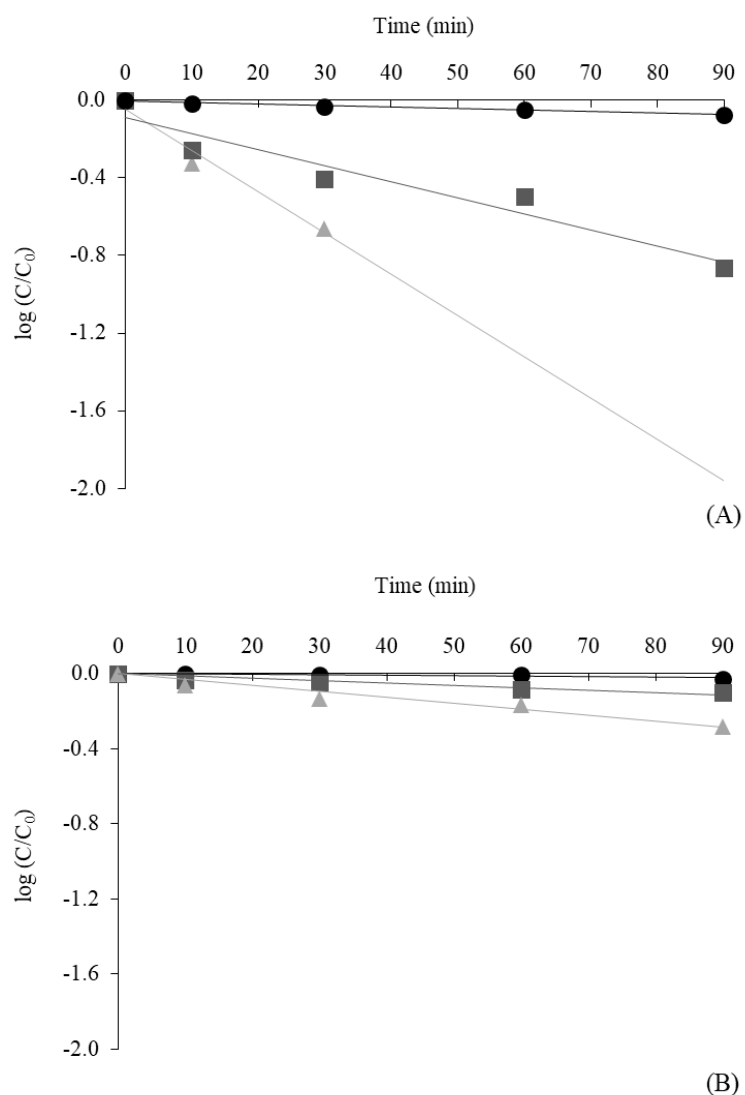


Figure 12.15 Fitting of carotenoids degradation data with the first-order kinetic model: non-encapsulated microbial oil (A) and oleogel-encapsulated microbial oil (B) during thermal treatment at 50 °C (●), 70 °C (■) and 90 °C (▲). First-order kinetic model for the degradation rate of carotenoids contained in free form (A) and in oleogel (B) at 50 °C (black line), 70 °C (dark grey line) and 90 °C (light grey line).

Table 12.6 First-order kinetic parameters: Carotenoids degradation constant values (k) and R^2 values of non-encapsulated microbial oil and oleogel-encapsulated microbial oil during thermal treatment at 50, 70 and 90 °C.

Temperature (°C)	Microbial oil		Oleogel	
	k	R^2	k	R^2
50	0.0009	0.9882	0.0002	0.9193
70	0.0096	0.9624	0.0013	0.9657
90	0.0231	0.9822	0.0032	0.9780

The thermal sensitivity of carotenoids for both non-encapsulated and oleogel-encapsulated formulations, were addressed by assessing the E_a according to the Arrhenius equation. **Figure 12.16** illustrates the trend lines and corresponding equations for the thermal sensitivity of carotenoids. The E_a of non-encapsulated carotenoids was 79.77 kJ/ mol, whereas the E_a for carotenoids within the oleogel was found to be 68.01 kJ/ mol. The reduced E_a suggested that oleogels-encapsulated carotenoids exhibited decreased thermal sensitivity, indicating once again that oleogel formulation can provide enhanced protection during thermal treatments.

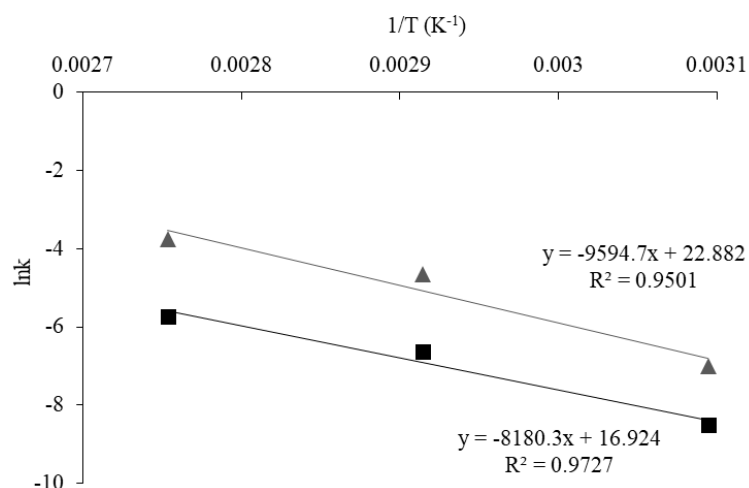


Figure 12.16 Fitting of carotenoids degradation data with the Arrhenius model for non-encapsulated microbial oil (▲) and oleogel-encapsulated microbial oil (■).

Recent studies have also demonstrated the importance of protecting thermo-sensitive carotenoids through encapsulation prior to the formation of the final food product. The encapsulation efficiency of these techniques under storage or heating conditions is among the most critical factors investigated. To the best of our knowledge, this is the first study to evaluate the thermal stability of carotenoids following oleogelation. The obtained results are comparable to, or even exceed, those achieved with other encapsulation techniques (**Table 12.7**). Esposto et al., (2022) reported approximately 50% degradation of total carotenoids extracted from carrots and encapsulated in liposomes, after heating at 70 °C for 40 min. Furthermore, encapsulation of carotenoid extract from Goji berry fruit in a nanoemulsion resulted in a 48.3% carotenoid yield after 60 min at 85 °C (Liu et al., 2022). Additionally, thermal heating at 100 °C for 30 min was applied to microcapsules formed using a combination of microencapsulation techniques; microemulsion and freeze-drying. These microcapsules, which contained lutein extracted from pumpkin, resulted in an 86.2% carotenoid yield (Shi et al., 2023).

Table 12.7 Carotenoids (%) under thermal processing at temperatures above 70 °C.

Carotenoid	Source	Encapsulation method	Carotenoids stability (%)	Reference
Carotenoid extract	Carrots (<i>Daucus carota</i> L.)	Liposome	70 °C, 40 min: ~ 50.0%	Esposito et al. (2022)
Fucoxanthin	Synthetic	Electrospinning	80 °C, 60 min: 74.6%	Tian et al. (2024)
Carotenoid extract	Goji berry fruit (<i>Lycium barbarum</i> L.)	Nanoemulsion	85 °C, 60 min: 48.3%	Liu et al. (2022)
Lutein	Pumpkin	Microemulsion - Freeze drying	80 °C, 30 min: 90.5%	Shi et al. (2023)
Lutein	Pumpkin	Microemulsion - Freeze drying	100 °C, 30 min: 86.2%	
Total carotenoids	<i>Rhodospiridium paludigenum</i> 2663	Oleogelation	70 °C, 60 min: 82.78% 70 °C, 90 min: 78.81%	This study
Total carotenoids	<i>Rhodospiridium paludigenum</i> 2663	Oleogelation	90 °C, 60 min: 67.81% 90 °C, 90 min: 52.30%	

12.4 Conclusions

The results of this chapter demonstrated the effectiveness of two different methods for encapsulating carotenoids-rich microbial oil. The microemulsion electrospinning technique showed that an improved encapsulation efficiency of microbial oil could be achieved by the use of EPS in the encapsulation matrix. This case demonstrated the potential of using *Rhodospiridium paludigenum* as a single-cell factory for the simultaneous production of carotenoids, microbial oil, and EPS, which can be utilized for the development of novel electrosprayed particles.

In the context of using oleogels as protective carriers for bioactive compounds, oleogels showed high potential for protecting carotenoids and oil from harsh thermal conditions. Furthermore, characterization of the physical properties of oleogels confirmed the production of a spreadable product with solid-like features, which make it a good candidate for several food applications. Literature-cited results concerning

the development of oleogel as delivery vehicles of carotenoids are limited, with most studies focusing on the incorporation of carotenoids into emulsions or emulgels. However, recent research efforts have focused on the development of astaxanthin-loaded oleogels with promising outcomes including enhanced bioavailability and bioaccessibility of the carotenoid (Shuai et al., 2024; Zhao et al., 2023). This underscores the need for further investigation into oleogelation for potential applications in functional food systems.

Overall, further optimization of both encapsulation processes, as well as additional evaluation of the final product, such as its toxicological effects, could enhance their potential for industrial-scale production of functional foods.

CHAPTER 13

Conclusions and future perspectives

13 Conclusions and Future Perspectives

The importance of natural carotenoids, especially regarding their health-promoting properties, is thoroughly presented in Chapter 2. Additionally, the biotechnological production of value-added compounds, including carotenoids, microbial oil, and exopolysaccharides, is explored in Chapter 3, with an emphasis on utilizing renewable resources as fermentation feedstocks. Various green extraction techniques, particularly the use of the supercritical CO₂ extraction method, are detailed in Chapter 4, being an imperative step for incorporating biotechnologically produced metabolites into food formulations. Finally, Chapter 5 assesses different encapsulation processes, emphasizing the need to protect carotenoids from environmental degradation prior to their addition into food products.

Initially, this thesis examined the ability of two *Rhodospiridium kratochvilovae* strains (Y-42 and Y-43) to produce carotenoids under varying carbon sources and C/N ratios (Chapter 8). Both strains showed more promising results when cultivated on glucose or a glucose-galactose mixture, while a C/N ratio equal to 80 (g/g) favored carotenoid production. Notably, the optimal C/N ratio for microbial oil production differed, as a higher ratio of 120 (g/g) was more preferable for this metabolite. Although the carotenoid profile varied when different carbon sources were employed, β -carotene consistently emerged as the predominant compound, highlighting the potential of these strains as efficient β -carotene producers.

In Chapter 9, the biotechnological production of carotenoids from *Rhodospiridium kratochvilovae* strains (Y-42 and Y-43) was evaluated using cheese whey as the fermentation substrate. Hydrolysis of cheese whey with fermented wheat bran significantly enhanced carotenoid synthesis and shifted the carotenoid pathway, leading to the predominant production of lycopene. The effects of temperature and C/FAN ratio were also examined with the maximum carotenoid concentration (36.35 mg/L) achieved at a temperature of 33 °C and at a C/FAN ratio of 40 (g/g) from strain Y-42. Moreover, the produced lycopene exhibited significant antioxidant activity with an IC₅₀ of 0.89 mg/mL.

The fermentative behavior of two novel *Rhodospiridium paludigenum* yeast strains (2663 and 2664) cultivated on glucose, fructose, sucrose, and a glucose-galactose mixture was also evaluated (Chapter 10). Strain *Rhodospiridium paludigenum* 2663 was able to produce three metabolites when grown on sucrose; carotenoids, microbial oil, and EPS. Therefore, the next step of the thesis was to assess both commercial sucrose and molasses as substrates on a bioreactor scale, showing higher titers for all metabolites when a fed-batch strategy was applied with molasses as the substrate. Particularly, maximum carotenoid production reached 3.3 mg/L, the achieved microbial oil accumulation was 10.9 g/L (50.7%, w/w), and the highest EPS concentration was 3 g/L. A mixture of β -carotene, γ -carotene, torulene, and torularhodin was produced, with β -carotene being the dominant carotenoid in all cases. The antioxidant and antimicrobial properties of these extracts were evaluated, revealing a strong antioxidant activity with an IC_{50} of 0.14 mg/mL, along with significant antimicrobial efficacy against various common pathogenic and spoilage bacteria and fungi. Analysis of the produced microbial oil indicated a high content of unsaturated fatty acids, with C18:1 as the primary component.

Chapter 11 evaluates the co-extraction of biotechnologically produced microbial oil and carotenoids from yeast biomass using SFE-CO₂. This investigation emphasizes the critical importance of employing the adequate cell disruption methods to improve process efficiency. High speed homogenization was proven to be the optimal method for disrupting red yeast cells. The use of ethanol as a co-solvent was also crucial for the efficient extraction of carotenoids. Cell disruption with high speed homogenization coupled with SFE-CO₂ resulted in substantial extraction yields, with recovery rates of 80% for carotenoids and 83% for microbial oil. Further evaluation of the extraction kinetics for both metabolites revealed that approximately 60% of carotenoids were recovered at the beginning of the extraction process, while an 80% yield of microbial oil was achieved after 60 min, indicating that this method can be promising also in terms of energy efficiency.

Finally, Chapter 12 focused on the development of novel food ingredients using the carotenoid-rich microbial oil through two different approaches; electrospinning and oleogelation. These techniques, also considered as encapsulation methods, were

evaluated for their effectiveness in encapsulating and protecting carotenoid-rich microbial oil. In the electrospinning process, the encapsulation matrix played a significant role on the encapsulation yield of carotenoids. The highest encapsulation efficiencies of 71.15% and 42.20%, for microbial oil and carotenoids, respectively, were achieved when the EPS fraction (produced by *Rhodospiridium paludigenum*) was incorporated in the encapsulation matrix. Additionally, oleogelation proved an effective method for the encapsulation of the carotenoid-rich microbial oil, protecting them from high-temperature thermal processing. Moreover, rheological analyses highlighted the strong gel properties and spreadability of the oleogel, underscoring its suitability for developing novel functional food products.

This thesis presents novel findings on the biotechnological production of valuable metabolites, with a particular emphasis on carotenoids. Additionally, it provides innovative insights into their encapsulation broadening their application in functional food systems. Future research could focus on the following topics:

- ❖ Investigation of the mechanism by which renewable resource-based media influence yeast metabolism, with an ultimate goal to sustainably produce specific carotenoids.
- ❖ Further optimization of the fermentation conditions on bioreactor scale in order to achieve higher concentrations of microbial metabolites from red yeast strains.
- ❖ Optimization of electrospinning process and further evaluation of the physical and functional properties of the electrosprayed particles.
- ❖ Development of novel food products using carotenoid-rich oleogels
- ❖ Evaluation of the encapsulated carotenoids for their bioavailability and bioaccessibility in the gastrointestinal tract.
- ❖ Evaluation of the toxicological effect of carotenoids to enhance their potential industrialization.

References

References

- Adbhai, A. R., Dewanjee, S., Patel, K. G., Karmakar, N. 2022. Sugar beet molasses production and utilization, in sugar beet cultivation, management and processing. Springer Nature Singapore. pp. 885-904. https://doi.org/10.1007/978-981-19-2730-0_44
- Aguilar, F., Autrup, H., Barlow, S., Castle, L., Crebelli, R., Engel, K., Gontard, N., Gott, D., Grilli, S., Gürtler, R., Larsen, C., Leclercq, C., Leblanc, J., Malcata, F.X., Mennes, W., Milana, M.R., Pratt, I., Rietjens, I., Tobback, P., Toldrá, F., 2008. Scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food adopted on 7 March 2008. EFSA J. 80, 1-29
- Ahangari, H., King, J.W., Ehsani, A., Yousefi, M., 2021. Supercritical fluid extraction of seed oils – A short review of current trends. Trends Food Sci. Technol. 111, 249-260. <https://doi.org/10.1016/j.tifs.2021.02.066>
- Aharoni, S., Lati, Y., Aviram, M., Fuhrman, B., 2015. Pomegranate juice polyphenols induce a phenotypic switch in macrophage polarization favoring a M2 anti-inflammatory state. BioFactors. 41(1), 44-51. <https://doi.org/10.1002/biof.1199>
- Aksu, Z., Tuğba Eren, A., 2005. Carotenoids production by the yeast *Rhodotorula mucilaginosa*: Use of agricultural wastes as a carbon source. Process Biochem. 40(9), 2985-2991. <https://doi.org/10.1016/j.procbio.2005.01.011>
- Alakraa, F., Saygün, A., Yeşilçubuk, N., 2020. Biotechnological production of lipids and carotenoids from *Rhodosporidium toruloides* Y27012. Eur. J. Sci. Technol. 156-164. <https://doi.org/10.31590/ejosat.708556>
- Alamgir, A.N.M., 2018. Biotechnology, in vitro production of natural bioactive compounds, herbal preparation, and disease management (treatment and prevention), progress in drug research. Springer Int. Publishing. 74, 585-664. https://doi.org/10.1007/978-3-319-92387-1_7

Alexandri, M., Kachrimanidou, V., Papapostolou, H., Papadaki, A., Kopsahelis, N., 2022. Sustainable food systems: The case of functional compounds towards the development of clean label food products. *Foods*. 11, 2796.

<https://doi.org/10.3390/foods11182796>

Ali, A., Rehman, A., Jafari, S.M., Ranjha, M.M.A.N., Shehzad, Q., Shahbaz, H.M., Khan, S., Usman, M., Kowalczewski, P.Ł., Jarzębski, M., Xia, W., 2022. Effect of co-encapsulated natural antioxidants with modified starch on the oxidative stability of β -carotene loaded within nanoemulsions. *Appl. Sci.* 12(3), 1070.

<https://doi.org/10.3390/12031070>

Allahkarami, S., Sepahi, A.A., Hosseini, H., Razavi, M.R., 2021. Isolation and identification of carotenoid-producing *Rhodotorula* sp. from Pinaceae forest ecosystems and optimization of in vitro carotenoid production. *Biotechnol. Reports*. 32, e00687. <https://doi.org/10.1016/j.btre.2021.e00687>

Ambrico, A., Larocca, V., Trupo, M., Martino, M., Magarelli, R.A., Spagnoletta, A., Balducchi, R., 2024. A new method for selective extraction of torularhodin from red yeast using CO₂-SFE technique. *Appl. Biochem. Biotechnol.*

<https://doi.org/10.1007/s12010-024-04884-9>

Anandharamakrishnan, C., Rielly, C.D., Stapley, A.G.F., 2010. Spray-freeze-drying of whey proteins at sub-atmospheric pressures. *Dairy Sci. Technol.* 90(2-3), 321-334. <https://doi.org/10.1051/dst/2010013>

Anticon, M., Blesa, J., Frigola, A., Esteve, M.J., 2020. High biological value compounds extraction from citruswaste with non-conventional methods. *Foods*. 9(6), 811. <https://doi.org/10.3390/foods9060811>

Antolak, H., Oracz, J., Otlewska, A., Zyzelewicz, D., Kregiel, D., 2017. Identification of carotenoids and isoprenoid quinones from *Asaia lannensis* and *Asaia bogorensis*. *Molecules*. 22(10), 1-17. <https://doi.org/10.3390/molecules22101608>

Ashokkumar, V., Flora, G., Sevanan, M., Sripriya, R., Chen, W.H., Park, J., Rajesh, J., Kumar, G., 2023. Technological advances in the production of carotenoids and their applications – A critical review. *Bioresour. Technol.* 367, 128215.

