AGRICULTURAL UNIVERSITY OF ATHENS

DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION

LABORATORY OF FOOD PROCESS ENGINEERING

Valorisation of renewable resources produced by sugarcane mills and oilseed processing industries via microbial and enzymatic processes for the production of fumaric acid, wax esters, biolubricants and oleogels

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ

ΕΡΓΑΣΤΗΡΙΟ ΜΗΧΑΝΙΚΗΣ ΤΡΟΦΙΜΩΝ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Αξιοποίηση ανανεώσιμων πρώτων υλών από βιομηχανίες επεξεργασίας ζαχαροκάλαμου και σπόρων πλούσιων σε φυτικά έλαια μέσω μικροβιακών και ενζυμικών διεργασιών για την παραγωγή φουμαρικού οξέος, κηρών, βιολιπαντικών και ελαιοπηκτωμάτων

ΑΙΚΑΤΕΡΙΝΗ Κ. ΠΑΠΑΔΑΚΗ

ΑΘΗΝΑ 2018

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ

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Abstract

This PhD thesis focuses on the processing and valorization of food industry raw materials, including very high polarity (VHP) cane sugar, molasses, soybean cake, and reneable resources, such as fatty acid distillates derived from vegetable oil refining processes, towards the development of novel biorefinery concepts. The aim was the production of value-added bio-economy products, which can be applied in the food industry, through bioprocess development. The chemical composition of all raw materials was initially quantified, including soluble sugars, nitrogen, protein, phenolic compounds, ash, moisture content, trace minerals and fibers. Thereafter, the proper processing methods were developed for the biotechnological production of intermediate products, such as microbial oils, and food additives, such as fumaric acid and carotenoids. Additionally, fatty acid esters and oleogels, as novel bio-based products for the food industry, were produced in the last part of this thesis.

Initially, the research focused on the soybean cake processing method via the enzymatic route, aiming to the valorization of the protein content. A two-stage bioprocess was developed employing solid state fungal fermentation for the production of crude enzymes that were used for enzymatic hydrolysis of soybean cake to produce a nutrient-rich fermentation feedstock. The effect of temperature and initial enzyme activity on the hydrolysis process was evaluated through the determination of free amino nitrogen (FAN) and inorganic phosphorus concentration in the produced hydrolysate.

Soybean cake hydrolysate was utilized as pre-culture fermentation medium in the cultivation of the fungal strain *Rhizopus arrhizus* for the production of fumaric acid. Batch and fed-batch fermentations were conducted in shake flasks for the production of fumaric acid using different raw materials, such as mixtures of glucose and fructose, VHP cane sugar and molasses. The highest fumaric acid concentration (40 g/L) with a sugar conversion yield of 0.86 g/g was achieved in a fermentation medium containing VHP cane sugar inoculated with a pre-culture medium produced with soybean cake hydrolysate at initial FAN concentration of 200 mg/L. The utilization of molasses showed that its high content in specific phenolic compounds and trace elements was inhibitory for the production of fumaric acid.

Microbial oil and carotenoids production by the oleaginous yeast *Rhodosporidium toruloides* DSM 4444 was assessed using VHP cane sugar and molasses. Shake flask fermentations using VHP cane sugar led to microbial oil concentration of 8.1 g/L and total dry weight of 23.8 g/L. The effect of supplementation with phosphate salts, trace elements and commercial nitrogen sources in a molasses-based medium was evaluated in shake flask

cultures for microbial oil production. Experimental results showed that 12.7 g/L microbial oil was achieved with an intracellular content of 59% (w/w), when the medium contained all the supplements. Fed-batch fermentations in bioreactor were also carried out using molasses with either commercial nitrogen sources or soybean cake hydrolysate. The highest microbial oil concentration (18.4 g/L) with an intracellular lipid content of 49.8% (w/w) and carotenoids content of 88.9 μg/g was achieved when soybean cake hydrolysate was used.