<https://doi.org/doi.org/10.1016/j.biortech.2022.128215>

Aswathanarayan, J.B., Vittal, R.R., 2019. Nanoemulsions and their potential applications in food industry. *Front. Sustain. Food Syst.* 3, 1-21.

<https://doi.org/10.3389/fsufs.2019.00095>

Bagade, S.B., Patil, M., 2021. Recent advances in microwave assisted extraction of bioactive compounds from complex herbal samples: A review. *Crit. Rev. Anal. Chem.* 51(2), 138-149. <https://doi.org/10.1080/10408347.2019.1686966>

Bai, S.K., Lee, S.J., Na, H.J., Ha, K.S., Han, J.A., Lee, H., Kwon, Y.G., Chung, C.K., Kim, Y.M., 2005. β -carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages by suppressing redox-based NF- κ B activation. *Exp. Mol. Med.* 37(4), 323-334. <https://doi.org/10.1038/emm.2005.42>

Baraki, S.Y., Jiang, Y., Li, X., Debeli, D.K., Wang, B., Feng, X., Mao, Z., Sui, X., 2021. Stable sunflower oil oleogel from oil/water pickering emulsion with regenerated chitin. *Lwt.* 146, 111483. <https://doi.org/10.1016/j.lwt.2021.111483>

Barth, A., 2007. Infrared spectroscopy of proteins. *Biochim. Biophys. Acta - Bioenerg.* 1767(9), 1073-1101. <https://doi.org/10.1016/j.bbabbio.2007.06.004>

Barzee, T.J., Cao, L., Pan, Z., Zhang, R., 2021. Fungi for future foods. *J. Futur. Foods* 1(1), 25-37. <https://doi.org/10.1016/j.jfutfo.2021.09.002>

Bertacchi, S., Bettiga, M., Porro, D., Branduardi, P., 2020. *Camelina sativa* meal hydrolysate as sustainable biomass for the production of carotenoids by *Rhodospiridium toruloides*. *Biotechnol. Biofuels.* 13(1), 47.

<https://doi.org/10.1186/s13068-020-01682-3>

Bhosale, P.B., Gadre, R. V., 2001. Production of β -carotene by a mutant of *Rhodotorula glutinis*. *Appl. Microbiol. Biotechnol.* 55(4), 423-427.

<https://doi.org/10.1007/s002530000570>

Bi, D., Li, M., Yao, L., Zhu, N., Fang, W., Guo, W., Wu, Y., Xu, H., Hu, Z., Xu, X., 2023. Enhancement of the chemical stability of nanoemulsions loaded with curcumin by unsaturated mannuronate oligosaccharide. Food Chem. 414, 1-9.

<https://doi.org/10.1016/j.foodchem.2023.135670>

Bocker, R., Silva, E.K., 2022. Pulsed electric field assisted extraction of natural food pigments and colorings from plant matrices. Food Chem. X. 15, 100398.

<https://doi.org/10.1016/j.fochx.2022.100398>

Bogacz-Radomska, L., Harasym, J., Piwowar, A., 2019. Commercialization aspects of carotenoids, in: Carotenoids: properties, processing and applications. Elsevier Inc. 327-357. <https://doi.org/10.1016/B978-0-12-817067-0.00010-5>

Böhm, V., 2017. Carotenoids as antioxidants: Possible health implications. Eurocaroten (CA15136), Scientific Newsletter. 4, 1-6.

Boonpratum, C., Naemchanthara, P., Limsuwan, P., Naemchanthara, K., 2022. Effects of chitosan and Tween 80 addition on the properties of nanofiber mat through the electrospinning. E-Polymers. 22(1), 234-248. <https://doi.org/10.1515/epoly-2022-0029>

Both, E.M., Boom, R.M., Schutyser, M.A.I., 2020. Particle morphology and powder properties during spray drying of maltodextrin and whey protein mixtures. Powder Technol. 363, 519-524. <https://doi.org/10.1016/j.powtec.2020.01.001>

Boukroufa, M., Boutekedjiret, C., Chemat, F., 2017. Development of a green procedure of citrus fruits waste processing to recover carotenoids. Resour. Technol. 3(3), 252-262. <https://doi.org/10.1016/j.reffit.2017.08.007>

Boviatsi, E., Papadaki, A., Efthymiou, M.N., Nychas, G.J.E., Papanikolaou, S., da Silva, J.A.C., Freire, D.M.G., Koutinas, A., 2020. Valorisation of sugarcane molasses for the production of microbial lipids via fermentation of two *Rhodospiridium* strains for enzymatic synthesis of polyol esters. J. Chem. Technol. Biotechnol. 95(2), 402-407. <https://doi.org/10.1002/jctb.5985>

Breierová, E., Čertík, M., Márová, I., Vadkertiová, R., 2018. The effect of Zn (II) ions and reactive oxygen on the uptake of zinc and production of carotenoids by selected red yeasts. Chem. Biodivers. 15(6), e1800069.

<https://doi.org/10.1002/cbdv.201800069>

Britton, G., 2020. Carotenoid research: History and new perspectives for chemistry in biological systems. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1865(11), 158699. <https://doi.org/10.1016/j.bbalip.2020.158699>

Britton, G., Jensen, S.L., Pfander, H., 2004. Carotenoid handbook. Birkhauser Verlag, Basel, Boston.

Bubalo, M.C., Vidović, S., Redovniković, I.R., Jokić, S., 2018. New perspective in extraction of plant biologically active compounds by green solvents. Food Bioprod. Process. 109, 52-73. <https://doi.org/10.1016/j.fbp.2018.03.001>

Bushway, R.J., 1986. Determination of α - and β -carotene in some raw fruits and vegetables by high-performance liquid chromatography. J. Agric. Food Chem. 34(3), 409-412. <https://doi.org/10.1021/jf00069a006>

Buzzini, P., Innocenti, M., Turchetti, B., Libkind, D., Van Broock, M., Mulinacci, N., 2007. Carotenoid profiles of yeasts belonging to the genera *Rhodotorula*, *Rhodospiridium*, *Sporobolomyces*, and *Sporidiobolus*. Can. J. Microbiol. 53(8), 1024-1031. <https://doi.org/10.1139/W07-068>

Byrtusová, D., Shapaval, V., Holub, J., Šimanský, S., Rapta, M., Szotkowski, M., Kohler, A., Márová, I., 2020. Revealing the potential of lipid and β -glucans coproduction in *Basidiomycetes* yeast. Microorganisms. 8(7), 1-19. <https://doi.org/10.3390/microorganisms8071034>

Byrtusová, D., Szotkowski, M., Kurowska, K., Shapaval, V., Márová, I., 2021. *Rhodotorula kratochvilovae* CCY 20-2-26- the source of multifunctional metabolites. Microorganisms. 9(6), 1280. <https://doi.org/10.3390/microorganisms9061280>

Cabezas-Terán, K., Grootaert, C., Ortiz, J., Donoso, S., Ruales, J., Van Bockstaele, F., Van Camp, J., Van de Wiele, T., 2023. In vitro bioaccessibility and uptake of β -carotene from encapsulated carotenoids from mango by-products in a coupled gastrointestinal digestion/Caco-2 cell model. Food Res. Int. 164, 112301.

<https://doi.org/10.1016/j.foodres.2022.112301>

Cardoso, L.A.C., Jäckel, S., Karp, S.G., Framboisier, X., Chevalot, I., Marc, I., 2016. Improvement of *Sporobolomyces ruberrimus* carotenoids production by the use of raw glycerol. Bioresour. Technol. 200, 374-379.

<https://doi.org/10.1016/j.biortech.2015.09.108>

Carfora, V., Cavallo, C., Catellani, P., Giudice, T.D, Cicia, G., 2021. Why do consumers intend to purchase natural food? Integrating theory of planned behavior, value-belief-norm theory, and trust. Nutrients. 13(6), 1904.

<https://doi.org/10.3390/nu13061904>

Cavaliere, A., Ventura, V., 2018. Mismatch between food sustainability and consumer acceptance toward innovation technologies among millennial students: The case of shelf life extension. J. Clean. Prod. 175, 641-650.

<https://doi.org/10.1016/j.jclepro.2017.12.087>

Cazetta, M.L., Celligoi, M.A.P.C., Buzato, J.B., Scarmino, I.S., 2007. Fermentation of molasses by *Zymomonas mobilis*: Effects of temperature and sugar concentration on ethanol production. Bioresour. Technol. 98(15), 2824-2828.

<https://doi.org/10.1016/j.biortech.2006.08.026>

Chaari, M., Theochari, I., Papadimitriou, V., Xenakis, A., Ammar, E., 2018. Encapsulation of carotenoids extracted from halophilic *Archaea* in oil-in-water (O/W) micro- and nano-emulsions. Coll. Surf. B Biointerfaces. 161, 219-227.

<https://doi.org/10.1016/j.colsurfb.2017.10.042>

Chalermthai, B., Giwa, A., Moheimani, N., Taher, H., 2022. Techno-economic strategies for improving economic viability of β -carotene extraction using natural oil and supercritical solvent: A comparative assessment. Algal Res. 68, 102875.

<https://doi.org/10.1016/j.algal.2022.102875>

Chang, H.I., Shao, C.W., Huang, E., Huang, K.Y., 2022. Development of astaxanthin-loaded nanosized liposomal formulation to improve bone health. *Pharmaceuticals*. 15(4), 490. <https://doi.org/10.3390/ph15040490>

Chaves, M.A., Filho, P.L.O., Jange, C.G., Sinigaglia-Coimbra, R., Oliveira, C.L.P., Pinho, S.C., 2018. Structural characterization of multilamellar liposomes coencapsulating curcumin and vitamin D₃. *Coll. Surf. A Physicochem. Eng. Asp.* 549, 112-121. <https://doi.org/10.1016/j.colsurfa.2018.04.018>

Chemat, F., Abert-Vian, M., Fabiano-Tixier, A.S., Strube, J., Uhlenbrock, L., Gunjevic, V., Cravotto, G., 2019. Green extraction of natural products. Origins, current status, and future challenges. *Trends Anal. Chem.* 118, 248-263. <https://doi.org/10.1016/j.trac.2019.05.037>

Chen, F., Wang, H., Lin, Z., Hu, J., Wu, Y., Shi, L., Wang, J., Xiu, Y., Lin, S., 2022. Enzymatic and non-enzymatic bioactive compounds, and antioxidant and antimicrobial activities of the extract from one selected wild berry (*Rubus coreanus*) as novel natural agent for food preservation. *Lwt.* 171, 114133. <https://doi.org/10.1016/j.lwt.2022.114133>

Chen, K., Chou, W., Liu, L., Cui, Y., Xue, P., Jia, M., 2019. Electrochemical sensors fabricated by electrospinning technology: An overview. *Sensors*. 19, 3676. [doi:10.3390/s19173676](https://doi.org/10.3390/s19173676)

Chen, L., Xiang, M., Wu, F., Jiang, Y., Wu, Q., Zhang, W., Guo, W., Cai, B., Liang, L., Li, S., Chen, Y., Du, X., 2023. Encapsulation of lycopene into electrospun nanofibers from whey protein isolate-*Tricholoma lobayense* polysaccharide complex stabilized emulsions: Structural characterization, storage stability, in vitro release, and cellular evaluation. *Int. J. Biol. Macromol.* 238, 123993. <https://doi.org/10.1016/j.ijbiomac.2023.123993>

Chen, X.W., Fu, S.Y., Hou, J.J., Guo, J., Wang, J.M., Yang, X.Q., 2016. Zein based oil-in-glycerol emulgels enriched with β -carotene as margarine alternatives. *Food Chem.* 211, 836-844. <https://doi.org/10.1016/j.foodchem.2016.05.133>

Cheng, J., Balbuena, E., Miller, B., Eroglu, A., 2021. The role of β -carotene in colonic inflammation and intestinal barrier integrity. *Front. Nutr.* 8, 1-13. <https://doi.org/10.3389/fnut.2021.723480>

Cheng, Y.T., Yang, C.F., 2016. Using strain *Rhodotorula mucilaginosa* to produce carotenoids using food wastes. *J. Taiwan Inst. Chem. Eng.* 61, 270-275. <https://doi.org/10.1016/j.jtice.2015.12.027>

Cho, D.H., Chae, H.J., Kim, E.Y., 2001. Synthesis and characterization of a novel extracellular polysaccharide by *Rhodotorula glutinis*. *Appl. Biochem. Biotechnol.* 95, 183-193. <https://doi.org/10.1385/ABAB:95:3:183>

Choudhari, S.M., Ananthanarayan, L., Singhal, R.S., 2008. Use of metabolic stimulators and inhibitors for enhanced production of β -carotene and lycopene by *Blakeslea trispora* NRRL 2895 and 2896. *Bioresour. Technol.* 99(8), 3166-3173. <https://doi.org/10.1016/j.biortech.2007.05.051>

Cipolatti, E.P., Remedi, R.D., dos Santos Sá, C., Rodrigues, A.B., Ramos, J.M.G., Burkert, C.A.V., Furlong, E.B., de Medeiros Burkert, J.F., 2019. Use of agroindustrial byproducts as substrate for production of carotenoids with antioxidant potential by wild yeasts. *Biocatal. Agric. Biotechnol.* 20, 101208. <https://doi.org/10.1016/j.bcab.2019.101208>

Coelho, T.L.S., Silva, D.S.N., dos Santos Junior, J.M., Dantas, C., de Araujo Nogueira, A.R., Júnior, C.A.L., Vieira, E.C., 2022. Multivariate optimization and comparison between conventional extraction (CE) and ultrasonic-assisted extraction (UAE) of carotenoid extraction from cashew apple. *Ultrason. Sonochem.* 84, 105980. <https://doi.org/10.1016/j.ultsonch.2022.105980>

Coniglio, S., Shumskaya, M., Vassiliou, E., 2023. Unsaturated fatty acids and their immunomodulatory properties. *Biology.* 12(2), 279. <https://doi.org/10.3390/biology12020279>

Correia-Filho, L.C., Lourenço, M.M., Moldão-Martins, M., Alves, V.D., 2019. Microencapsulation of β -carotene by spray drying: Effect of wall material concentration and drying inlet temperature. *Int. J. Food Sci.* 2019.

<https://doi.org/10.1155/2019/8914852>

Correia-Filho, L.C., Santos, D.I., Brito, L., Moldão-Martins, M., Alves, V.D., 2022. Storage stability and in vitro bioaccessibility of microencapsulated tomato (*Solanum Lycopersicum L.*) pomace extract. *Bioengineering*. 9(7), 311.

<https://doi.org/10.3390/bioengineering9070311>

Couto, R.M., Simões, P.C., Reis, A., Da Silva, T.L., Martins, V.H., Sánchez-Vicente, Y., 2010. Supercritical fluid extraction of lipids from the heterotrophic microalga *Cryptothecodinium cohnii*. *Eng. Life Sci.* 10(2), 158-164.

<https://doi.org/10.1002/elsc.200900074>

Custom Market Insights, 2023. Global Carotenoid Market.

<https://www.gminsights.com/industry-analysis/lycopene-market>

da Costa, W.A., de Araújo Padilha, C.E., de Oliveira Júnior, S.D., da Silva, F.L.H., Silva, J., Ancântara, M.A., Ferrari, M., dos Santos, E.S., 2020. Oil-lipids, carotenoids and fatty acids simultaneous production by *Rhodotorula mucilaginosa* CCT 3892 using sugarcane molasses as carbon source. *Brazilian J. Food Technol.* 23, 1-11.

<https://doi.org/10.1590/1981-6723.06419>

de Andrade Lima, M., Kestekoglou, I., Charalampopoulos, D., Chatzifragkou, A., 2019. Supercritical fluid extraction of carotenoids from vegetable waste matrices. *Molecules*. 24(3), 466. <https://doi.org/10.3390/molecules24030466>

De Baets, S., Du Laing, S., Vandamme, E., 2002. Yeast extracellular polysaccharides. In “Biopolymers Series”: (Vol 6), Polysaccharides from Eukaryotes.

de Lima, P.M., Dacanal, G.C., Pinho, L.S., de Sá, S.H.G., Thomazini, M., Favaro-Trindade, C.S., 2022. Combination of spray-chilling and spray-drying techniques to protect carotenoid-rich extracts from pumpkin (*Cucurbita moschata*) byproducts, aiming at the production of a powdered natural food dye. *Molecules*. 27(21), 7530. <https://doi.org/10.3390/molecules27217530>

De Oliveira Meira, A.C.F., De Moraes, L.C., De Abreu Figueiredo, J., Veríssimo, L.A.A., Botrel, D.A., De Resende, J.V., 2023. Microencapsulation of β -carotene using barley residue proteins from beer waste as coating material. *J. Microencapsul.* 40(3), 171-185. <https://doi.org/10.1080/02652048.2023.2183277>

de Oliveira, G.L.R., Medeiros, I., da Cruz Nascimento, S.S., Viana, R.L.S., Porto, D.L., Rocha, H.A.O., Aragão, C.F.S., Maciel, B.L.L., de Assis, C.F., de Araújo Moraes, A.H., Passos, T.S., 2021. Antioxidant stability enhancement of carotenoid rich-extract from cantaloupe melon (*Cucumis melo* L.) nanoencapsulated in gelatin under different storage conditions. *Food Chem.* 348, 129055. <https://doi.org/10.1016/j.foodchem.2021.129055>

de Queiroz, J.L.C., Medeiros, I., Trajano, A.C., Piuvezam, G., de França Nunes, A.C., Passos, T.S., de Araújo Moraes, A.H., 2022. Encapsulation techniques perfect the antioxidant action of carotenoids: A systematic review of how this effect is promoted. *Food Chem.* 385, 132593. <https://doi.org/10.1016/j.foodchem.2022.132593>

del Pilar Sánchez-Camargo, A., Gutiérrez, L.F., Vargas, S.M., Martínez-Correa, H.A., Parada-Alfonso, F., Narváez-Cuenca, C.E., 2019. Valorisation of mango peel: Proximate composition, supercritical fluid extraction of carotenoids, and application as an antioxidant additive for an edible oil. *J. Supercrit. Fluids*. 152, 104574. <https://doi.org/10.1016/j.supflu.2019.104574>

Demirci, M., Caglar, M.Y., Cakir, B., Gülseren, I., 2017. Encapsulation by nanoliposomes. *Nanoencapsulation Technol. Food Nutraceutical Ind.* 74-113. <https://doi.org/10.1016/B978-0-12-809436-5.00003-3>

Dias, C., Silva, C., Freitas, C., Reis, A., da Silva, T.L., 2016. Effect of medium pH on *Rhodospiridium toruloides* NCYC 921 carotenoid and lipid production evaluated by flow cytometry. Appl. Biochem. Biotechnol. 179, 776-787.