Microbial oils and fatty acid distillates were subsequently used as raw materials for the production of value-added bio-based products. An enzymatic bioprocess was developed for wax esters production using oleyl, cetyl and behenyl alcohols. The enzymatic process was optimized, regarding temperature and initial enzyme concentration, using commercial (Novozyme 435, Lipozyme) and non-commercial lipases. Conversion yields up to 94% were achieved. The properties acid value, iodine value, saponification value, viscosity and thermal behavior, using differential scanning calorimetry (DSC), of the produced wax esters were evaluated, which showed comparable properties to natural waxes (e.g. carnauba wax, beeswax). Furthermore, polyol ester production was carried out using microbial oils with neopentyl glycol or trimethylolpropane polyols. The reaction was catalyzed by commercial lipases (Lipomod 34MDP) resulting inconversion yields up to 88%. The physicochemical properties of the produced polyol esters showed that they could be used as biolubricants.

Finally, the produced bio-based was esters were evaluated as novel oleogelator agents for the production of oleogels. Oleogellation process was developed using extra virgin olive oil, soybean oil and microbial oil as base oils. The produced oleogels were evaluated through the analysis of color, crystal morphology, using polarised light microscopy (PLM), texture, using texture analyser, rheological and thermal behavior, using rheometer and differential scanning calorimetry, respectively. The novel oleogels produced in this study showed similar properties with spreadable fat-based products and could be a potential substitute of saturated and *trans* fatty acids in food products.

Keywords: Circular Economy; Biorefineries; Food and by-products processing; Bioprocessing; Biotechnology; Fermentation; Enzymes; Microbial oil; Carotenoids; food additives; Oleogels.

Περίληψη

Η παρούσα διδακτορική διατριβή εστίασε στην επεξεργασία πρώτων υλών της βιομηχανίας τροφίμων, όπως ακατέργαστη ζάχαρη, μελάσα, σογιάλευρο και παράπλευρα ρεύματα από την επεξεργασία εξευγενισμού των βρώσιμων ελαίων, στα πλαίσια ανάπτυξης καινοτόμων βιοδιυλιστηρίων. Ο στόχος ήταν η ανάπτυξη των κατάλληλων βιοδιεργασιών που θα οδηγήσουν στην παραγωγή νέων προϊόντων βιο-οικονομίας, με ευρεία εφαρμογή στη βιομηχανία τροφίμων. Ειδικότερα, πραγματοποιήθηκε ποσοτικός προσδιορισμός της χημικής σύστασης των πρώτων υλών με σκοπό να προσδιοριστούν τα κυριότερα συστατικά αυτών, όπως ελεύθερα σάκχαρα, άζωτο, πρωτεΐνη, φαινολικά συστατικά, τέφρα, περιεκτικότητα σε νερό, ιχνοστοιχεία και διαιτητικές ίνες. Με βάση τη χημική σύσταση των πρώτων υλών επιλέχθηκαν οι κατάλληλες μέθοδοι επεξεργασίας και αξιοποίησης αυτών για την βιοτεχνολογική παραγωγή ενδιάμεσων προϊόντων, όπως μικροβιακά λιπίδια, και πρόσθετων και βιολειτουργικών συστατικών τροφίμων, όπως φουμαρικό οξύ και καροτενοειδή. Επίσης, στο τελικό στάδιο της μελέτης παρήχθησαν καινοτόμα προϊόντα με ευρεία εφαρμογή στη βιομηχανία τροφίμων, όπως εστέρες λιπαρών οξέων και ελαιοπηκτές.