<https://doi.org/10.1007/s12010-016-2030-y>

Dias, C., Sousa, S., Caldeira, J., Reis, A., da Silva, T.L., 2015. New dual-stage pH control fed-batch cultivation strategy for the improvement of lipids and carotenoids production by the red yeast *Rhodospiridium toruloides* NCYC 921. Bioresour. Technol. 189, 309-318. <https://doi.org/10.1016/j.biortech.2015.04.009>

Dias, M.I., Ferreira, I.C.F.R., Barreiro, M.F., 2015. Microencapsulation of bioactives for food applications. Food Funct. 6(4), 1035-2052.

<https://doi.org/10.1039/c4fo01175a>

dos Passos Ramos, S., da Trindade, L.G., Mazzo, T.M., Longo, E., Bonsanto, F.P., de Rosso, V.V., Braga, A.R.C., 2022. Electrospinning composites as carriers of natural pigment: Screening of polymeric blends. Processes. 10(12), 2737.

<https://doi.org/10.3390/pr10122737>

dos Santos, R.R., Kunigami, C.N., Gomes Aranda, D.A., Lapa Teixeira, C.M.L., 2016. Assessment of triacylglycerol content in *Chlorella vulgaris* cultivated in a two-stage process. Biomass and Bioenergy. 92, 55-60.

<https://doi.org/10.1016/j.biombioe.2016.05.014>

Drosou, C., Krokida, M., Biliaderis, C.G., 2022. Encapsulation of β -carotene into food-grade nanofibers via coaxial electrospinning of hydrocolloids: Enhancement of oxidative stability and photoprotection. Food Hydrocoll. 133, 107949.

<https://doi.org/10.1016/j.foodhyd.2022.107949>

Du, Y., Bao, C., Huang, J., Jiang, P., Jiao, L., Ren, F., Li, Y., 2019. Improved stability, epithelial permeability and cellular antioxidant activity of β -carotene via encapsulation by self-assembled α -lactalbumin micelles. Food Chem. 271, 707-714.

<https://doi.org/10.1016/j.foodchem.2018.07.216>

Dutta, D., Dutta, D., 2024. Designing a nanoliposome loaded with provitamin A xanthophyll β -cryptoxanthin from *K. marina* DAGII to target a population suffering from hypovitaminosis A. *Process Biochem.* 138, 97-100.

<https://doi.org/10.1016/j.procbio.2024.01.003>

Dutta, R.S., Elhassan, G.O., Devi, T.B., Bhattacharjee, B., Singh, M., Jana, B.K., Sahu, S., Mazumder, B., Sahu, R.K., Khan, J., 2024. Enhanced efficacy of β -carotene loaded solid lipid nanoparticles optimized and developed via central composite design on breast cancer cell lines. *Heliyon.* 10(7), e28457.

<https://doi.org/10.1016/j.heliyon.2024.e28457>

Dyaa, A., Soliman, H., Abdelrazak, A., Samra, B.N., Khojah, E., Ahmed, A.F., El-Esawi, M.A., Elsayed, A., 2022. Optimization of carotenoids production from *Rhodotorula sp.* strain ATL72 for enhancing its biotechnological applications. *J. Fungi.* 8(2), 160. <https://doi.org/10.3390/jof8020160>

El-Banna, A.A., El-Razek, A.M.A., El-Mahdy, A.R., 2012. Some factors affecting the production of carotenoids by *Rhodotorula glutinis* var. *glutinis*. *Food Nutr. Sci.* 3(1), 64-71. <https://doi.org/10.4236/fns.2012.31011>

Elfeky, N., Elmahmoudy, M., Bao, Y., 2020. Manipulation of culture conditions: Tool for correlating/improving lipid and carotenoid production by *Rhodotorula glutinis*. *Processes.* 8(2), 140. <https://doi.org/10.3390/pr8020140>

Elfeky, N., Elmahmoudy, M., Zhang, Y., Guo, J., Bao, Y., 2019. Lipid and carotenoid production by *Rhodotorula glutinis* with a combined cultivation mode of nitrogen, sulfur, and aluminium stress. *Appl. Sci.* 9(12), 2444.

<https://doi.org/10.3390/APP9122444>

Elik, A., Yanık, D.K., Göğüş, F., 2020. Microwave-assisted extraction of carotenoids from carrot juice processing waste using flaxseed oil as a solvent. *Lwt.* 123, 109100. <https://doi.org/10.1016/j.lwt.2020.109100>

Elkholy, N.S., Hariri, M.L.M., Mohammed, H.S., Shafaa, M.W., 2023. Lutein and β -carotene characterization in free and nanodispersion forms in terms of antioxidant activity and cytotoxicity. *J. Pharm. Innov.* 18(4), 1727-1744.

<https://doi.org/10.1007/s12247-023-09745-2>

Elsanhoty, R.M., Al-Turki, A.I., El-Razik, M.M.A., 2017. Production of carotenoids from *Rhodotorula mucilaginosa* and their applications as colorant agent in sweet candy. *J. Food, Agric. Environ.* 15(2), 21-26.

Erickson, B., Nelson, J.E., Winters, P., 2012. Perspective on opportunities in industrial biotechnology in renewable chemicals. *Biotechnol. J.* 7(2), 176-185.

<https://doi.org/10.1002/biot.201100069>

Eroglu, A., Harrison, E.H., 2013. Carotenoid metabolism in mammals, including man: Formation, occurrence, and function of apocarotenoids. *J. Lipid Res.* 54(7), 1719-1730. <https://doi.org/10.1194/jlr.R039537>

Espert, M., Hernández, M.J., Sanz, T., Salvador, A., 2022. Rheological properties of emulsion templated oleogels based on xanthan gum and different structuring agents. *Curr. Res. Food Sci.* 5, 564-570. <https://doi.org/10.1016/j.crfs.2022.03.001>

Esposito, B.S., Pinho, S.G.B., Thomazini, M., Ramos, A.P., Tapia-Blácido, D.R., Martelli-Tosi, M., 2022. TPP-chitosomes as potential encapsulation system to protect carotenoid-rich extract obtained from carrot by-product: A comparison with liposomes and chitosomes. *Food Chem.* 397, 133857.

<https://doi.org/10.1016/j.foodchem.2022.133857>

European Commission, 2019. Commission Implementing Decision (EU). Off. J. Eur. Union.

European Commission, 2021. Functional Foods. Publication Office of the EU.

European Medicines Agency, 2019. ICH guideline Q3C (R6) on impurities: Guideline for Residual Solvents.

Fact.MR, 2023. Carotenoid Market <https://www.factmr.com/report/1196/carotenoids-market>

Falsafi, S.R., Rostamabadi, H., Nishinari, K., Amani, R., Jafari, S.M., 2022. The role of emulsification strategy on the electrospinning of β -carotene-loaded emulsions stabilized by gum Arabic and whey protein isolate. Food Chem. 374, 131826. <https://doi.org/10.1016/j.foodchem.2021.131826>

Fan, Y., Meng, H.M., Hu, G.R., Li, F.L., 2018. Biosynthesis of nervonic acid and perspectives for its production by microalgae and other microorganisms. Appl. Microbiol. Biotechnol. 102(7), 3027-3035. <https://doi.org/10.1007/s00253-018-8859-y>

Fărcaș, A.C., Socaci, S.A., Nemeș, S.A., Salanță, L.C., Chiș, M.S., Pop, C.R., Borșa, A., Diaconeasa, Z., Vodnar, D.C., 2022. Cereal waste valorization through conventional and current extraction techniques- An up-to-date overview. Foods. 11(16), 2454. <https://doi.org/10.3390/foods11162454>

Farruggia, C., Kim, M.B., Bae, M., Lee, Y., Pham, T.X., Yang, Y., Han, M.J., Park, Y.K., Lee, J.Y., 2018. Astaxanthin exerts anti-inflammatory and antioxidant effects in macrophages in NRF2-dependent and independent manners. J. Nutr. Biochem. 62, 202-209. <https://doi.org/10.1016/j.jnutbio.2018.09.005>

Ferreira, A.S., Mendonça, I., Póvoa, I., Carvalho, H., Correia, A., Vilanova, M., Silva, T.H., Coimbra, M.A., Nunes, C., 2021. Impact of growth medium salinity on galactoxylan exopolysaccharides of *Porphyridium purpureum*. Algal Res. 59, 102439. <https://doi.org/10.1016/j.algal.2021.102439>

Ferreira, J., Braga, M.Q., da Gama, R.C.N., Magalhães, I.B., Marangon, B.B., de Siqueira Castro, J., Lorentz, J.F., Henriques, B.S., de Paula Pereira, Paula, A.S.A., Assemany, P.P., Calijuri, M.L., 2024. Carotenoids from wastewater-grown microalgae biomass: Life cycle assessment and techno-economical analysis. J. Clean. Prod. 434, 140526. <https://doi.org/10.1016/j.jclepro.2023.140526>

Ferreira, L.M., de Arce Velasquez, A., Schaffazick, S.R., Cruz, L., 2015. Pullulan: An advantageous natural polysaccharide excipient to formulate tablets of alendronate-loaded microparticles. *Brazilian J. Pharm. Sci.* 51(1), 27-34.

<https://doi.org/10.1590/S1984-82502015000100003>

Fidan, O., Zhan, J., 2019. Discovery and engineering of an endophytic *Pseudomonas* strain from *Taxus chinensis* for efficient production of zeaxanthin diglucoside. *J. Biol. Eng.* 13(1), 1-18. <https://doi.org/10.1186/s13036-019-0196-x>

Filippousi, R., Diamantopoulou, P., Stavropoulou, M., Makris, D. P., Papanikolaou, S. 2022. Lipid production by *Rhodospiridium toruloides* from biodiesel-derived glycerol in shake flasks and bioreactor: Impact of initial C/N molar ratio and added onion-peel extract. *Process Biochem.* 123, 52-62.

<https://doi.org/10.1016/j.procbio.2022.10.008>

Folch, J., Lees, M., Stanley, G.H.S., 1987. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 55, 999-1033.

Foo, S.C., Yusoff, F.M., Khong, N.M.H., 2024. Storage and degradation kinetics of physicochemical and bioactive attributes in microalgal-derived fucoxanthin-rich microcapsules. *J. Agric. Food Res.* 15, 100823.

<https://doi.org/10.1016/j.jafr.2023.100823>

Freitas, C., Parreira, T.M., Roseiro, J., Reis, A., da Silva, T.L., 2014. Selecting low-cost carbon sources for carotenoid and lipid production by the pink yeast *Rhodospiridium toruloides* NCYC 921 using flow cytometry. *Bioresour. Technol.* 158, 355-359. <https://doi.org/10.1016/j.biortech.2014.02.071>

Frengova, G.I., Simova, E.D., Beshkova, D.M., 2006. β -carotene-rich carotenoid-protein preparation and exopolysaccharide production by *Rhodotorula rubra* GED8 grown with a yogurt starter culture. *Zeitschrift für Naturforsch. C.* 61(7-8), 571-577. <https://doi.org/10.1515/ZNC-2006-7-817>

FSIS, 2020. FSIS Compliance Guideline for Label. Approval July 2020. 1-28.

Gayathri, S., Radhika, S.R.R., Suman, T.Y., Aranganathan, L., 2018. Ultrasound-assisted microextraction of β , ϵ -carotene-3, 3'-diol (lutein) from marine microalgae *Chlorella salina*: Effect of different extraction parameters. *Biomass Convers. Biorefinery*. 8(4), 791-797. <https://doi.org/10.1007/s13399-018-0331-9>

Geow, C.H., Tan, M.C., Yeap, S.P., Chin, N.L., 2021. A review on extraction techniques and its future applications in industry. *Eur. J. Lipid Sci. Technol.* 123(4), 1-10. <https://doi.org/10.1002/ejlt.202000302>

Gervasi, T., Pellizzeri, V., Benameur, Q., Gervasi, C., Santini, A., Cicero, N., Dugo, G., 2018. Valorization of raw materials from agricultural industry for astaxanthin and β -carotene production by *Xanthophyllomyces dendrorhous*. *Nat. Prod. Res.* 32(13), 1554-1561. <https://doi.org/10.1080/14786419.2017.1385024>

Ghazala, I., Bouassida, M., Krichen, F., Benito, J.M., Ellouz-Chaabouni, S., Haddar, A., 2017. Anionic lipopeptides from *Bacillus mojavensis* I4 as effective antihypertensive agents: Production, characterization, and identification. *Eng. Life Sci.* 17(12), 1244-1253. <https://doi.org/10.1002/elsc.201700020>

Ghilardi, C., Negrete, P.S., Carelli, A.A., Borroni, V., 2020. Evaluation of olive mill waste as substrate for carotenoid production by *Rhodotorula mucilaginosa*. *Bioresour. Bioprocess.* 7(1), 52. <https://doi.org/10.1186/s40643-020-00341-7>

Gientka, I., Blazejak, S., Stasiak-Rózanska, L., Chlebowska-Smigiel, A., 2015. Exopolysaccharides from yeast: Insight into optimal conditions for biosynthesis, chemical composition and functional properties - review. *Acta Sci. Pol. Technol. Aliment.* 14, 283–292. <https://doi.org/10.17306/J.AFS.2015.4.29>

Gientka, I., Wirkowska-Wojdyła, M., Ostrowska-Ligęza, E., Janowicz, M., Reczek, L., Synowiec, A., Błażej, S., 2022. Enhancing red yeast biomass yield and lipid biosynthesis by using waste nitrogen source by glucose fed-batch at low temperature. *Microorganisms*. 10(6), 1253. <https://doi.org/10.3390/microorganisms10061253>

Global Market Insights, 2023a. Beta-carotene Market Size. <https://www.gminsights.com/industry-analysis/beta-carotene-market>

Global Market Insights, 2023b. Carotenoids Market.

<https://www.gminsights.com/industry-analysis/carotenoids-market>

Gomes, A., Costa, A.L.R., do Amaral Sobral, P.J., Cunha, R.L., 2023. Delivering β -carotene from O/W emulsion-based systems: Influence of phase ratio and carrier lipid composition. Food Hydrocoll. Heal. 3, 100125.

<https://doi.org/10.1016/j.fhfh.2023.100125>

Gong, X., Smith, J.R., Swanson, H.M., Rubin, L.P., 2018. Carotenoid lutein selectively inhibits breast cancer cell growth and potentiates the effect of chemotherapeutic agents through ROS-mediated mechanisms. Molecules. 23(4), 1-18.

<https://doi.org/10.3390/molecules23040905>

Goodwin, T.W., 1965. Chemistry and biochemistry of plant pigments. Academic Press.

Gopi, S., Amalraj, A., Jacob, J., Kalarikkal, N., Thomas, S., Guo, Q., 2018. Preparation, characterization and in vitro study of liposomal curcumin powder by cost effective nanofiber weaving technology. New J. Chem. 42(7), 5117-5127.

<https://doi.org/10.1039/c7nj05029a>

Gordillo Sierra, A.R., Alper, H.S., 2020. Progress in the metabolic engineering of bio-based lactams and their ω -amino acids precursors. Biotechnol. Adv. 43, 107587.

<https://doi.org/10.1016/j.biotechadv.2020.107587>

Gorrini, C., Harris, I.S., Mak, T.W., 2013. Modulation of oxidative stress as an anticancer strategy. Nat. Rev. Drug Discov. 12(12), 931-947.

<https://doi.org/10.1038/nrd4002>

Gorte, O., Nazarova, N., Papachristou, I., Wüstner, R., Leber, K., Syldatk, C., Ochsenreither, K., Frey, W., Silve, A., 2020. Pulsed electric field treatment promotes lipid extraction on fresh oleaginous yeast *Saitozyma podzolica* DSM 27192. Front. Bioeng. Biotechnol. 8, 1-14. <https://doi.org/10.3389/fbioe.2020.575379>

Gosalawit, C., Imsoonthornruksa, S., Gilroyed, B.H., Mcnea, L., Boontawan, A., Ketudat-Cairns, M., 2021. The potential of the oleaginous yeast *Rhodotorula paludigena* CM33 to produce biolipids. J. Biotechnol. 329, 56-64.
<https://doi.org/10.1016/j.jbiotec.2021.01.021>

Grand View Research, 2023. Functional Food Market Size.
<https://www.grandviewresearch.com/industry-analysis/functional-food-market>

Grand View Research, 2023a. Carotenoids market size, share and growth report, 2030. <https://www.grandviewresearch.com/industry-analysis/carotenoids-market>

Griep, P., da Silva, L.M., Tres, B.P., Colet, R., Junges, A., Steffens, C., Zeni, J., Valduga, E., 2023. Effects of encapsulating agents and temperature in the microencapsulation of carotenogenic extracts from *Sporidiobolus Salmonicolor* CBS 2636 and storage stability. Biointerface Res. Appl. Chem. 13(5), 449.
<https://doi.org/10.33263/BRIAC135.449>

Griffin, W.C., 1954. Calculation of HLB values of non-ionic surfactants.

Grigorova, D., Pavlova, K., Panchev, I., 1999. Preparation and preliminary characterization of exopolysaccharides by yeast *Rhodotorula acheniorum* MC. Appl. Biochem. Biotechnol. 81(3), 181-191. <https://doi.org/10.1385/abab:81:3:181>

Gul, K., Tak, A., Singh, A.K., Singh, P., Yousuf, B., Wani, A.A., 2015. Chemistry, encapsulation, and health benefits of β -carotene - A review. Cogent Food Agric. 1(1), 1018696. <https://doi.org/10.1080/23311932.2015.1018696>

Gullón, P., Gullón, B., Román, A., Rocchetti, G., Lorenzo, J.M., 2020. Smart advanced solvents for bioactive compounds recovery from agri-food by-products: A review. Trends Food Sci. Technol. 101, 182-197.
<https://doi.org/10.1016/j.tifs.2020.05.007>

Guo, Q., Bayram, I., Shu, X., Su, J., Liao, W., Wang, Y., Gao, Y., 2022. Improvement of stability and bioaccessibility of β -carotene by curcumin in pea protein isolate-based complexes-stabilized emulsions: Effect of protein complexation by pectin and small molecular surfactants. *Food Chem.* 367(17), 130726.

<https://doi.org/10.1016/j.foodchem.2021.130726>

Hamadou, A.H, Huang, W.C., Xue, C., Mao, X., 2020. Comparison of β -carotene loaded marine and egg phospholipids nanoliposomes. *J. Food Eng.* 283, 110055.

<https://doi.org/10.1016/j.jfoodeng.2020.110055>

Hamidi, M., Gholipour, A.R., Delattre, C., Sedighi, F., Seveiri, R.M., Pasdaran, A., Kheirandish, S., Pierre, G., Kozani, P.S., Kozani, P.S., Karimitabar, F., 2020. Production, characterization and biological activities of exopolysaccharides from a new cold-adapted yeast: *Rhodotorula mucilaginosa* sp. GUMS16. *Int. J. Biol. Macromol.* 151, 268-277. <https://doi.org/10.1016/j.ijbiomac.2020.02.206>

Han, G., Duan, X., Jiang, B., Li, Y., Li, B., Yang, J., Pan, S., Liu, F., 2023. Emulsifying properties, in vitro digestive characteristics, and β -carotene bioaccessibility of mandarin peel pectin emulsions prepared with different carrier oil phases. *Int. J. Biol. Macromol.* 242, 124961.