Το πρώτο στάδιο της μελέτης εστίασε στην επεξεργασία του σογιαλεύρου με ενζυμικές βιοδιεργασίες, με στόχο την αξιοποίηση των πρωτεϊνών του σογιαλεύρου για την παραγωγή θρεπτικού μέσου κατάλληλου για μικροβιακές ζυμώσεις. Η διεργασία αυτή περιελάμβανε την παραγωγή ακατέργαστων πρωτεολυτικών ενζύμων μέσω ζύμωσης στερεής κατάστασης και χρήση των ενζύμων αυτών για την υδρόλυση του σογιαλεύρου. Η ενζυμική υδρόλυση βελτιστοποιήθηκε όσον αφορά τη θερμοκρασία υδρόλυσης και την αρχική ενζυμική ενεργότητα. Το παραχθέν υδρόλυμα σογιαλεύρου αξιολογήθηκε μέσω του χημικού προσδιορισμού του αζώτου των ελεύθερων αμινομάδων των αμινοξέων και των πεπτιδίων (free amino nitrogen - FAN) και του ανόργανου φωσφόρου.

Το υδρόλυμα σογιαλεύρου χρησιμοποιήθηκε ως θρεπτικό μέσο στην προκαλλιέργεια ανάπτυξης του μυκητιακού στελέχους *Rhizopus arrhizus* NRRL 2582 με στόχο τη βιοτεχνολογική παραγωγή φουμαρικού οξέος. Σε αυτό το στάδιο της μελέτης πραγματοποιήθηκαν ασυνεχείς και ημι-συνεχείς ζυμώσεις σε αναδευόμενες φιάλες χρησιμοποιώντας διαφορετικές πρώτες ύλες, όπως μείγμα εμπορικής γλυκόζης-φρουκτόζης, ακατέργαστη ζάχαρη και μελάσα. Τα αποτελέσματα έδειξαν ότι η μεγαλύτερη παραγωγή φουμαρικού οξέος (40 g/L) με απόδοση 0,86 g/g επιτεύχθηκε στο υπόστρωμα ακατέργαστης ζάχαρης. Ο συνδυασμός υδρολύματος σογιαλεύρου και μελάσας δεν επέφερε ανάλογα αποτελέσματα, καθώς ο μεταβολισμός του μύκητα *R. arrhizus* στράφηκε προς την παραγωγή

αιθανόλης. Επιπρόσθετα, ο ποσοτικός προσδιορισμός της σύστασης της μελάσας έδειξε ότι περιέχει σημαντικές ποσότητες συγκεκριμένων φαινολικών συστατικών και αυξημένη περιεκτικότητα ιχνοστοιχείων που επηρεάζουν το μεταβολισμό των μυκήτων του γένους *Rhizopus*.

Στη συνέχεια μελετήθηκε η δυνατότητα παραγωγής μικροβιακών λιπιδίων και καροτενοειδών από την ζύμη *Rhodosporidium toruloides* DSM 4444. Κατά την μελέτη ασυνεχών ζυμώσεων που πραγματοποιήθηκαν σε αναδευόμενες φιάλες με υπόστρωμα ακατέργαστης ζάχαρης, η μεγαλύτερη παραγωγή μικροβιακών λιπιδίων ήταν 8,1 g/L με 23,8 g/L ξηρή κυτταρική μάζα. Ακολούθησαν ασυνεχείς ζυμώσεις με στόχο την μελέτη της επίδρασης διαφορετικών θρεπτικών συστατικών (π.χ. φωσφορικών αλάτων και ιχνοστοιχείων) στην παραγωγή μικροβιακών λιπιδίων. Τα καλύτερα αποτελέσματα (12,7 g/L συγκέντρωση μικροβιακών λιπιδίων με 59% κ.β. λιποπεριεκτικότητα) σημειώθηκαν με την προσθήκη τόσο των φωσφορικών αλάτων όσο και του μείγματος των ιχνοστοιχείων. Στη συνέχεια, πραγματοποιήθηκαν ημι-συνεχείς ζυμώσεις σε βιοαντιδραστήρα σε υπόστρωμα μελάσας με χρήση εμπορικών πηγών αζώτου ή υδρολύματος σογιαλεύρου. Η χρήση υδρολύματος σογιαλεύρου σε ημι-συνεχείς ζυμώσεις οδήγησε στη μεγαλύτερη παραγωγή μικροβιακών λιπιδίων (18,4 g/L) με ταυτόχρονη παραγωγή καροτενοειδών με μέγιστη περιεκτικότητα 88,9 μg/g ξηρής κυτταρικής μάζας.