<https://doi.org/10.1016/j.ijbiomac.2023.124961>

Han, H., Lim, J.W., Kim, H., 2019. Lycopene inhibits activation of epidermal growth factor receptor and expression of cyclooxygenase-2 in gastric cancer cells. *Nutrients.* 11(9), 2113. <https://doi.org/10.3390/nu11092113>

Han, X., Zhao, R., Zhang, G., Jiao, Y., Wang, Y., Wang, D., Cai, H., 2022. Association of retinol and carotenoids content in diet and serum with risk for colorectal cancer: A meta-analysis. *Front. Nutr.* 9, 918777.

<https://doi.org/10.3389/fnut.2022.918777>

Hartmann, C., Siegrist, M., 2017. Consumer perception and behaviour regarding sustainable protein consumption: A systematic review. *Trends Food Sci. Technol.* 61, 11-25. <https://doi.org/10.1016/j.tifs.2016.12.006>

Hasan, M., Azhar, M., Nangia, H., Bhatt, P.C., Panda, B.P., 2015. Influence of high-pressure homogenization, ultrasonication, and supercritical fluid on free astaxanthin extraction from β -glucanase treated *Phaffia rhodozyma* cells. Prep. Biochem. Biotechnol. 46(2), 116-122. <https://doi.org/10.1080/10826068.2014.995807>

Hazewindus, M., Haenen, G.R.M.M., Weseler, A.R., Bast, A., 2012. The anti-inflammatory effect of lycopene complements the antioxidant action of ascorbic acid and α -tocopherol. Food Chem. 132(2), 954-958. <https://doi.org/10.1016/j.foodchem.2011.11.075>

Hernández-Almanza, A., Montañez-Sáenz, J., Martínez-Ávila, C., Rodríguez-Herrera, R., Aguilar, C.N., 2014. Carotenoid production by *Rhodotorula glutinis* YB-252 in solid-state fermentation. Food Biosci. 7, 31-36. <https://doi.org/10.1016/j.fbio.2014.04.001>

Hidalgo, T., Giménez-Marqués, M., Bellido, E., Avila, J., Asensio, M.C., Salles, F., Lozano, M. V., Guillevic, M., Simón-Vázquez, R., González-Fernández, A., Serre, C., Alonso, M.J., Horcajada, P., 2017. Chitosan-coated mesoporous MIL-100(Fe) nanoparticles as improved bio-compatible oral nanocarriers. Sci. Rep. 7, 1-14. <https://doi.org/10.1038/srep43099>

Himanath, G., Shruthy, R., Preetha, R., Sreejit, V., 2021. Nanoemulsion with coconut oil and soy lecithin as a stable delivery system for lycopene and its incorporation into yogurt to enhance antioxidant properties and maintain quality. ACS Food Sci. Technol. 1(9), 1538-1549. <https://doi.org/10.1021/acsfoodscitech.1c00117>

Hong, J., Park, S.H., Kim, S., Kim, S.W., Hahn, J.S., 2019. Efficient production of lycopene in *Saccharomyces cerevisiae* by enzyme engineering and increasing membrane flexibility and NADPH production. Appl. Microbiol. Biotechnol. 103(1), 211-223. <https://doi.org/10.1007/s00253-018-9449-8>

Hong, T., Yin, J.Y., Nie, S.P., Xie, M.Y., 2021. Applications of infrared spectroscopy in polysaccharide structural analysis: Progress, challenge and perspective. Food Chem. X. 12, 100168. <https://doi.org/10.1016/j.fochx.2021.100168>

- Hoondede, P., Phuengjayaem, S., Kingkaew, E., Rojsitthisak, P., Sritularak, B., Thompho, S., Pornputtpong, N., Thitikornpong, W., Tanasupawat, S., 2024. Comparative genomic analysis and optimization of astaxanthin production of *Rhodotorula paludigena* TL35-5 and *Rhodotorula sampaioana* PL61-2. PLoS One. 19(7), 1-22. <https://doi.org/10.1371/journal.pone.0304699>
- Horuz, T.İ., Belibağlı, K.B., 2019. Nanoencapsulation of carotenoids extracted from tomato peels into zein fibers by electrospinning. J. Sci. Food Agric. 99(2), 759-766. <https://doi.org/10.1002/jsfa.9244>
- Hu, B., Zheng, Q., Weng, Z., Xiao, J., Cao, Y., Lan, Y., 2022. Non-isothermal crystallization kinetics study of multi-component oleogels. Food Chem. 389, 133123. <https://doi.org/10.1016/j.foodchem.2022.133123>
- Hua, X., Xu, S., Wang, M., Chen, Y., Yang, H., Yang, R., 2017. Effects of high-speed homogenization and high-pressure homogenization on structure of tomato residue fibers. Food Chem. 232, 443-449. <https://doi.org/10.1016/j.foodchem.2017.04.003>
- Human, C., De Beer, D., Van Der Rijst, M., Aucamp, M., Joubert, E., 2019. Electrospraying as a suitable method for nanoencapsulation of the hydrophilic bioactive dihydrochalcone, aspalathin. Food Chem. 276, 467-474. <https://doi.org/10.1016/j.foodchem.2018.10.016>
- Iacobazzi, V., Infantino, V., 2014. Citrate-new functions for an old metabolite. Biol. Chem. 395(4), 387-399. <https://doi.org/10.1515/hsz-2013-0271>
- Ielchishcheva, I., Stachowiak, B., Szwengiel, A., Bozhkov, A., 2018. Growth and carotenogenesis in *Rhodospiridium diobovatum* IMB Y-5023: Effects of culture medium and illumination intensity. FEMS Microbiol. Lett. 365(1), 1–8. <https://doi.org/10.1093/femsle/fnx261>
- Igreja, W.S., de Andrade Maia, F., Lopes, A.S., Chisté, R.C., 2021. Biotechnological production of carotenoids using low cost-substrates is influenced by cultivation parameters: A review. Int. J. Mol. Sci. 22(16), 8819. <https://doi.org/10.3390/ijms22168819>

İnan-Çinkır, N., Ağcam, E., Altay, F., Akyıldız, A., 2023. Emulsion electrospinning of zein or gelatin-pectin with carotenoid from watermelon. *Food Chem. Adv.* 3, 100346. <https://doi.org/10.1016/j.focha.2023.100346>

Ishika, T., Moheimani, N.R., Bahri, P.A., Laird, D.W., Blair, S., Parlevliet, D., 2017. Halo-adapted microalgae for fucoxanthin production: Effect of incremental increase in salinity. *Algal Res.* 28, 66-73. <https://doi.org/10.1016/j.algal.2017.10.002>

Iurkov, A.M., Vustin, M.M., Tiaglov, B. V., Maksimova, I.A., Sineokiy, S.P., 2008. Pigmented basidiomycete yeasts are a promising source of carotenoids and ubiquinone Q₁₀. *Microbiology.* 77(1), 5-10. <https://doi.org/10.1134/s0026261708010013>

Jain, A., Sharma, G., Thakur, K., Raza, K., Shivhare, U.S., Ghoshal, G., Katare, O.P., 2019. Beta-carotene-encapsulated solid lipid nanoparticles (BC-SLNs) as promising vehicle for cancer: An investigative assessment. *AAPS PharmSciTech.* 20(3), 1-7. <https://doi.org/10.1208/s12249-019-1301-7>

Jamir, L., Kumar, V., Kaur, J., Kumar, S., Singh, H., 2021. Composition, valorization and therapeutical potential of molasses: a critical review. *Environ. Technol. Rev.* 10(1), 131-142. <https://doi.org/10.1080/21622515.2021.1892203>

Janiszewska-Turak, E., 2017. Carotenoids microencapsulation by spray drying method and supercritical micronization. *Food Res. Int.* 99, 891-901. <https://doi.org/10.1016/j.foodres.2017.02.001>

Ji, Y., Wang, Z., Ju, X., Deng, F., Yang, F., He, R., 2023. Co-encapsulation of rutinoid and β -carotene in liposomes modified by rhamnolipid: Antioxidant activity, antibacterial activity, storage stability, and in vitro gastrointestinal digestion. *J. Food Sci.* 88(5), 2064-2077. <https://doi.org/10.1111/1750-3841.16548>

Jiru, T.M., Groenewald, M., Pohl, C., Steyn, L., Kiggundu, N., Abate, D., 2017. Optimization of cultivation conditions for biotechnological production of lipid by *Rhodotorula kratochvilovae* (syn, *Rhodosporidium kratochvilovae*) SY89 for biodiesel preparation. 3 Biotech. 7(2), 145. <https://doi.org/10.1007/s13205-017-0769-7>

Kachrimanidou, V., Alexandri, M., Nascimento, M.F., Alimpoumpa, D., Faria, N.T., Papadaki, A., Ferreira, F.C., Kopsahelis, N., 2022. Lactobacilli and *Moesziomyces* biosurfactants: Toward a closed-loop approach for the dairy industry. Fermentation. 8(10), 517. <https://doi.org/10.3390/fermentation8100517>

Kachrimanidou, V., Alimpoumpa, D., Papadaki, A., Lappa, I., Alexopoulos, K., Kopsahelis, N., 2022. Cheese whey utilization for biosurfactant production: evaluation of bioprocessing strategies using novel *Lactobacillus* strains. Biomass Convers. Bioref. 12, 4621-4635. <https://doi.org/10.1007/s13399-022-02767-9>

Kanzy, H., Nasr, F.N., El-Shazly, H.A.M., Barakat, O.S., 2015. Optimization of Carotenoids production by yeast strains of *Rhodotorula* using salted cheese whey. Int. J. Curr. Microbiol. Appl. Sci. 4(1), 456-469.

Karpiński, T.M., Adamczak, A., 2019. Fucoxanthin-an antibacterial carotenoid. Antioxidants. 8(8), 1-8. <https://doi.org/10.3390/antiox8080239>

Katouzian, I., Esfanjani, A.F., Jafari, S.M., Akhavan, S., 2017. Formulation and application of a new generation of lipid nano-carriers for the food bioactive ingredients. Trends Food Sci. Technol. 68, 14-25. <https://doi.org/10.1016/j.tifs.2017.07.017>

Kavitake, D., Tiwari, S., Devi, P.B., Shah, I.A., Reddy, G.B., Shetty, P.H., 2024. Production, purification, and functional characterization of glucan exopolysaccharide produced by *Enterococcus hirae* strain OL616073 of fermented food origin. Int. J. Biol. Macromol. 259, 129105. <https://doi.org/10.1016/j.ijbiomac.2023.129105>

Kawata, A., Murakami, Y., Suzuki, S., Fujisawa, S., 2018. Anti-inflammatory activity of β -carotene, lycopene and tri-*n*-butylborane, a scavenger of reactive oxygen species. *In Vivo*. 32(2), 255-264. <https://doi.org/10.21873/invivo.11232>

Kelly, M.E., Ramkumar, S., Sun, W., Ortiz, C.C., Kiser, P.D., Golczak, M., von Lintig, J., 2018. The biochemical basis of vitamin A production from the asymmetric carotenoid β -cryptoxanthin. *ACS Chem Biol*. 13(8), 2121-2129. <https://doi.org/10.1021/acschembio.8b00290>

Khan, R.U., Khan, A., Naz, S., Ullah, Q., Puvača, N., Laudadio, V., Mazzei, D., Seidavi, A., Ayasan, T., Tufarelli, V., 2023. Pros and cons of dietary vitamin A and its precursors in poultry health and production: A comprehensive review. *Antioxidants*. 12(5), 1131. <https://doi.org/10.3390/antiox12051131>

Kharat, M., Skrzynski, M., Decker, E.A., McClements, D.J., 2020. Enhancement of chemical stability of curcumin-enriched oil-in-water emulsions: Impact of antioxidant type and concentration. *Food Chem*. 320, 126653. <https://doi.org/10.1016/j.foodchem.2020.126653>

Khoomrung, S., Chumnanpuen, P., Jansa-Ard, S., Ståhlman, M., Nookaew, I., Borén, J., Nielsen, J., 2013. Rapid quantification of yeast lipid using microwave-assisted total lipid extraction and HPLC-CAD. *Anal. Chem*. 85(10), 4912-4919. <https://doi.org/10.1021/ac3032405>

Kim, J.H., Lee, J., Choi, I.J., Kim, Y. I., Kwon, O., Kim, H., Kim, J., 2018. Dietary carotenoids intake and the risk of gastric cancer: A case-control study in Korea. *Nutrients*. 10(8), 1-19. <https://doi.org/10.3390/nu10081031>

Koo, S.Y., Hwang, K.T., Hwang, S., Choi, K.Y., Park, Y.J., Choi, J.H., Truong, T.Q., Kim, S.M., 2023. Nanoencapsulation enhances the bioavailability of fucoxanthin in microalga *Phaeodactylum tricornutum* extract. *Food Chem*. 403, 134348. <https://doi.org/10.1016/j.foodchem.2022.134348>

Kot, A.M., Błażej, S., Gientka, I., Kieliszek, M., Bryś, J., 2018. Torulene and torularhodin: “New” fungal carotenoids for industry?. *Microb. Cell Fact.* 17(1), 49. <https://doi.org/10.1186/s12934-018-0893-z>

Kot, A.M., Błażej, S., Kieliszek, M., Gientka, I., Bryś, J., 2019. Simultaneous production of lipids and carotenoids by the red yeast *Rhodotorula* from waste glycerol fraction and potato wastewater. *Appl. Biochem. Biotechnol.* 189(2), 589-607. <https://doi.org/10.1007/s12010-019-03023-z>

Kot, A.M., Błażej, S., Kieliszek, M., Gientka, I., Bryś, J., Reczek, L., Pobiega, K., 2019. Effect of exogenous stress factors on the biosynthesis of carotenoids and lipids by *Rhodotorula* yeast strains in media containing agro-industrial waste. *World J. Microbiol. Biotechnol.* 35, 157. <https://doi.org/10.1007/s11274-019-2732-8>

Kot, A.M., Błażej, S., Kieliszek, M., Gientka, I., Piwowarek, K., Brzezińska, R., 2020. Production of lipids and carotenoids by *Rhodotorula gracilis* ATCC 10788 yeast in a bioreactor using low-cost wastes. *Biocatal. Agric. Biotechnol.* 26, 101634. <https://doi.org/10.1016/j.bcab.2020.101634>

Koutinas, M., Vasquez, M.I., Nicolaou, E., Pashali, P., Kyriakou, E., Loizou, E., Papadaki, A., Koutinas, A.A., Vyrides, I., 2019. Biodegradation and toxicity of emerging contaminants: Isolation of an exopolysaccharide-producing *Sphingomonas* sp. for ionic liquids bioremediation. *J. Hazard. Mater.* 365, 88-96. <https://doi.org/10.1016/j.jhazmat.2018.10.059>

Kruglyakov, P.M., Mal'kov, V.D., Sedova, T.P., 2002. The effect of fatty alcohols and valeric acid on the regions of existence of a three-phase system containing water, oil, and surfactant. *Colloid J.* 64(6), 796-801.

Kumar, A., Nirmal, P., Kumar, M., Jose, A., Tomer, V., Oz, E., Proestos, C., Zeng, M., Elobeid, T., Sneha, V., Oz, F., 2023. Major phytochemicals: Recent advances in health benefits and extraction method. *Molecules.* 28(2), 1-41. <https://doi.org/10.3390/molecules28020887>

Kumari, B., Tiwari, B.K., Hossain, M.B., Brunton, N.P., Rai, D.K., 2018. Recent advances on application of ultrasound and pulsed electric field technologies in the extraction of bioactives from agro-industrial by-products. *Food Bioprocess Technol.* 11(2), 223-241. <https://doi.org/10.1007/s11947-017-1961-9>

Kyriakoudi, A., Tsiouras, A., Mourtzinou, I., 2022. Extraction of lycopene from tomato using hydrophobic natural deep eutectic solvents based on terpenes and fatty acids. *Foods*. 11(17), 2645. <https://doi.org/10.3390/foods11172645>

Lakshmidhevi, R., Ramakrishnan, B., Ratha, S.K., Bhaskar, S., Chinnasamy, S., 2021. Valorisation of molasses by oleaginous yeasts for single cell oil (SCO) and carotenoids production. *Environ. Technol. Innov.* 21, 101281. <https://doi.org/10.1016/j.eti.2020.101281>

Langi, P., Kiokias, S., Varzakas, T., Proestos, C., 2018. Carotenoids: From plants to food and feed industries. *Methods Mol. Biol.* 1852, 57-71. https://doi.org/10.1007/978-1-4939-8742-9_3

Lappa, I.K., Kachrimanidou, V., Papadaki, A., Stamatiou, A., Ladakis, D., Eriotou, E., Kopsahelis, N., 2021. A comprehensive bioprocessing approach to foster cheese whey valorization: On-site β -galactosidase secretion for lactose hydrolysis and sequential bacterial cellulose production. *Fermentation*. 7(3), 184. <https://doi.org/10.3390/fermentation7030184>

Lappa, I.K., Papadaki, A., Kachrimanidou, V., Terpou, A., Koulougliotis, D., Eriotou, E., Kopsahelis, N., 2019. Cheese whey processing: Integrated biorefinery concepts and emerging food applications. *Foods*. 8(8), 347. <https://doi.org/10.3390/foods8080347>

Lara-Abia, S., Lobo, G., Pérez-Pascual, N., Welti-Chanes, J., Cano, M.P., 2023. Improvement in the stability and bioaccessibility of carotenoid and carotenoid esters from a papaya by-product using O/W emulsions. *Foods*. 12(14), 2654. <https://doi.org/10.3390/foods12142654>

Larocca, V., Martino, M., Trupo, M., Magarelli, R.A., Spagnoletta, A., Ambrico, A., 2023. Evaluation of carbon dioxide supercritical fluid extraction (CO₂-SFE) on carotenoids recovery from red yeast cells. *Biomass Convers. Bioref.* <https://doi.org/10.1007/s13399-023-04434-z>

Lazaridou, A., Roukas, T., Biliaderis, C.G., Vaikousi, H., 2002. Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. *Enzyme Microb. Technol.* 31(1-2), 122-132. [https://doi.org/10.1016/S0141-0229\(02\)00082-0](https://doi.org/10.1016/S0141-0229(02)00082-0)

Lee, J.J.L., Chen, L., Shi, J., Trzcinski, A., Chen, W.N., 2014. Metabolomic profiling of *Rhodospiridium toruloides* grown on glycerol for carotenoid production during different growth phases. *J. Agric. Food Chem.* 62(41), 10203-10209. <https://doi.org/10.1021/jf502987q>

Leema, J.T.M., Jothy, T.P., Dharani, G., 2022. Rapid green microwave assisted extraction of lutein from *Chlorella sorokiniana* (NIOT-2) – Process optimization. *Food Chem.* 372, 131151. <https://doi.org/10.1016/j.foodchem.2021.131151>

Li, C., Xie, Z., Zhao, D., Li, B., Wang, D., Chang, L., Feng, F., Zheng, L., Wang, X., Shao, M., Miao, S., Sun, Y., Cheng, P., Yu, G., 2024. Multi-omics analysis provides insights into the enhancement of β -carotene and torularhodin production in oleaginous red yeast *Sporobolomyces pararoseus* under H₂O₂-induced oxidative stress. *Lwt.* 197, 115947. <https://doi.org/10.1016/j.lwt.2024.115947>

Li, C., Xu, Y., Li, Z., Cheng, P., Yu, G., 2022. Transcriptomic and metabolomic analysis reveals the potential mechanisms underlying the improvement of β -carotene and torulene production in *Rhodospiridiobolus colostri* under low temperature treatment. *Food Res. Int.* 156, 111158. <https://doi.org/10.1016/j.foodres.2022.111158>

Li, C., Zhang, N., Li, B., Xu, Q., Song, J., Wei, N., Wang, W., Zou, H., 2017. Increased torulene accumulation in red yeast *Sporidiobolus pararoseus* NGR as stress response to high salt conditions. *Food Chem.* 237, 1041-1047. <https://doi.org/10.1016/j.foodchem.2017.06.033>

- Li, X., Guo, C., Zhang, Y., Yu, L., Ma, F., Wang, X., Zhang, L., Li, P., 2023. Contribution of different food types to vitamin A intake in the chinese diet. *Nutrients*. 15(18), 1-10. <https://doi.org/10.3390/nu15184028>
- Liang, X., Ma, C., Yan, X., Liu, X., Liu, F., 2019. Advances in research on bioactivity, metabolism, stability and delivery systems of lycopene. *Trends Food Sci. Technol.* 93, 185-196. <https://doi.org/10.1016/j.tifs.2019.08.019>
- Lie, S., 1973. The EBC-ninhydrin method for determination of free alpha amino nitrogen. *J. Inst. Brew.* 79(1), 37-41. <https://doi.org/10.1002/J.20500416.1973.TB03495.X>
- Lim, J.R., Chua, L.S., Mustaffa, A.A., 2022. Ionic liquids as green solvent and their applications in bioactive compounds extraction from plants. *Process Biochem.* 122, 292-306. <https://doi.org/10.1016/j.procbio.2022.10.024>
- Lin, Y., Pangloli, P., Dia, V.P., 2021. Physicochemical, functional and bioactive properties of hempseed (*Cannabis sativa* L.) meal, a co-product of hempseed oil and protein production, as affected by drying process. *Food Chem.* 350, 129188. <https://doi.org/10.1016/j.foodchem.2021.129188>
- Lin-Vien, D., Colthup, N.B., Fateley, W.G., Grasselli, J.G., 1991. Alkanes, in: *The handbook of infrared and raman characteristic frequencies of organic molecules*. pp. 9–28.
- Liu, C., Guo, Y., Cheng, Y., Qian, H., 2022. Bilosomes: A controlled delivery system for the sustained release of torularhodin during digestion in the small intestine both in vitro and in vivo. *Coll. Surf. A Physicochem. Eng. Asp.* 654, 130055. <https://doi.org/10.1016/j.colsurfa.2022.130055>
- Liu, C., Guo, Y., Cheng, Y., Qian, H., 2023. A colon-targeted delivery system of torularhodin encapsulated in electrospinning microspheres, and its co-metabolic regulation mechanism of gut microbiota. *Food Hydrocoll.* 135, 108189. <https://doi.org/10.1016/j.foodhyd.2022.108189>

- Liu, D., Liu, H., Qi, H., Guo, X.J., Jia, B., Zhang, J.L., Yuan, Y.J., 2019. Constructing yeast chimeric pathways to boost lipophilic terpene synthesis. *ACS Synth. Biol.* 8(4), 724-733. <https://doi.org/10.1021/acssynbio.8b00360>
- Liu, H., Zhang, C., Zhang, X., Tan, K., Zhang, H., Cheng, D., Ye, T., Li, S., Ma, H., Zheng, H., 2020. A novel carotenoids-producing marine bacterium from noble scallop *Chlamys nobilis* and antioxidant activities of its carotenoid compositions. *Food Chem.* 320, 126629. <https://doi.org/10.1016/j.foodchem.2020.126629>
- Liu, M., Zhang, W.J., Yao, J., Niu, J., 2021. Production, purification, characterization, and biological properties of *Rhodospiridium paludigenum* polysaccharide. *PLoS One.* 16, 1-23. <https://doi.org/10.1371/journal.pone.0246148>
- Liu, Y., Zhang, C., Cui, B., Zhou, Q., Wang, Y., Chen, X., Fu, H., Wang, Y., 2022. Effect of emulsifier composition on oil-in-water nano-emulsions: Fabrication, structural characterization and delivery of zeaxanthin dipalmitate from *Lycium barbarum* L. *Lwt.* 161, 113353. <https://doi.org/10.1016/j.lwt.2022.113353>
- Liu, Y.S., Wu, J.Y., 2007. Optimization of cell growth and carotenoid production of *Xanthophyllomyces dendrorhous* through statistical experiment design. *Biochem. Eng. J.* 36(2), 182-189. <https://doi.org/10.1016/j.bej.2007.02.014>
- Liu, Z., Feist, A.M., Dragone, G., Mussatto, S.I., 2020. Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts. *J. Clean. Prod.* 249, 119308. <https://doi.org/10.1016/j.jclepro.2019.119308>
- Ljungdahl, P.O., Daignan-Fornier, B., 2012. Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. *Genetics.* 190(3), 885-929. <https://doi.org/10.1534/genetics.111.133306>
- Lobo, R.E., Gómez, M.I., de Valdez, G.F., Torino, M.I., 2019. Physicochemical and antioxidant properties of a gastroprotective exopolysaccharide produced by *Streptococcus thermophilus* CRL1190. *Food Hydrocoll.* 96, 625-633. <https://doi.org/10.1016/j.foodhyd.2019.05.036>

Lopes, N.A., Remedi, R.D., dos Santos Sá, C., Burkert, C.A.V., de Medeiros Burkert, J.F., 2017. Different cell disruption methods for obtaining carotenoids by *Sporodiobolus pararoseus* and *Rhodotorula mucilaginosa*. Food Sci. Biotechnol. 26(3), 759-766. <https://doi.org/10.1007/s10068-017-0098-y>

Lucarini, M., Durazzo, A., Sánchez del Pulgar, J., Gabrielli, P., Lombardi-Boccia, G., 2018. Determination of fatty acid content in meat and meat products: The FTIR-ATR approach. Food Chem. 267, 223–230. <https://doi.org/10.1016/j.foodchem.2017.11.042>

Ludwig, K., Rihko-Struckmann, L., Brinitzer, G., Unkelbach, G., Sundmacher, K., 2021. β -carotene extraction from *Dunaliella salina* by supercritical CO₂. J. Appl. Phycol. 33(3), 1435-1445. <https://doi.org/10.1007/s10811-021-02399-y>

Luengo, E., Álvarez, I., Raso, J., 2014. Improving carotenoid extraction from tomato waste by pulsed electric fields. Front. Nutr. 1, 1-10. <https://doi.org/10.3389/fnut.2014.00012>

Ma, W., Chen, X., Wang, B., Lou, W., Chen, X., Hua, J., Sun, Y.J., Zhao, Y., Peng, T., 2018. Characterization, antioxidativity, and anti-carcinoma activity of exopolysaccharide extract from *Rhodotorula mucilaginosa* CICC 33013. Carbohydr. Polym. 181, 768-777. <https://doi.org/10.1016/j.carbpol.2017.11.080>

Machado, W.R.C., Murari, C.S., Duarte, A.L.F., Del Bianchi, V.L., 2022. Optimization of agro-industrial coproducts (molasses and cassava wastewater) for the simultaneous production of lipids and carotenoids by *Rhodotorula mucilaginosa*. Biocatal. Agric. Biotechnol. 42, 1-13. <https://doi.org/10.1016/j.bcab.2022.102342>

Macías-Sánchez, M.D., Serrano, C.M., Rodríguez, M.R., de la Ossa, E.M., 2009. Kinetics of the supercritical fluid extraction of carotenoids from microalgae with CO₂ and ethanol as cosolvent. Chem. Eng. J. 150(1), 104-113. <https://doi.org/10.1016/j.cej.2008.12.006>

Mahadev Gaikwad, K., Pansare, G.E., Madane, M.P., Mulay, Y.R., 2022. Studies on exopolysaccharide production from *Aureobasidium pullulans* spi 10 and its application as a green inhibitor for corrosion mitigation. J. Appl. Biol. Biotechnol. 10(3), 34-43. <https://doi.org/10.7324/JABB.2022.100306>

Mahalakshmi, L., Leena, M.M., Moses, J.A., Anandharamakrishnan, C., 2020. Micro- and nano-encapsulation of β -carotene in zein protein: size-dependent release and absorption behavior. Food Funct. 11(2), 1647-1660. <https://doi.org/10.1039/c9fo02088h>

Maitah, M., Procházka, P., Rojík, S., Činčalová, S., Malec, K., Smutka, L., Gebeltová, Z., Koželský, R., Maitah, K., Tomšík, K., 2024. Future development of sugar market in the European Union in the period 2023-2032. Sugar Tech. 26(1), 295-304. <https://doi.org/10.1007/s12355-023-01342-4>

Mandelli, F., Miranda, V.S., Rodrigues, E., Mercadante, A.Z., 2012. Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. World J. Microbiol. Biotechnol. 28, 1781-1790. <https://doi.org/10.1007/s11274-011-0993-y>

Mantzouridou, F., 2020. Newsletter Eurocaroten: Production of carotenoids. 1-8.

Maoka, T., 2020. Carotenoids as natural functional pigments. J. Nat. Med. 74(1), 1-16. <https://doi.org/10.1007/s11418-019-01364-x>

Market.us Report, 2023. Carotenoids Market. <https://market.us/report/carotenoids-market/>

Markets and Markets, 2024. Carotenoids Market. <https://www.marketsandmarkets.com/Market-Reports/carotenoid-market-158421566.html>

Marova, I., Carnecka, M., Halienova, A., Certik, M., Dvorakova, T., Haronikova, A., 2012. Use of several waste substrates for carotenoid-rich yeast biomass production. J. Environ. Manage. 95, S338-S342. <https://doi.org/10.1016/j.jenvman.2011.06.018>

Martínez, J.M., Schottroff, F., Haas, K., Fauster, T., Sajfrtová, M., Álvarez, I., Raso, J., Jaeger, H., 2020. Evaluation of pulsed electric fields technology for the improvement of subsequent carotenoid extraction from dried *Rhodotorula glutinis* yeast. Food Chem. 323, 126824. <https://doi.org/10.1016/j.foodchem.2020.126824>

Martins, A.J., Vicente, A.A., Pastrana, L.M., Cerqueira, M.A., 2020. Oleogels for development of health-promoting food products. Food Sci. Hum. Wellness 9(1), 31-39. <https://doi.org/10.1016/j.fshw.2019.12.001>

Mata-Gómez, L.C., Mapelli-Brahm, P., Meléndez-Martínez, A.J., Méndez-Zavala, A., Morales-Oyervides, L., Montañez, J., 2023. Microbial carotenoid synthesis optimization in goat cheese whey using the robust Taguchi method: A sustainable approach to help tackle vitamin A deficiency. Foods. 12(3), 658. <https://doi.org/10.3390/foods12030658>

Mata-Gómez, L.C., Montañez, J.C., Méndez-Zavala, A., Aguilar, C.N., 2014. Biotechnological production of carotenoids by yeasts: An overview. Microb. Cell Fact. 13(12). <http://www.microbialcellfactories.com/content/13/1/12>

Maza, D.D., Viñarta, S.C., Su, Y., Guillamón, J.M., Aybar, M.J., 2020. Growth and lipid production of *Rhodotorula glutinis* R4, in comparison to other oleaginous yeasts. J. Biotechnol. 310, 21-31. <https://doi.org/10.1016/j.jbiotec.2020.01.012>

McClements, D.J., 2015. Food Emulsions, 3rd ed. <https://doi.org/https://doi.org/10.1201/b18868>

McClements, D.J., Rao, J., 2011. Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. Crit. Rev. Food Sci. Nutr. 51(4), 285-330. <https://doi.org/10.1080/10408398.2011.559558>

Mehariya, S., Iovine, A., Di Sanzo, G., Larocca, V., Martino, M., Leone, G.P., Casella, P., Karatza, D., Marino, T., Musmarra, D., Molino, A., 2019. Supercritical fluid extraction of lutein from *Scenedesmus almeriensis*. Molecules. 24(7), 1-15. <https://doi.org/10.3390/molecules24071324>

Mehri, D., Perendeci, N.A., Goksungur, Y., 2021. Utilization of whey for red pigment production by *Monascus purpureus* in submerged fermentation. *Fermentation*. 7(2), 75. <https://doi.org/10.3390/fermentation7020075>

Meléndez-Martínez, A.J., Mandić, A.I., Bantis, F., Böhm, V., Borge, G.I.A., Brnčić, M., Bysted, A., Cano, M.P., Dias, M.G., Elgersma, A., Fikselová, M., García-Alonso, J., Giuffrida, D., Gonçalves, V.S.S., Hornero-Méndez, D., Kljak, K., Lavelli, V., Manganaris, G.A., Mapelli-Brahm, P., Marounek, M., Olmedilla-Alonso, B., Periago-Castón, M.J., Pintea, A., Sheehan, J.J., Šaponjac, V.T., Valšíková-Frey, M., Van Meulebroek, L., O'Brien, N., 2022. A comprehensive review on carotenoids in foods and feeds: *status quo*, applications, patents, and research needs. *Crit. Rev. Food Sci. Nutr.* 62(8), 1999-2049. <https://doi.org/10.1080/10408398.2020.1867959>

Metwally, R.A., El-Sersy, N.A., El Sikaily, A., Sabry, S.A., Ghozlan, H.A., 2022. Optimization and multiple in vitro activity potentials of carotenoids from marine *Kocuria* sp. RAM1. *Sci. Rep.* 12(1), 1-19. <https://doi.org/10.1038/s41598-022-22897-4>

Michelon, M., de Matos de Borba, T., da Silva Rafael, R., Burkert, C.A.V., de Medeiros Burkert, J.F., 2012. Extraction of carotenoids from *Phaffia rhodozyma*: A comparison between different techniques of cell disruption. *Food Sci. Biotechnol.* 21(1), 1-8. <https://doi.org/10.1007/s10068-012-0001-9>

Miller, K.K., Alper, H.S., 2019. *Yarrowia lipolytica*: more than an oleaginous workhorse. *Appl. Microbiol. Biotechnol.* 103(23-24), 9251-9262. <https://doi.org/10.1007/s00253-019-10200-x>

Mirzaie, M., Saei-Dehkordi, S.S., Levin, D.B., 2019. Lipid and β -carotene production by *Rhodospiridium diobovatum* cultured with different carbon to nitrogen ratios. *J. Food Qual. Hazards Control.* 6(2), 58-65. <https://doi.org/10.18502/jfqhc.6.2.956>

Mohammed, Y.M.M., Saad, M.M.G., Abdelgaleil, S.A.M., 2021. Production, characterization and bio-emulsifying application of exopolysaccharides from *Rhodotorula mucilaginosa* YMM19. *3 Biotech.* 11(7), 1-12. <https://doi.org/10.1007/s13205-021-02898-2>

Molino, A., Larocca, V., Di Sanzo, G., Martino, M., Casella, P., Marino, T., Karatza, D., Musmarra, D., 2019. Extraction of bioactive compounds using supercritical carbon dioxide. *Molecules*. 24(4), 782. <https://doi.org/10.3390/molecules24040782>

Molino, A., Mehariya, S., Iovine, A., Larocca, V., Di Sanzo, G., Martino, M., Casella, P., Chianese, S., Musmarra, D., 2018. Extraction of astaxanthin and lutein from microalga *Haematococcus pluvialis* in the red phase using CO₂ supercritical fluid Extraction technology with ethanol as co-solvent. *Mar. Drugs*. 16(11), 432. <https://doi.org/10.3390/md16110432>

Montero-Calderon, A., Cortes, C., Zulueta, A., Frigola, A., Esteve, M.J., 2019. Green solvents and ultrasound-assisted extraction of bioactive orange (*Citrus sinensis*) peel compounds. *Sci. Rep.* 9(1), 1-8. <https://doi.org/10.1038/s41598-019-52717-1>

More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernández-Jerez, A., Hougaard Bennekou, S., Koutsoumanis, K., Lambré, C., Machera, K., Naegeli, H., Nielsen, S., Schlatter, J., Schrenk, D., Silano, V., Turck, D., Younes, M., Castenmiller, J., Chaudhry, Q., Cubadda, F., Franz, R., Gott, D., Mast, J., Mortensen, A., Oomen, A.G., Weigel, S., Barthelemy, E., Rincon, A., Tarazona, J., Schoonjans, R., 2021. Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health. *EFSA J.* 19(8), 6768. <https://doi.org/10.2903/j.efsa.2021.6768>

Moreira, M.D., Melo, M.M., Coimbra, J.M., dos Reis, K.C., Schwan, R.F., Silva, C.F., 2018. Solid coffee waste as alternative to produce carotenoids with antioxidant and antimicrobial activities. *Waste Manag.* 82, 93-99. <https://doi.org/10.1016/j.wasman.2018.10.017>

Morón-Ortiz, Á., Mapelli-Brahm, P., Meléndez-Martínez, A.J., 2024. Sustainable green extraction of carotenoid pigments: Innovative technologies and bio-based solvents. *Antioxidants*. 13(2), 239. <https://doi.org/10.3390/antiox13020239>

Morton, S., Pencheon, D., Squires, N., 2017. Sustainable Development Goals (SDGs), and their implementation. *Br. Med. Bull.* 124(1), 81-90. <https://doi.org/10.1093/bmb/ldx031>

Mosaddik, A., Ravinayagam, Vijaya Elanthikkal, S., Fessi, H., Badri, W., Elaissari, A., 2018. Development and use of polymeric nanoparticles for the encapsulation and administration of plant extracts, in: Natural products as source of molecules with therapeutic potential. pp. 391-463.

Mouahid, A., Seengeon, K., Martino, M., Crampon, C., Kramer, A., Badens, E., 2020. Selective extraction of neutral lipids and pigments from *Nannochloropsis salina* and *Nannochloropsis maritima* using supercritical CO₂ extraction: Effects of process parameters and pre-treatment. J. Supercrit. Fluids. 165, 104934.

<https://doi.org/10.1016/j.supflu.2020.104934>

Muniz, N.O., Vechietti, F.A., Anesi, G.R., Guinea, G. V., dos Santos, L.A.L., 2020. Blend-based fibers produced via centrifugal spinning and electrospinning processes: Physical and rheological properties. J. Mater. Res. 35(21), 2905-2916.

<https://doi.org/10.1557/jmr.2020.189>

Murador, D.C., Braga, A.R.C., Martins, P.L.G., Mercadante, A.Z., de Rosso, V. V., 2019. Ionic liquid associated with ultrasonic-assisted extraction: A new approach to obtain carotenoids from orange peel. Food Res. Int. 126, 108653.