Ακολούθως, η ερευνητική μελέτη επικεντρώθηκε στην παραγωγή διαφόρων χημικών προϊόντων υψηλής προστιθέμενης αξίας, ως καινοτόμες εφαρμογές της χρήσης μικροβιακών λιπιδίων αλλά και της αξιοποίησης και επεξεργασίας των παράπλευρων ρευμάτων που προκύπτουν από τον εξευγενισμό των φυτικών ελαίων. Αρχικά, η ενζυμική μετατροπή των μικροβιακών και φυτικής προέλευσης λιπιδίων για την παραγωγή εστέρων μελετήθηκε με την χρήση ελαϊκής, παλμιτικής και βεχενυλικής αλκοόλης. Οι εστέρες αυτοί που παράγονται από αλκοόλες μεγάλης ανθρακικής αλυσίδας ονομάζονται κηροί. Πραγματοποιήθηκε βελτιστοποίηση της ενζυμικής διεργασίας ως προς την θερμοκρασία και την αρχική ενζυμική ενεργότητα με χρήση εμπορικών (Novozyme 435, Lipozyme) και μη εμπορικών λιπασών, που οδήγησαν σε εξαιρετικά υψηλές αποδόσεις βιομετατροπής, έως και 94%. Μελετήθηκαν οι χημικές και φυσικές ιδιότητες των παραγόμενων βιογενών κηρών, όπως οξύτητα, αριθμός ιωδίου, αριθμός σαπωνοποποίησης, ιξώδες και θερμική συμπεριφορά με χρήση θερμιδομετρίας σάρωσης (differential scanning calorimetry, DSC), και βρέθηκαν παρόμοιες με αυτές των ευρέως χρησιμοποιούμενων φυτικών κηρών (π.χ. καρναουβικός κηρός, κηρός μέλισας κ.α.). Εν συνεχεία, η παραγωγή εστέρων με βιολιπαντικές ιδιότητες από μικροβιακά λιπίδια πραγματοποιήθηκε με χρήση των πολυολών νεοπεντυλογλυκόλη και

τριμεθυλοπροπάνιο. Η ενζυμική αντίδραση καταλύθηκε από εμπορικές λιπάσες (Lipomod 34MDP) και τα αποτελέσματα έδειξαν ότι ο μέγιστος βαθμός εστεροποίησης που επιτεύχθηκε ήταν 88%, ενώ η καταλληλότητά τους για χρήση ως βιολιπαντικές ουσίες επιβεβαιώθηκε μέσω της μελέτης των ιδιοτήτων των εν λόγω εστέρων.

Στο τελευταίο στάδιο της διδακτορικής διατριβής μελετήθηκε η ανάπτυξη ελαιοπηκτωμάτων ως καινοτόμος εφαρμογή των παραχθέντων βιογενών κηρών. Οι ελαιώδεις βάσεις για την παραγωγή ελαιοπηκτωμάτων ήταν ελαιόλαδο, σογιέλαιο και μικροβιακά λιπίδια, ενώ οι βιογενείς κηροί που χρησιμοποιήθηκαν προέρχονταν είτε από μικροβιακά λιπίδια είτε από παράπλευρα ρεύματα εξευγενισμένων ελαίων. Η αξιολόγηση των ελαιοπηκτωμάτων πραγματοποιήθηκε μέσω της μελέτης των ιδιοτήτων τους, όπως το χρώμα, η κρυσταλλική μορφή μέσω μικροσκοπίου πολωμένου φωτός (polarised light microscopy, PLM), η υφή μέσω αναλυτή υφής (texture analyser), η ρεολογική και η θερμική συμπεριφορά, με χρήση ρεομέτρου και θερμιδομετρία σάρωσης, αντίστοιχα. Τα αποτελέσματα έδειξαν ότι οι ιδιότητες των παραχθέντων ελαιοπηκτών ομοιάζουν με εκείνες τροφίμων, όπως λιπαρές ύλες επικάλυψης, και δύναται να χρησιμοποιηθούν ως υποκατάστατα κορεσμένων και *trans* λιπαρών υλών σε προϊόντα τροφίμων.