<https://doi.org/10.1016/j.foodres.2019.108653>

Mussagy, C.U., Gonzalez-Miquel, M., Santos-Ebinuma, V.C., Pereira, J.F.B., 2023. Microbial torularhodin-A comprehensive review. Crit. Rev. Biotechnol. 43(4), 540-558. <https://doi.org/doi.org/10.1080/07388551.2022.2041540>

Mussagy, C.U., Guimarães, A.A.C., Rocha, L.V.F., Winterburn, J., de Carvalho Santos-Ebinuma, V., Pereira, J.F.B., 2021. Improvement of carotenoids production from *Rhodotorula glutinis* CCT-2186. Biochem. Eng. J. 165, 107827.

<https://doi.org/10.1016/j.bej.2020.107827>

Mussagy, C.U., Silva, P.G.P., Amantino, C.F., Burkert, J.F.M., Primo, F.L., Pessoa Jr, A., Santos-Ebinuma, V.C., 2022. Production of natural astaxanthin by *Phaffia rhodozyma* and its potential application in textile dyeing. Biochem. Eng. J. 187, 108658. <https://doi.org/10.1016/j.bej.2022.108658>

- Mussagy, C.U., Winterburn, J., Santos-Ebinuma, V.C., Pereira, J.F.B., 2019. Production and extraction of carotenoids produced by microorganisms. *Appl. Microbiol. Biotechnol.* 103, 1095–1114. <https://doi.org/10.1007/S00253-018-9557-5>
- Nagal, S., Raja, A., Gupta, I., Adin, S.N., Panda, B.P., 2023. Screening and development of β -carotene enriched *Phaffia rhodozyma* cell by culture media engineering. *Microbiol.* 92(1), 36-46. <https://doi.org/10.1134/S002626172210068X>
- Nakata, H., Tamura, M., Shintani, T., Gomi, K., 2014. Evaluation of baker's yeast strains exhibiting significant growth on Japanese beet molasses and compound analysis of the molasses types. *J. Biosci. Bioeng.* 117(6), 715-719. <https://doi.org/10.1016/j.jbiosc.2013.11.009>
- Nasirian, N., Mirzaie, M., Cicek, N., Levin, D.B., 2018. Lipid and carotenoid synthesis by *Rhodospiridium diobovatum*, grown on glucose versus glycerol, and its biodiesel properties. *Can. J. Microbiol.* 64(4), 277-289. <https://doi.org/10.1139/cjm-2017-0613>
- Nasr, G., Greige-Gerges, H., Elaissari, A., Khreich, N., 2020. Liposomal membrane permeability assessment by fluorescence techniques: Main permeabilizing agents, applications and challenges. *Int. J. Pharm.* 580, 119198. <https://doi.org/10.1016/j.ijpharm.2020.119198>
- Niu, B., Shao, P., Sun, P., 2020. Ultrasound-assisted emulsion electrosprayed particles for the stabilization of β -carotene and its nutritional supplement potential. *Food Hydrocoll.* 102, 105634. <https://doi.org/10.1016/j.foodhyd.2019.105634>
- Nunes, A.N., Monte, J., Rodríguez-Rojo, S., Nogueira, I.D., Gouveia, L.F., Brazinha, C., Matias, A.A., 2024. Development of a carotenoid-rich microalgae colorant by microencapsulation. *Colorants.* 3(1), 39-52. <https://doi.org/10.3390/colorants3010003>
- Ochoa-Viñals, N., Alonso-Estrada, D., Faife-Pérez, E., Chen, Z., Michelena-Alvarez, G., Martínez-Hernández, J.L., García-Cruz, A., Iliina, A., 2024. β -carotene production from sugarcane molasses by a newly isolated *Rhodotorula toruloides* L/24-26-1. *Arch. Microbiol.* 206(6), 1-10. <https://doi.org/10.1007/s00203-024-03973-x>

Olbrich, H., 2006. The molasses, in: Principles of sugar technology, Vol. III. Elsevier Publishing. N.Y.

Oleksy-Sobczak, M., Klewicka, E., 2020. Optimization of media composition to maximize the yield of exopolysaccharides production by *Lactobacillus rhamnosus* strains. Probiotics Antimicrob. Proteins. 12(2), 774-783.

<https://doi.org/10.1007/s12602-019-09581-2>

Osanlou, R., Emtiazjoo, M., Banaei, A., Hesarinejad, M.A., Ashrafi, F., 2022. Preparation of solid lipid nanoparticles and nanostructured lipid carriers containing zeaxanthin and evaluation of physicochemical properties. Coll. Surf. A Physicochem. Eng. Asp. 641, 128588. <https://doi.org/10.1016/j.colsurfa.2022.128588>

Osman, M.E., Abdel-Razik, A.B., Zaki, K.I., Mamdouh, N., El-Sayed, H., 2022. Isolation, molecular identification of lipid-producing *Rhodotorula diobovata*: Optimization of lipid accumulation for biodiesel production. J. Genet. Eng. Biotechnol. 20(1), 32. <https://doi.org/10.1186/s43141-022-00304-9>

Ozdal, M., Kurbanoglu, E.B., 2019. Use of chicken feather peptone and sugar beet molasses as low cost substrates for xanthan production by *Xanthomonas campestris* MO-03. Fermentation. 5(1), 8-10. <https://doi.org/10.3390/fermentation5010009>

Paliwal, C., Rehmanji, M., Shaikh, K.M., Zafar, S.U., Jutur, P.P., 2022. Green extraction processing of lutein from *Chlorella saccharophila* in water-based ionic liquids as a sustainable innovation in algal biorefineries. Algal Res. 66, 102809. <https://doi.org/10.1016/j.algal.2022.102809>

Pang, S.F., Yusoff, M.M., Gimbin, J., 2014. Assessment of phenolic compounds stability and retention during spray drying of *Orthosiphon stamineus* extracts. Food Hydrocoll. 37, 159-165. <https://doi.org/10.1016/j.foodhyd.2013.10.022>

Panić, M., Radić Stojković, M., Kraljić, K., Škevin, D., Radojčić Redovniković, I., Gaurina Srček, V.G., Radošević, K., 2019. Ready-to-use green polyphenolic extracts from food by-products. Food Chem. 283, 628-636.

<https://doi.org/10.1016/j.foodchem.2019.01.061>

Papadaki, A., Alexandri, M., Kachrimanidou, V., Tzora, P., Kopsahelis, N., 2023. Enhancing hydrophobic and antioxidant properties of whey protein films through the inclusion of oil and oleogel sourced from spent coffee grounds. *Sustain. Chem. Pharm.* 36, 101293. <https://doi.org/10.1016/j.scp.2023.101293>

Papadaki, A., Cipolatti, E.P., Aguiéiras, E.C.G., Pinto, M.C.C., Kopsahelis, N., Freire, D.M.G., Mandala, I., Koutinas, A.A., 2019a. Development of microbial oil wax-based oleogel with potential application in food formulations. *Food Bioprocess Technol.* 12(6), 899-909. <https://doi.org/10.1007/s11947-019-02257-3>

Papadaki, A., Kopsahelis, N., Mallouchos, A., Mandala, I., Koutinas, A.A., 2019b. Bioprocess development for the production of novel oleogels from soybean and microbial oils. *Food Res. Int.* 126, 108684. <https://doi.org/10.1016/j.foodres.2019.108684>

Papadaki, A., Lappa, I.K., Manikas, A.C., Pastore Carbone, M.G., Natsia, A., Kachrimanidou, V., Kopsahelis, N., 2024. Grafting bacterial cellulose nanowhiskers into whey protein/essential oil film composites: Effect on structure, essential oil release and antibacterial properties of films. *Food Hydrocoll.* 147, 109374. <https://doi.org/10.1016/j.foodhyd.2023.109374>

Papanikolaou, S., Rontou, M., Belka, A., Athenaki, M., Gardeli, C., Mallouchos, A., Kalantzi, O., Koutinas, A.A., Kookos, I.K., Zeng, A.P., Aggelis, G., 2017. Conversion of biodiesel-derived glycerol into biotechnological products of industrial significance by yeast and fungal strains. *Eng. Life Sci.* 17(3), 262-281. <https://doi.org/10.1002/elsc.201500191>

Papapostolou, H., Kachrimanidou, V., Alexandri, M., Plessas, S., Papadaki, A., Kopsahelis, N., 2023. Natural carotenoids: Recent advances on separation from microbial biomass and methods of analysis. *Antioxidants.* 12, 1030. <https://doi.org/10.3390/antiox12051030>

Park, C.H., Xu, F.H., Roh, S.S., Song, Y.O., Uebaba, K., Noh, J.S., Yokozawa, T., 2015. Astaxanthin and corni fructus protect against diabetes-induced oxidative stress, inflammation, and advanced glycation end product in livers of streptozotocin-induced diabetic rats. *J. Med. Food.* 18(3), 337-344. <https://doi.org/10.1089/jmf.2014.3174>

Park, K.M., Song, M.W., Lee, J.H., 2008. Production of carotenoids by β -ionone-resistant mutant of *Xanthophyllomyces dendrorhous* using various carbon sources. *Biotechnol. Bioprocess Eng.* 13(2), 197-203. <https://doi.org/10.1007/s12257-007-0160-3>

Pasandide, B., Khodaiyan, F., Mousavi, Z.E., Hosseini, S.S., 2017. Optimization of aqueous pectin extraction from *Citrus medica* peel. *Carbohydr. Polym.* 178, 27-33. <https://doi.org/10.1016/j.carbpol.2017.08.098>

Pataro, G., Carullo, D., Falcone, M., Ferrari, G., 2020. Recovery of lycopene from industrially derived tomato processing by-products by pulsed electric fields-assisted extraction. *Innov. Food Sci. Emerg. Technol.* 63, 102369. <https://doi.org/10.1016/j.ifset.2020.102369>

Patel, A., Pravez, M., Deeba, F., Pruthi, V., Singh, R.P., Pruthi, P.A., 2014. Boosting accumulation of neutral lipids in *Rhodospiridium kratochvilovae* HIMPA1 grown on hemp (*Cannabis sativa* Linn) seed aqueous extract as feedstock for biodiesel production. *Bioresour. Technol.* 165, 214-222. <https://doi.org/10.1016/j.biortech.2014.03.142>

Patel, A., Pruthi, V., Singh, R.P., Pruthi, P.A., 2015. Synergistic effect of fermentable and non-fermentable carbon sources enhances TAG accumulation in oleaginous yeast *Rhodospiridium kratochvilovae* HIMPA1. *Bioresour. Technol.* 188, 136-144. <https://doi.org/10.1016/j.biortech.2015.02.062>

Patel, A.S., Kar, A., Dash, S., Dash, S.K., 2019. Supercritical fluid extraction of β -carotene from ripe bitter melon pericarp. *Sci. Rep.* 9(1), 1-10. <https://doi.org/10.1038/s41598-019-55481-4>

Paul, D., Kumari, P.K., Siddiqui, N., 2023. Yeast carotenoids: Cost-effective fermentation strategies for health care applications. *Fermentation*. 9(2), 147.
<https://doi.org/10.3390/fermentation9020147>

Pegklidou, K., Mantzouridou, F., Tsimidou, M.Z., 2008. Lycopene production using *Blakeslea trispora* in the presence of 2-methyl imidazole: Yield, selectivity, and safety aspects. *J. Agric. Food Chem.* 56(12), 4482-4490.
<https://doi.org/10.1021/jf800272k>

Perța-Crișan, S., Ursachi, C. Ș., Chereji, B.D., Tolan, I., Munteanu, F.D., 2023. Food-grade oleogels: Trends in analysis, characterization, and applicability. *Gels*. 9(5), 386.
<https://doi.org/10.3390/gels9050386>

Phuengjayaem, S., Kingkaew, E., Hoonddee, P., Rojsitthisak, P., Sritularak, B., Thitikornpong, W., Thompho, S., Pornputtpong, N., Tanasupawat, S., 2023. Diversity, astaxanthin production, and genomic analysis of *Rhodotorula paludigena* SP9-15. *Heliyon*. 9(7), e18280. <https://doi.org/10.1016/j.heliyon.2023.e18280>

Pink, D.L., Loruthai, O., Ziolk, R.M., Wasutrasawat, P., Terry, A.E., Lawrence, M.J., Lorenz, C.D., 2019. On the structure of solid lipid nanoparticles. *Small*. 15(45), 1903156. <https://doi.org/10.1002/sml.201903156>

Pinna, N., Ianni, F., Selvaggini, R., Urbani, S., Codini, M., Grispoldi, L., Cenci-Goga, B.T., Cossignani, L., Blasi, F., 2023. Valorization of pumpkin byproducts: Antioxidant activity and carotenoid characterization of extracts from peel and filaments. *Foods*. 12(21), 1-13. <https://doi.org/10.3390/foods12214035>

Plazzotta, S., Calligaris, S., Manzocco, L., 2020. Structural characterization of oleogels from whey protein aerogel particles. *Food Res. Int.* 132, 109099.
<https://doi.org/10.1016/j.foodres.2020.109099>

Ponce Del Castillo, A.M., 2010. The EU approach to regulating nanotechnology. ETUI.

Poonia, A., Pandey, S., 2023. Production of microbial pigments from whey and their applications: A review. *Nutr. Food Sci.* 53(2), 265-284. <https://doi.org/10.1108/NFS-02-2022-0055>

Puscas, A., Muresan, V., Socaciu, C., Muste, S., 2020. Oleogels in food: A review of current and potential applications. *Foods*. 9(1), 1-27. <https://doi.org/10.3390/foods9010070>

Qadariyah, L., Mahfud, M., Sulistiawati, E., Swastika, P., 2018. Natural dye extraction from teak leaves (*Tectona Grandis*) using ultrasound assisted extraction method for dyeing on cotton fabric. *MATEC Web Conf.* 156, 05004. <https://doi.org/10.1051/mateconf/201815605004>

Qi, F., Shen, P., Hu, R., Xue, T., Jiang, X., Qin, L., Chen, Y., Huang, J., 2020. Carotenoids and lipid production from *Rhodospiridium toruloides* cultured in tea waste hydrolysate. *Biotechnol. Biofuels*. 13(1), 74. <https://doi.org/10.1186/s13068-020-01712-0>

Quilaqueo, M., Millao, S., Luzardo-Ocampo, I., Campos-Vega, R., Acevedo, F., Shene, C., Rubilar, M., 2019. Inclusion of piperine in β -cyclodextrin complexes improves their bioaccessibility and in vitro antioxidant capacity. *Food Hydrocoll.* 91, 143-152. <https://doi.org/10.1016/j.foodhyd.2019.01.011>

Qumsani, A.T., 2024. The contribution of microorganisms to sustainable development: Towards a green future through synthetic biology and systems biology. *J. Umm Al-Qura Univ. Appl. Sci.* 1-17. <https://doi.org/10.1007/s43994-024-00180-8>

Radzali, S.A., Baharin, B.S., Othman, R., Markom, M., Rahman, R.A., 2014. Co-solvent selection for supercritical fluid extraction of astaxanthin and other carotenoids from *Penaeus monodon* waste. *J. Oleo Sci.* 63(8), 769-777. <https://doi.org/10.5650/jos.ess13184>

Rahbar Saadat, Y., Khosroushahi, A.Y., Pourghassem Gargari, B., 2021. Yeast exopolysaccharides and their physiological functions. *Folia Microbiol.* 66(2), 171-182. <https://doi.org/10.1007/s12223-021-00856-2>

Rajabi, H., Ghorbani, M., Jafari, S.M., Sadeghi Mahoonak, A., Rajabzadeh, G., 2015. Retention of saffron bioactive components by spray drying encapsulation using maltodextrin, gum Arabic and gelatin as wall materials. *Food Hydrocoll.* 51, 327-337. <https://doi.org/10.1016/j.foodhyd.2015.05.033>

Ramezani, M., Martín-Belloso, O., Salvia-Trujillo, L., 2024. Influence of oleogel composition on lipid digestibility and β -carotene bioaccessibility during in vitro digestion. *Food Chem.* 456, 139978. <https://doi.org/10.1016/j.foodchem.2024.139978>

Rani, A., Saini, K.C., Bast, F., Mehariya, S., Bhatia, S.K., Lavecchia, R., Zuorro, A., 2021. Microorganisms: A potential source of bioactive molecules for antioxidant applications. *Molecules.* 26(4), 1142. <https://doi.org/10.3390/molecules26041142>

Razani, S.H., Mousavi, S.M., Yeganeh, H.M.M., Marc, I., 2007. Fatty acid and carotenoid production by *Sporobolomyces ruberrimus* when using technical glucerol and ammonium sulfate. *J. Microbiol. Biotechnol.* 17(10), 1591-1597.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26(9-10), 1231-1237. [https://doi.org/10.1016/S0891-5849\(98\)00315-3](https://doi.org/10.1016/S0891-5849(98)00315-3)

Ribeiro, D., Freitas, M., Silva, A.M.S., Carvalho, F., Fernandes, E., 2018. Antioxidant and pro-oxidant activities of carotenoids and their oxidation products. *Food Chem. Toxicol.* 120, 681-699. <https://doi.org/10.1016/j.fct.2018.07.060>

Ribeiro, R.M.M.G.P., Picão, B.W., Gonçalves, D.O., Scontri, M., Mazziero, V.T., Mussagy, C.U., Raghavan, V., Astudillo-Castro, C., Córdova, A., Cerri, M.O., Tambourgi, E.B., 2023. Synergistic effects of stirring and aeration rate on carotenoid production in yeast *Rhodotorula toruloides* CCT 7815 envisioning their application as soap additives. *Fermentation.* 9(9), 828. <https://doi.org/10.3390/fermentation9090828>

Rizvi, S.Z.H., Shah, F.A., Khan, N., Muhammad, I., Ali, K.H., Ansari, M.M., ud Din, F., Qureshi, O.S., Kim, K.W., Choe, Y.H., Kim, J.K., Zeb, A., 2019. Simvastatin-loaded solid lipid nanoparticles for enhanced anti-hyperlipidemic activity in hyperlipidemia animal model. *Int. J. Pharm.* 560, 136-143.

<https://doi.org/10.1016/j.ijpharm.2019.02.002>

Rizzardi, N., Pezzolesi, L., Samorì, C., Senese, F., Zalambani, C., Pitacco, W., Calonghi, N., Bergamini, C., Prata, C., Fato, R., 2022. Natural astaxanthin is a green antioxidant able to counteract lipid peroxidation and ferroptotic cell death. *Int. J. Mol. Sci.* 23(23), 15137. <https://doi.org/10.3390/ijms232315137>

Rodrigues, L.A., Pereira, C. V., Leonardo, I.C., Fernández, N., Gaspar, F.B., Silva, J.M., Reis, R.L., Duarte, A.R.C., Paiva, A., Matias, A.A., 2020. Terpene-based natural deep eutectic systems as efficient solvents to recover astaxanthin from brown crab shell residues. *ACS Sustain. Chem. Eng.* 8(5), 2246-2259.