Λέξεις-κλειδιά: Κυκλική οικονομία; Βιοδιυλιστήρια; Επεξεργασία τροφίμων και παραπροϊόντων; Βιοδιεργασίες; Βιοτεχνολογία; Ζύμωση; Ένζυμα; Μικροβιακά λιπίδια; Καροτενοειδή; Πρόσθετα τροφίμων; Ελαιοπηκτές.

Dissemination activities and research visits

List of publications - related to this thesis

1. **Papadaki A.**, Cipolatti E.P., Aguieiras E.C.G., Pinto M.C.C., Kopsahelis N., Freire D.M.G., Mandala I., Koutinas A.A.[*] (2018). Development of microbial oil wax-based oleogel with potential application in food formulations. *Food and Bioprocess Technology*, In Press.

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3. **Papadaki A.**, Papapostolou H., Alexandri M., Kopsahelis N., Papanikolaou S., de Castro A.M., Freire D.M.G., Koutinas A.A.[*] (2018). Fumaric acid production using renewable resources from biodiesel and cane sugar production processes. *Environmental Science and Pollution Research*, 25: 35960-35970.

4. Fernandes K.V., **Papadaki A.**, da Silva Cavalcanti J.A., Fernandez-Lafuente R., Koutinas A.A.[*], Freire D.M.G.[*] (2018). Enzymatic esterification of palm fatty acids distillate for the production of polyol esters with biolubricant properties. *Industrial Crops and Products*, 116: 90-96.

5. **Papadaki A.**, Mallouchos A., Efthymiou M.-N., Gardeli C., Kopsahelis N., Papanikolaou S., Koutinas A.A.[*] (2017). Production of wax esters via microbial oil synthesis from food industry waste and by-product streams. *Bioresource Technology*, 245, Part A, 274-282.

6. **Papadaki A.**, Androutsopoulos N., Patsalou M., Koutinas M., Kopsahelis N., de Castro A.M., Papanikolaou S., Koutinas A.A.[*] (2017). Biotechnological Production of Fumaric Acid: The effect of morphology of *Rhizopus arrhizus* NRRL 2582. *Fermentation*, 3(3):33.

List of publications - not related to this thesis

1. Boviatsi E., **Papadaki A.***, Efthymiou M.-N., Nychas G.-J. E., Papanikolaou S., da Silva J.A.C., Freire D.M.G., Koutinas A.* (2018). Valorisation of sugarcane molasses for the production of microbial lipids via fermentation of two *Rhodosporidium* strains for enzymatic synthesis of polyol esters. *Journal of Chemical Technology and Biotechnology*, In Press.
2. Koutinas M.*, Vasquez M.I., Nicolaou E., Pashali P., Kyriakou E., Loizou E., **Papadaki A**., Koutinas A.A., Vyrides I. (2018). Biodegradation and toxicity of emerging contaminants: Isolation of an exopolysaccharide-producing *Sphingomonas* sp. for ionic liquids bioremediation. *Journal of Hazardous Materials*, In Press.
3. Kopsahelis N.*, Dimou C., **Papadaki A.**, Xenopoulos E., Kyraleou M., Kallithraka S., Kotseridis G., Papanikolaou S., Kookos K. I., Koutinas A.A.* (2018). Refining of wine lees and cheese whey for the production of microbial oil, polyphenol-rich extracts and value-added co-products. *Journal of Chemical Technology and Biotechnology,* 93: 257–268.
4. Dimou C., Kopsahelis N., **Papadaki A.**, Papanikolaou S., Kookos K. I., Mandala I., Koutinas A.A.* (2015). Wine lees valorization: Biorefinery development including production of a generic fermentation feedstock employed for poly(3-hydroxybutyrate) synthesis. *Food Research International*, 73:81-87.

Conference oral presentations - related to this thesis

1. **Papadaki A.**, Aguieiras E.C.G., Kopsahelis N., Papanikolaou S., Freire D.M.G., Mandala I., Koutinas A. A. (2018). Bioprocess development for the production of oleogels as novel food formulation using oleaginous yeast via the valorization of soybean and sugarcane mills side streams. 4th Iberoamerican Conference on Biorefineries (4-CIAB), 22nd - 24th October, Jaen, Spain.
2. **Papadaki A**., Boviatsi E., Kyriakou M., Nychas G.J., da Silva J.A.C., Freire D.M.G., Koutinas A. A. (2018). Valorisation of molasses for the production of biolubricants via fermentation using oleaginous yeasts. 7th International Conference on Engineering for Waste and Biomass Valorisation. 2nd - 5th July, Prague, Czech Republic.

3. **Papadaki A.**, Boviatsi E., Kopsahelis N., Nychas G.J., da Silva J.A.C., Freire D.M.G., Koutinas A. A. (2018). Biolubricants production via enzymatic conversion of microbial oil produced through the valorisation of sugarcane molasses. 6th International Conference on Sustainable Solid Waste Management, 13th - 15th June, Naxos, Greece.
4. **Papadaki A.**, Kopsahelis N., Patsalou M., Koutinas M., Machado de Castro A., Papanikolaou S., Koutinas A. A. (2017). Fumaric acid production through valorization of molasses and soybean cake derived from biodiesel and sugarcane industries. 5th International Conference on Sustainable Solid Waste Management, 21st - 24th June, Athens, Greece.
5. **Papadaki A.**, Efthymiou M.-N., Mallouchos A., Gardeli Chr., Kopsahelis N., Papanikolaou S., Koutinas A. A. (2016). Wax esters synthesis as innovative application of microbial oil produced from food industry by-products. 38th Symposium on Biotechnology for Fuels & Chemicals (SBFC). 25th - 28th April, Baltimore, Maryland, U.S.A.
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Chapter 1

Introduction

1 Introduction

1.1 Generation of food industry wastes

According to FAO (2013), theproduction of food waste has the following definitions:

- "*Food loss* refers to a decrease in mass (dry basis) or nutritional value (quality) of food that was originally intended for human consumption. These losses are mainly caused by inefficiencies in the food supply chains, such as poor infrastructure and logistics, lack of technology, insufficient skills, knowledge and management capacity of supply chain actors, lack of access to markets and natural disasters."
- "*Food waste* refers to the discarded food which was for human consumption, whether or not after it is kept beyond its expiry date or left to spoil. Often this is because food has spoiled but it can be for other reasons such as oversupply due to markets, or individual consumer shopping/eating habits."
- "*Food wastage* refers to any food lost by deterioration or waste and thereafter the term "wastage" encompasses both food loss and food waste."

In 2011, the global food losses and food waste were estimated annually around one-third of the food produced for human consumption (FAO, 2011)and it is related to 4×10^6 t of greenhouse gas emissions, vast quantities of water consumption (200×10^6 m^3), fertilizer usage (28×10^6 t) and avoidable food production. In the developing world, food waste occurs after harvesting (e.g. transportation), whereas in the developed world, food waste is related with consumer's consumption attitude. Considering the fact that due to the increasing world population the demand for food production in 2050 will be increased by 60% as compared to 2005/2007 (Alexandratos and Bruinsma, 2012), the production of uneaten food will have significant environmental and economic costs.According to FAO, food losses and waste accounts to US$ 680 billion in industrialized countries and US$ 310 billion in developing countries (Anonymous, 2018a). In the EU, the annual food waste was around 89 million twith the expectation to reach 126 million t by 2020 (Figure 1.1).