<https://doi.org/10.1021/acssuschemeng.9b06283>

Rodrigues, T.V.D., Teixeira, E.C., Macedo, L.P., dos Santos, G.M., Burkert, C.A.V., de Medeiros Burkert, J.F., 2022. Agroindustrial byproduct-based media in the production of microbial oil rich in oleic acid and carotenoids. *Bioprocess Biosyst. Eng.* 45(4), 721-732. <https://doi.org/10.1007/s00449-022-02692-1>

Ropciuc, S., Dranca, F., Oroian, M.A., Leahu, A., Codină, G.G., Prisacaru, A.E., 2023. Structuring of cold pressed oils: Evaluation of the physicochemical characteristics and microstructure of white beeswax oleogels. *Gels.* 9(3), 216. <https://doi.org/10.3390/gels9030216>

Rostami, H., Hamed, H., Yolmeh, M., 2016. Some biological activities of pigments extracted from *Micrococcus roseus* (PTCC 1411) and *Rhodotorula glutinis* (PTCC 5257). *Int. J. Immunopathol. Pharmacol.* 29(4), 684-695.

<https://doi.org/10.1177/0394632016673846>

Roukas, T., Varzakakou, M., Kotzekidou, P., 2015. From cheese whey to carotenes by *Blakeslea trispora* in a bubble column reactor. *Appl. Biochem. Biotechnol.* 175(1), 182-193. <https://doi.org/10.1007/s12010-014-1260-0>

Ruggeri, G., Corsi, S., 2019. An analysis of the Fairtrade cane sugar small producer organizations network. J. Clean. Prod. 240, 118191.

<https://doi.org/10.1016/j.jclepro.2019.118191>

Rukavina, I., Rodrigues, M.J., Pereira, C.G., Mansinhos, I., Romano, A., Slusarczyk, S., Matkowski, A., Custódio, L., 2021. Greener is better : First approach for the use of natural deep eutectic solvents (NADES) to extract antioxidants from the medicinal halophyte *Polygonum maritimum* L. Molecules. 26, 6136.

<https://doi.org/10.3390/molecules26206136>

Sahin, S., Sumnu, S.G., 2007. Surface properties of foods. Phys. Prop. Foods. 229-250. https://doi.org/10.1007/0-387-30808-3_6

Saifullah, M., Shishir, M.R.I., Ferdowsi, R., Tanver Rahman, M.R., Van Vuong, Q., 2019. Micro and nano encapsulation, retention and controlled release of flavor and aroma compounds: A critical review. Trends Food Sci. Technol. 86, 230-251. <https://doi.org/10.1016/j.tifs.2019.02.030>

Saini, A., Panesar, P.S., Dilbaghi, N., Prasad, M., Bera, M.B., 2022. Lutein extract-loaded nanoemulsions: Preparation, characterization, and application in dairy product. J. Food Process. Preserv. 46(11), 1-18. <https://doi.org/10.1111/jfpp.17082>

Salvi, V.R., Pawar, P., 2019. Nanostructured lipid carriers (NLC) system: A novel drug targeting carrier. J. Drug Deliv. Sci. Technol. 51, 255-267. <https://doi.org/10.1016/j.jddst.2019.02.017>

Santos Assunção, L., Muniz Bezerra, P.Q., Poletto, V.S.H., de Oliveira Rios, A., Ramos, I.G., Ferreira Ribeiro, C.D., Souza Machado, B.A., Druzian, J.I., Vieira Costa, J.A., Nunes, I.L., 2021. Combination of carotenoids from *Spirulina* and PLA/PLGA or PHB: New options to obtain bioactive nanoparticles. Food Chem. 346, 128742. <https://doi.org/10.1016/j.foodchem.2020.128742>

Santos Ribeiro, J.E., da Silva Sant'Ana, A.M., Martini, M., Sorce, C., Andreucci, A., de Melo, D.J.N., da Silva, F.L.H., 2019. *Rhodotorula glutinis* cultivation on cassava wastewater for carotenoids and fatty acids generation. Biocatal. Agric. Biotechnol. 22, 101419. <https://doi.org/10.1016/j.bcab.2019.101419>

Sarnaik, A., Nambissan, V., Pandit, R., Lali, A., 2018. Recombinant *Synechococcus elongatus* PCC 7942 for improved zeaxanthin production under natural light conditions. Algal Res. 36, 139-151. <https://doi.org/10.1016/j.algal.2018.10.021>

Sathasivam, R., Radhakrishnan, R., Kim, J.K., Park, S.U., 2021. An update on biosynthesis and regulation of carotenoids in plants. South African J. Bot. 140, 290-302. <https://doi.org/10.1016/j.sajb.2020.05.015>

Scheiner, D., 1976. Determination of ammonia and Kjeldahl nitrogen by indophenol method. Water Res. 10(1), 31-36. [https://doi.org/10.1016/0043-1354\(76\)90154-8](https://doi.org/10.1016/0043-1354(76)90154-8)

Scholten, E., 2019. Edible oleogels: How suitable are proteins as a structurant? Curr. Opin. Food Sci. 27, 36-42. <https://doi.org/10.1016/j.cofs.2019.05.001>

Scioli Montoto, S., Muraca, G., Ruiz, M.E., 2020. Solid lipid nanoparticles for drug delivery: Pharmacological and biopharmaceutical aspects. Front. Mol. Biosci. 7, 1-24. <https://doi.org/10.3389/fmolb.2020.587997>

Selim, A., Kassem, M.M., Bayoumy, S.M.M., El- Sawah, M.M.A., Ali, D.F.I., 2013. Effect of some sugars and agro-industrial by-Products on carotenoids production by some yeast strains of *Rhodotorula* Spp. J. Plant Prod. 4(7), 1097-1107. <https://doi.org/10.21608/jpp.2013.73735>

Selvakumar, P., Sivashanmugam, P., 2019. Ultrasound assisted oleaginous yeast lipid extraction and garbage lipase catalyzed transesterification for enhanced biodiesel production. Energy Convers. Manag. 179, 141-151. <https://doi.org/10.1016/j.enconman.2018.10.051>

Sen, S., 2019. The chemistry and biology of lycopene : Antioxidant for human health. Int. J. Adv. Life Sci. Res. 2(4), 8-14. <https://doi.org/10.31632/ijalsr.2019v02i04.002>

Sencient Food Colors, 2020. Consumer preference for natural products. <https://sensientfoodcolors.com/en-eu/market-trends/the-undeniable-value-of-colour/>

Šeregelj, V., Pezo, L., Šovljanski, O., Lević, S., Nedović, V., Markov, S., Tomić, A., Čanadanović-Brunet, J., Vulić, J., Šaponjac, V.T., Ćetković, G., 2021. New concept of fortified yogurt formulation with encapsulated carrot waste extract. Lwt. 138, 110732. <https://doi.org/10.1016/j.lwt.2020.110732>

Seveiri, R.M., Hamidi, M., Delattre, Rahmani, B., Darzi, S., Pierre, G., Sedighian, H., Brasselet, C., Karimitabar, F., Pishkhan Dibazar, S., Ghasemi, D., Amani, J., 2019. Characterization of the exopolysaccharides from *Rhodotorula minuta* IBRC-M 30135 and evaluation of their emulsifying, antioxidant and antiproliferative activities. Medical Sci. 23(97), 381-389.

Seveiri, R.M., Hamidi, M., Delattre, Sedighian, H., Pierre, G., Rahmani, B., Darzi, S., Brasselet, C., Karimitabar, F., Razaghpour, A., Amani, J., 2020. Characterization and prospective applications of the exopolysaccharides produced by *Rhodospiridium babjevae*. Adv. Pharm. Bull. 10(2), 254-263. <https://doi.org/10.34172/apb.2020.030>

Shakeri, S., Khoshbasirat, F., Maleki, M., 2021. *Rhodospiridium* sp. DR37: A novel strain for production of squalene in optimized cultivation conditions. Biotechnol. Biofuels. 14(1), 95. <https://doi.org/10.1186/s13068-021-01947-5>

Shang, W., Sun, Y., Song, J., Zhang, P., Hou, Y., Wang, H., Tan, M., 2023. Novel high internal phase oleogels-in-water pickering emulsions stabilized solely by whey protein isolate for 3D printing and fucoxanthin delivery. Food Hydrocoll. 140, 108609. <https://doi.org/10.1016/j.foodhyd.2023.108609>

Shantha, N.C., Decker, E.A., 1994. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. J. AOAC Int. 77(2), 421-424. <https://doi.org/10.1093/jaoac/77.2.421>

Sharayei, P., Azarpazhooh, E., Einafshar, S., Zomorodi, S., Zare, F., Ramaswamy, H.S., 2024. Optimization of wall materials for astaxanthin powder production from shrimp shell extract using simplex lattice mixture design. *J. Food Process. Preserv.* 2024(1), 9794290. <https://doi.org/10.1155/2024/9794290>

Sharma, R., Ghoshal, G., 2020. Optimization of carotenoids production by *Rhodotorula mucilaginosa* (MTCC-1403) using agro-industrial waste in bioreactor: A statistical approach. *Biotechnol. Reports.* 25, e00407. <https://doi.org/10.1016/j.btre.2019.e00407>

Shen, Y., Hong, S., Li, Y., 2022. Pea protein composition, functionality, modification, and food applications: A review, in: *Advances in Food and Nutrition Research.* Elsevier Inc. 101, 71-127. <https://doi.org/10.1016/bs.afnr.2022.02.002>

Sheng, Y.N., Luo, Y.H., Liu, S. B., Xu, W.T., Zhang, Y., Zhang, T., Xue, H., Zuo, W.B., Li, Y.N., Wang, C.Y., Jin, C.H., 2020. Zeaxanthin induces apoptosis via ROS-regulated MAPK and Akt signaling pathway in human gastric cancer cells. *Oncotargets. Ther.* 13, 10995-11006. <https://doi.org/10.2147/OTT.S272514>

Shi, Z., Kong, G., Wang, F., Gao, H., Wei, A., Ren, S., Yan, X., 2023. Improvement in the stability and bioavailability of pumpkin lutein using β -cyclodextrin microcapsules. *Food Sci. Nutr.* 11(6), 3067-3074. <https://doi.org/10.1002/fsn3.3288>

Shields-Menard, S.A., Amirsadeghi, M., Sukhbaatar, B., Revellame, E., Hernandez, R., Donaldson, J.R., French, W.T., 2015. Lipid accumulation by *Rhodococcus rhodochrous* grown on glucose. *J. Ind. Microbiol. Biotechnol.* 42(5), 693-699. <https://doi.org/10.1007/s10295-014-1564-7>

Shuai, X., McClements, D.J., Dai, T., Geng, Q., Wei, C., Wang, W., Chen, J., Zhang, M., Du, L., 2024. Effect of different oleogelators on physicochemical properties, oxidative stability and astaxanthin delivery of macadamia oil-based oleogels. *Food Res. Int.* 196, 115131. <https://doi.org/10.1016/j.foodres.2024.115131>

Siddeeg, A., Manzoor, M.F., Ahmad, M.H., Ahmad, N., Ahmed, Z., Khan, M.K.I., Maan, A.A., Nisa, M.U., Zeng, X.A., Ammar, A.F., 2019. Pulsed electric field-assisted ethanolic extraction of date palm fruits: Bioactive compounds, antioxidant activity and physicochemical properties. *Processes*, 7(9), 585.

<https://doi.org/10.3390/pr7090585>

Siegrist, M., Hartmann, C., 2020. Consumer acceptance of novel food technologies. *Nat. Food*. 1(6), 343-350. <https://doi.org/10.1038/s43016-020-0094-x>

Silva, E.J., Rocha e Silva, N.M.P., Rufino, R.D., Luna, J.M., Silva, R.O., Sarubbo, L.A., 2014. Characterization of a biosurfactant produced by *Pseudomonas cepacia* CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. *Coll. Surf. B Biointerfaces*. 117, 36-41.

<https://doi.org/10.1016/j.colsurfb.2014.02.012>

Singh, G., Sinha, S., Bandyopadhyay, K.K., Lawrence, M., Paul, D., 2018. Triaxenic growth of an oleaginous red yeast *Rhodospiridium toruloides* on waste “extract” for enhanced and concomitant lipid and β -carotene production. *Microb. Cell Fact.* 17, 1-10. <https://doi.org/10.1186/s12934-018-1026-4>

Singh, R.S., Kaur, N., 2018. Biochemical and molecular characterization of a new pullulan producer *Rhodospiridium paludigenum* PUPY-06. *J. Appl. Biol. Biotechnol.* 6(1), 28-37. <https://doi.org/10.7324/jabb.2018.60106>

Sinha, S., Das, S., Saha, B., Paul, D., Basu, B., 2023. Anti-microbial, anti-oxidant, and anti-breast cancer properties unraveled in yeast carotenoids produced via cost-effective fermentation technique utilizing waste hydrolysate. *Front. Microbiol.* 13, 1-8. <https://doi.org/10.3389/fmicb.2022.1088477>

Siregar, T.M., Margareta, M., 2019. Microencapsulation of carotenoids from red melinjo (*Gnetum gnemon* L.) peels extract. *J. Phys. Conf. Ser.* 1351, 012031.

<https://doi.org/10.1088/1742-6596/1351/1/012031>

- Sirikhet, J., Chanmahasathien, W., Raiwa, A., Kiattisin, K., 2021. Stability enhancement of lycopene in *Citrullus lanatus* extract via nanostructured lipid carriers. Food Sci. Nutr. 9(3), 1750-1760. <https://doi.org/10.1002/fsn3.2156>
- Sjölin, M., Thuvander, J., Wallberg, O., Lipnizki, F., 2020. Purification of sucrose in sugar beet molasses by utilizing ceramic nanofiltration and ultrafiltration membranes. Membranes. 10(1), 5. <https://doi.org/10.3390/membranes10010005>
- Solomon S. 2011. Sugarcane by-products based industries. India. Sugar Tech. 13(4), 408-416. <https://doi.org/10.1007/s12355-011-0114-0>
- Sosnik, A., Seremeta, K.P., 2015. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv. Colloid Interface Sci. 223, 40-54. <https://doi.org/10.1016/j.cis.2015.05.003>
- Sowmya, P.R.R., Arathi, B.P., Vijay, K., Baskaran, V., Lakshminarayana, R., 2017. Astaxanthin from shrimp efficiently modulates oxidative stress and allied cell death progression in MCF-7 cells treated synergistically with β -carotene and lutein from greens. Food Chem. Toxicol. 106, 58-69. <https://doi.org/10.1016/j.fct.2017.05.024>
- Stahl, W., Sies, H., 2003. Antioxidant activity of carotenoids. Mol. Aspects Med. 24(6), 345-351. [https://doi.org/10.1016/S0098-2997\(03\)00030-X](https://doi.org/10.1016/S0098-2997(03)00030-X)
- Stinco, C.M., Sentandreu, E., Mapelli-Brahm, P., Navarro, J.L., Vicario, I.M., Meléndez-Martínez, A.J., 2020. Influence of high pressure homogenization and pasteurization on the in vitro bioaccessibility of carotenoids and flavonoids in orange juice. Food Chem. 331, 127259. <https://doi.org/10.1016/j.foodchem.2020.127259>
- Stupar, A., Šregelj, V., Dias Ribeiro, B., Pezo, L., Cvetanović, A., Mišan, A., Marrucho, I., 2021. Recovery of β -carotene from pumpkin using switchable natural deep eutectic solvents. Ultrason. Sonochem. 76, 105638. <https://doi.org/10.1016/j.ultsonch.2021.105638>

Sun, T., Rao, S., Zhou, X., Li, L., 2022. Plant carotenoids: Recent advances and future perspectives. *Mol. Hortic.* 2(1), 1-21. <https://doi.org/10.1186/s43897-022-00023-2>

Sundaramahalingam, M.A., Sivashanmugam, P., 2023. Production of microbial carotenoid using innate inherent of the food industry wastewater. *J. Mol. Liq.* 388, 122772. <https://doi.org/10.1016/j.molliq.2023.122772>

Szotkowski, M., Plhalová, Ž., Sniegoňová, P., Holub, J., Chujanov, O., Špačková, D., Blažková, J., Márová, I., 2023. Conversion of mixed waste food substrates by carotenogenic yeasts of *Rhodotorula* sp. genus. *Microorganisms*. 11(4), 1013. <https://doi.org/10.3390/microorganisms11041013>

Tan, C., Xue, J., Lou, X., Abbas, S., Guan, Y., Feng, B., Zhang, X., Xia, S., 2014. Liposomes as delivery systems for carotenoids: Comparative studies of loading ability, storage stability and *in vitro* release. *Food Funct.* 5(6), 1232-1240. <https://doi.org/10.1039/c3fo60498e>

Tansel, B., Tansel, D.Z., 2013. Adhesion strength and spreading characteristics of EPS on membrane surfaces during lateral and central growth. *Coll. Surf. B Biointerfaces*. 111, 594-599. <https://doi.org/10.1016/j.colsurfb.2013.07.005>

Tarannum, N., Hossain, T.J., Ali, F., Das, T., Dhar, K., Nafiz, I.H., 2023. Antioxidant, antimicrobial and emulsification properties of exopolysaccharides from lactic acid bacteria of bovine milk: Insights from biochemical and genomic analysis. *Lwt.* 186, 115263. <https://doi.org/10.1016/j.lwt.2023.115263>

Tchakouteu, S.S., Kopsahelis, N., Chatzifragkou, A., Kalantzi, O., Stoforos, N.G., Koutinas, A.A., Aggelis, G., Papanikolaou, S., 2017. *Rhodosporidium toruloides* cultivated in NaCl-enriched glucose-based media: Adaptation dynamics and lipid production. *Eng. Life Sci.* 17(3), 237-248. <https://doi.org/10.1002/elsc.201500125>

Teeranachaideekul, V., Boribalnukul, P., Morakul, B., Junyaprasert, V.B., 2022. Influence of vegetable oils on in vitro performance of lutein-loaded lipid carriers for skin delivery: Nanostructured lipid carriers vs. nanoemulsions. *Pharmaceutics*. 14(10), 2160. <https://doi.org/10.3390/pharmaceutics14102160>

The European Parliament and the council of the European Union, 2009. Directive 2009/32. Off. J. Eur. Union 3-11.

Thomas, C.E., Luu, H.N., Wang, R., Adams-Haduch, J., Jin, A., Koh, W.P., Yuan, J.M., 2020. Association between dietary tomato Intake and the risk of hepatocellular carcinoma: The singapore chinese health study. *Cancer Epidemiol. Biomarkers Prev.* 29(7), 1430-1435. <https://doi.org/10.1158/1055-9965.EPI-20-0051>

Thumkasem, N., On-mee, T., Kongsinkaew, C., Chittapun, S., Pornpukdeewattana, S., Ketudat-Cairns, M., Thongprajukaew, K., Antimanon, S., Charoenrat, T., 2024. Enhanced high β -carotene yeast cell production by *Rhodotorula paludigena* CM33 and in vitro digestibility in aquatic animals. *Sci. Rep.* 14(1), 1-14. <https://doi.org/10.1038/s41598-024-59809-7>

Tian, X., Li, J., Wang, K., Fei, S., Zhang, X., Wu, C., Tan, M., Su, W., 2024. Microfluidic fabrication of core-shell fucoxanthin nanofibers with improved environmental stability for reducing lipid accumulation in vitro. *Food Chem.* 442, 138474. <https://doi.org/10.1016/j.foodchem.2024.138474>

Tirgarian, B., Yadegari, H., Bagheri, A., Neshagaran, E., Mardani, M., Farmani, J., 2023. Reduced-fat chocolate spreads developed by water-in-oleogel emulsions. *J. Food Eng.* 337, 111233. <https://doi.org/10.1016/j.jfoodeng.2022.111233>

Tkáčová, J., Čaplová, J., Klempová, T., Čertík, M., 2017. Correlation between lipid and carotenoid synthesis in torularhodin-producing *Rhodotorula glutinis*. *Ann. Microbiol.* 67(8), 541-551. <https://doi.org/10.1007/S13213-017-1284-0>

Tkáčová, J., Klempová, T., Čertík, M., 2018. Kinetic study of growth, lipid and carotenoid formation in β -carotene producing *Rhodotorula glutinis*. *Chem. Pap.* 72(5), 1193-1203. <https://doi.org/10.1007/s11696-017-0368-4>

Tolve, R., Galgano, F., Caruso, M.C., Tchuenbou-Magaia, F.L., Condelli, N., Favati, F., Zhang, Z., 2016. Encapsulation of health-promoting ingredients: applications in foodstuffs. *Int. J. Food Sci. Nutr.* 67(8), 888-918. <https://doi.org/10.1080/09637486.2016.1205552>

Topolska, K., Florkiewicz, A., Filipiak-Florkiewicz, A., 2021. Functional food-Consumer motivations and expectations. *Int. J. Environ. Res. Public Health* 18(10), 5327. <https://doi.org/10.3390/ijerph18105327>

Toschkova, S., 2022. Structural characterization of an exopolysaccharide produced by *Lactobacillus plantarum* Ts. *Acta Sci. Nat.* 9(3), 71-83. <https://doi.org/10.2478/asn-2022-0022>

Tsakona, S., Skiadaresis, A.G., Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Kookos, I.K., Koutinas, A.A., 2016. Valorisation of side streams from wheat milling and confectionery industries for consolidated production and extraction of microbial lipids. *Food Chem.* 198, 85-92. <https://doi.org/10.1016/j.foodchem.2015.11.031>

Tsogtoo, B., Taarji, N., Melanie, H., Khalid, N., Tsolmon, S., Kobayashi, I., Neves, M.A., Nakajima, M., 2020. Emulsion-based extraction of β -sitosterol and carotenoids from sea buckthorn (*Hippophae rhamnoides*) pomace. *Int. Food Res. J.* 27(1), 56-65.

Tyle, P., 1993. Effect of size, shape and hardness of particles in suspension on oral texture and palatability. *Acta Psychol.* 84, 111-118. [https://doi.org/10.1016/0001-6918\(93\)90077-5](https://doi.org/10.1016/0001-6918(93)90077-5)

Uwineza, P.A., Waśkiewicz, A., 2020. Recent advances in supercritical fluid extraction of natural bioactive compounds from natural plant materials. *Molecules.* 25(17), 3847. <https://doi.org/10.3390/molecules25173847>

Van Hoang, D., Pham, N.M., Lee, A.H., Tran, D.N., Binns, C.W., 2018. Dietary carotenoid intakes and prostate cancer risk: A case-control study from Vietnam. *Nutrients.* 10(1), 1-11. <https://doi.org/10.3390/nu10010070>

Vargas-Sinisterra, A.F., Ramírez-Castrillón, M., 2021. Yeast carotenoids: Production and activity as antimicrobial biomolecule. *Arch. Microbiol.* 203(3), 873-888. <https://doi.org/10.1007/s00203-020-02111-7>

Varma, K., Jude, S., Nair, R.V.R., Varghese, B.A., Jacob, J., Amalraj, A., Kuttappan, S., 2021. Novel formulation of liposomal lutein using nanofiber weaving (NFW) technology: Antioxidant property and in vitro release studies. *Food Hydrocoll. Health.* 1, 100025. <https://doi.org/10.1016/j.fhfh.2021.100025>

Varmira, K., Habibi, A., Bahramian, E., Jamshidpour, S., 2016. Progressive agents for improvement of carotenogenesis in *Rhodotorula rubra*. *Orig. Res. Artic. J. Adv. Food Sci. Technol.* 3(2), 70-78.

Varzakakou, M., Roukas, T., Kotzekidou, P., 2010. Effect of the ratio of (+) and (-) mating type of *Blakeslea trispora* on carotene production from cheese whey in submerged fermentation. *World J. Microbiol. Biotechnol.* 26(12), 2151-2156. <https://doi.org/10.1007/s11274-010-0398-3>

Vazquez-Rodriguez, A., Vasto-Anzaldo, X.G., Perez, D.B., Vázquez-Garza, E., Chapoy-Villanueva, H., García-Rivas, G., Garza-Cervantes, J.A., Gómez-Lugo, J.J., Gomez-Loredo, A.E., Garza Gonzalez, M.T., Zarate, X., Morones-Ramirez, J.R., 2018. Microbial competition of *Rhodotorula mucilaginosa* UANL-001L and *E. coli* increase biosynthesis of non-toxic exopolysaccharide with applications as a wide-spectrum antimicrobial. *Sci. Rep.* 8(1), 1-14. <https://doi.org/10.1038/s41598-017-17908-8>

Vecino, X., Barbosa-Pereira, L., Devesa-Rey, R., Cruz, J.M., Moldes, A.B., 2015. Optimization of extraction conditions and fatty acid characterization of *L. pentosus* cell-bound biosurfactant/bioemulsifier. *J. Sci. Food Agric.* 95(2), 313-320. <https://doi.org/10.1002/jsfa.6720>

Viguera, M., Marti, A., Masca, F., Prieto, C., Calvo, L., 2016. The process parameters and solid conditions that affect the supercritical CO₂ extraction of the lipids produced by microalgae. *J. Supercrit. Fluids.* 113, 16-22. <https://doi.org/10.1016/j.supflu.2016.03.001>

Vila, E., Hornero-Méndez, D., Lareo, C., Saravia, V., 2020. Biotechnological production of zeaxanthin by an antarctic *Flavobacterium*: Evaluation of culture conditions. *J. Biotechnol.* 319, 54-60. <https://doi.org/10.1016/j.jbiotec.2020.05.014>

- Viñas-Ospino, A., López-Malo, D., Esteve, M.J., Frígola, A., Blesa, J., 2023. Green solvents: Emerging alternatives for carotenoid extraction from fruit and vegetable by-products. *Foods*. 12(4), 1-19. <https://doi.org/10.3390/foods12040863>
- Vladić, J., Jerković, I., Radman, S., Molnar Jazić, J., Ferreira, A., Maletić, S., Gouveia, L., 2022. Supercritical CO₂ extract from microalga *Tetrademus obliquus*: The effect of high-pressure pre-treatment. *Molecules*. 27, 3883. <https://doi.org/10.3390/molecules27123883>
- Vulić, J., Šeregelj, V., Šaponjac, V.T., Banjac, M.K., Kovacević, S., Šovljanski, O., Cetković, G., Canadanović-Brunet, J., Jevri, L., Podunavac-Kuzmanović, S., 2022. From sweet corn by-products to carotenoid-rich encapsulates for food applications. *Processes*. 10(8), 1616. <https://doi.org/10.3390/pr10081616>
- Wang, H.B., He, F., Lu, M.B., Zhao, C.F., Xiong, L., Yu, L.J., 2014. High-quality lycopene overaccumulation via inhibition of γ -carotene and ergosterol biosyntheses in *Blakeslea trispora*. *J. Funct. Foods*. 7, 435-442. <https://doi.org/10.1016/j.jff.2014.01.014>
- Wang, L., Liu, Z., Jiang, H., Mao, X., 2021. Biotechnology advances in β -carotene production by microorganisms. *Trends Food Sci. Technol.* 111, 322-332. <https://doi.org/10.1016/j.tifs.2021.02.077>
- Wang, Q., Espert, M., Larrea, V., Quiles, A., Salvador, A., Sanz, T., 2023. Comparison of different indirect approaches to design edible oleogels based on cellulose ethers. *Food Hydrocoll.* 134, 108007. <https://doi.org/10.1016/j.foodhyd.2022.108007>
- Wang, Y.X., Xin, Y., Yin, J.Y., Huang, X.J., Wang, J.Q., Hu, J.L., Geng, F., Nie, S.P., 2022. Revealing the architecture and solution properties of polysaccharide fractions from *Macrolepiota aluminosa* (Berk.) Pegler. *Food Chem.* 368, 130772. <https://doi.org/10.1016/j.foodchem.2021.130772>

Warjoto, R.E., Jennifer, J., Lay, B.W., 2020. Carotenoid production by *Rhodospiridium paludigenum* using orange peel extract as substrate. Biosaintifika J. Biol. Biol. Educ. 12(3), 319-328.

<https://doi.org/10.15294/biosaintifika.v12i3.24867>

Wei, C., Wu, T., Ao, H., Qian, X., Wang, Z., Sun, J., 2020. Increased torulene production by the red yeast, *Sporidiobolus pararoseus*, using citrus juice. Prep. Biochem. Biotechnol. 50(1), 66-73. <https://doi.org/10.1080/10826068.2019.1663533>

Wu, C.C., Honda, K., Kazuhito, F., 2023. Current advances in alteration of fatty acid profile in *Rhodotorula toruloides*: A mini-review. World J. Microbiol. Biotechnol. 39(9), 1-16. <https://doi.org/10.1007/s11274-023-03595-3>

Wu, X., Zha, J., Koffas, M.A.G., 2020. Microbial production of bioactive chemicals for human health. Curr. Opin. Food Sci. 32, 9-16.

<https://doi.org/10.1016/j.cofs.2019.12.007>

Xiang, H., Zhong, A., Wang, H., Xiao, L., Deng, T., Hu, T., Wen, P., 2022. Fabrication of alkali lignin-based emulsion electrospun nanofibers for the nanoencapsulation of beta-carotene and the enhanced antioxidant property. Int. J. Biol. Macromol. 218, 739-750. <https://doi.org/10.1016/j.ijbiomac.2022.07.121>

Xiao, Y., Huang, W., Li, D., Song, J., Liu, C., Wei, Q., Zhang, M., Yang, Q., 2018. Thermal degradation kinetics of all-*trans* and *cis*-carotenoids in a light-induced model system. Food Chem. 239, 360-368. <https://doi.org/10.1016/j.foodchem.2017.06.107>

Xu, T., Zhang, J., Jin, R., Cheng, R., Wang, X., Yuan, C., Gan, C., 2022. Physicochemical properties, antioxidant activities and in vitro sustained release behaviour of co-encapsulated liposomes as vehicle for vitamin E and β -carotene. J. Sci. Food Agric. 102, 5759-5767. <https://doi.org/10.1002/jsfa.11925>

- Xue, S.J., Li, X.C., Huang, X., Liu, J., Li, Y., Zhang, X.T., Zhang, J.Y., 2023. Diversity investigation of cultivable yeasts associated with honeycombs and identification of a novel *Rhodotorula toruloides* strain with the robust concomitant production of lipid and carotenoid. *Bioresour. Technol.* 370, 128573. <https://doi.org/10.1016/j.biortech.2022.128573>
- Yabuzaki, J., 2017. Carotenoids Database: structures, chemical fingerprints and distribution among organisms. *Database.* 2017, 1-11. <https://doi.org/10.1093/DATABASE/BAX004>
- Yang, G., Liu, G.L., Wang, S.J., Chi, Z.M., Chi, Z., 2020. Pullulan biosynthesis in yeast-like fungal cells is regulated by the transcriptional activator Msn2 and cAMP-PKA signaling pathway. *Int. J. Biol. Macromol.* 157, 591-603. <https://doi.org/10.1016/j.ijbiomac.2020.04.174>
- Yara-Varón, E., Fabiano-Tixier, A.S., Balcells, M., Canela-Garayoa, R., Bily, A., Chemat, F., 2016. Is it possible to substitute hexane with green solvents for extraction of carotenoids? A theoretical versus experimental solubility study. *RSC Adv.* 6(33), 27750-27759. <https://doi.org/10.1039/c6ra03016e>
- Yen, H.W., Palanisamy, G., Su, G.C., 2019. The Influences of supplemental vegetable oils on the growth and β -carotene accumulation of oleaginous yeast-*Rhodotorula glutinis*. *Biotechnol. Bioprocess Eng.* 24, 522-528. <https://doi.org/10.1007/s12257-019-0027-4>
- Yildiz, Z.I., Topuz, F., Kilic, M.E., Durgun, E., Uyar, T., 2023. Encapsulation of antioxidant beta-carotene by cyclodextrin complex electrospun nanofibers: Solubilization and stabilization of beta-carotene by cyclodextrins. *Food Chem.* 423, 136284. <https://doi.org/10.1016/j.foodchem.2023.136284>
- Yimyoo, T., Yongmanitchai, W., Limtong, S., 2011. Carotenoid production by *Rhodospiridium paludigenum* DMKU3-LPK4 using glycerol as the carbon source. *Kasetsart J. Nat. Sci.* 45(1), 90-100.

Yoon, S.H., Lee, S.H., Das, A., Ryu, H.K., Jang, H.J., Kim, J.Y., Oh, D.K., Keasling, J.D., Kim, S.W., 2009. Combinatorial expression of bacterial whole mevalonate pathway for the production of β -carotene in *E. coli*. *J. Biotechnol.* 140(3-4), 218-226. <https://doi.org/10.1016/j.jbiotec.2009.01.008>

Yousefbeyk, F., Vatandoost, H., Golfakhrabadi, F., Mirzaee, Z., Abai, M.R., Amin, G., Khanavi, M., 2018. Antioxidant and larvicidal activity of areal parts of *Scrophularia striata* against malaria vector *Anopheles stephensi*. *J. Arthropod. Borne. Dis.* 12(2), 119-126. <https://doi.org/10.18502/jad.v12i2.37>

Zampolli, J., De Giani, A., Di Canito, A., Sello, G., Di Gennaro, P., 2022. Identification of a novel biosurfactant with antimicrobial activity produced by *Rhodococcus opacus* R7. *Microorganisms.* 10(2), 475. <https://doi.org/10.3390/microorganisms10020475>

Zardini, A.A., Mohebbi, M., Farhoosh, R., Bolurian, S., 2018. Production and characterization of nanostructured lipid carriers and solid lipid nanoparticles containing lycopene for food fortification. *J. Food Sci. Technol.* 55(1), 287-298. <https://doi.org/10.1007/s13197-017-2937-5>

Zeng, Y., Wang, R., Liang, J., Zhang, H., Yi, J., Liu, Z., 2023. Strategies for recovery, purification and quantification of torularhodin produced by *Rhodotorula mucilaginosa* using different carbon sources. *Fermentation.* 9(9), 846. <https://doi.org/10.3390/fermentation9090846>

Zhang, J., Li, Q.R., Zhang, M.H., You, Y., Wang, Y., Wang, Y.H., 2019. Enhancement of carotenoid biosynthesis in *Phaffia rhodozyma* PR106 under stress conditions. *Biosci. Biotechnol. Biochem.* 83(12), 2375-2385. <https://doi.org/10.1080/09168451.2019.1650633>

Zhang, S., Lu, Y., He, X., Su, Y., Hu, F., Wei, X., Pan, M., Zhou, Q., Yang, W., 2023. Lutein inhibits tumor progression through the ATR/Chk1/p53 signaling pathway in non-small cell lung cancer. *Phyther. Res.* 37(4), 1260-1273. <https://doi.org/10.1002/ptr.7682>

Zhang, X., Fan, M., Yuan, Y., Dong, J., Yin, H., He, Y., Ma, L., Wang, D., Yu, J., 2022. Investigation of the optimal fabrication of a single-carrier encapsulated fucoxanthin based on colloidal nanoparticles. J. Ind. Eng. Chem. 114, 96-107.

<https://doi.org/10.1016/j.jiec.2022.06.035>

Zhang, Z., Guo, C., Jiang, H., Han, B., Wang, X., Li, S., Lv, Y., Lv, Z., Zhu, Y., 2020. Inflammation response after the cessation of chronic arsenic exposure and post-treatment of natural astaxanthin in liver: Potential role of cytokine-mediated cell-cell interactions. Food Funct. 11(10), 9252-9262. <https://doi.org/10.1039/d0fo01223h>

Zhao, D., Li, C., 2022. Multi-omics profiling reveals potential mechanisms of culture temperature modulating biosynthesis of carotenoids, lipids, and exopolysaccharides in oleaginous red yeast *Rhodotorula glutinis* ZHK. Lwt. 171, 114103.

<https://doi.org/10.1016/j.lwt.2022.114103>

Zhao, D., Li, C., 2023. Effects of TiO₂ and H₂O₂ treatments on the biosynthesis of carotenoids and lipids in oleaginous red yeast *Rhodotorula glutinis* ZHK. Lwt. 180, 114733. <https://doi.org/10.1016/j.lwt.2023.114733>

Zhao, L., Temelli, F., Curtis, J.M., Chen, L., 2017. Encapsulation of lutein in liposomes using supercritical carbon dioxide. Food Res. Int. 100, 168-179.

<https://doi.org/10.1016/j.foodres.2017.06.055>

Zhao, W., Wei, Z., Xue, C., Meng, Y., 2023. Development of food-grade oleogel via the aerogel-templated method: Oxidation stability, astaxanthin delivery and emulsifying application. Food Hydrocoll. 134, 108058.

<https://doi.org/10.1016/j.foodhyd.2022.108058>

Zhao, Y., Guo, L., Xia, Y., Zhuang, X., Chu, W., 2019. Isolation, identification of carotenoid-producing *Rhodotorula* sp. from marine environment and optimization for carotenoid production. Mar. Drugs 17(3), 161.

<https://doi.org/10.3390/md17030161>

Zheng, X., Hu, R., Chen, D., Chen, J., He, W., Huang, L., Lin, C., Chen, H., Chen, Y., Zhu, J., Qi, F., Xue, T., 2021. Lipid and carotenoid production by the *Rhodospiridium toruloides* mutant in cane molasses. *Bioresour. Technol.* 326, 124816. <https://doi.org/10.1016/j.biortech.2021.124816>

Zhou, L., Zhang, W., Wang, J., Zhang, R., Zhang, J., 2022. Comparison of oil-in-water emulsions prepared by ultrasound, high-pressure homogenization and high-speed homogenization. *Ultrason. Sonochem.* 82, 105885. <https://doi.org/10.1016/j.ultsonch.2021.105885>

Zia-Ul-Haq, M., Dewanjee, S., Riaz, M., 2021. Carotenoids: Structure and function in the human body. Springer. 1-42. <https://doi.org/10.1007/978-3-030-46459-2>