The largest share of food waste is produced by households(42%) followed by agriculture/food processing (39%), food service/catering (14%) and retail/wholesale (5%) (Anonymous, 2014). The global quantitative food losses and waste per year are 30% for cereals, 40-50% for root crops, fruits and vegetables, 20% for oilseeds, meat and dairy plus 35% for fish (Anonymous, 2018a). The valorisation of these kind of wastes could lead to reduction of unavoidable wastes, sustainable production of chemicals and functional food additives via novel bioprocessing, economic and social growth of communities. The double

advantage of the valorisation of these wastes is concentrated on the reduction of environmental pollution and the sustainable development of the agro-food sector through a rational use of the bioderived compounds from food wastes (Lin et al., 2013).

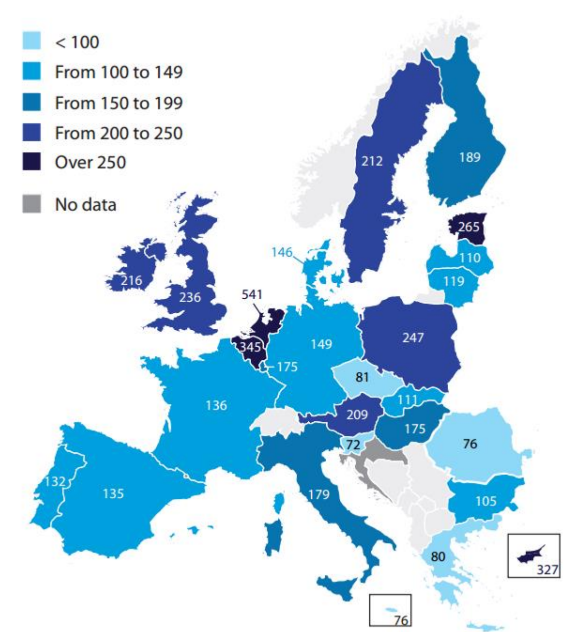

Figure 1.1 Estimated total food waste in the EU in 2010 (kg per capita) (Anonymous, 2014)

1.2 Valorisation of food industry derived wastestowards a circular bio-economy

The concept of Circular Economy has gained increasing interest in recent years, even though the principles started gaining popularity in Europe in the late 1990s. The aim of Circular Economy is the elimination of wastes and excessive resource use by turning goods at the end of their lifespan and waste generated during the manufacturing and use of goods into resources for the manufacturing of other products (Topi and Bilinska, 2017). Recently, the European Commission published the Circular Economy Package which consists of an EU action plan for the Circular Economy,indicating theEurope's transition towards a new sustainable development policy (European Commission, 2015).

The current food waste valorisation practices include animal feed, composting, incineration and landfill. The waste usage as animal feed is currently the most cost effective approach, whereas the disposal in landfill poses many environmental concerns.Around 4.2 t of CO_2 are emitted along the supply chain per t of food waste.The valorisation of wastes towards the production of biofuels, biomolecules and natural chemicals, biomaterials and food products has an economical advantage (Lin et al., 2013).Specifically, the development of

biorefinery concepts is considered essential for the successful valorisation of wastes, which can lead to a circular economy based on zero waste. The biorefinery concepts have attained great interest in the last years, which targets the production of several products using a wide variety of technologies (Lin et al., 2013).

As depicted in Figure 1.2, food waste could be regarded as renewable feedstock for sustainable chemical production (Pfaltzgraff et al. 2013).An integrated biorefinery scheme includes the extraction of valuable components such as, proteins, oils, and bioactive compounds among other biomass components,and the further food waste processing for the production of fermentation media aiming to the production ofvalue-added chemicals and polymers. For instance, Lin et al. (2013) have mentioned that whey could be fractionated into a protein-rich fraction for the production of whey protein concentrates or isolates and a lactose-rich fraction that could be used as carbon source for the production of various chemicals via fermentation.

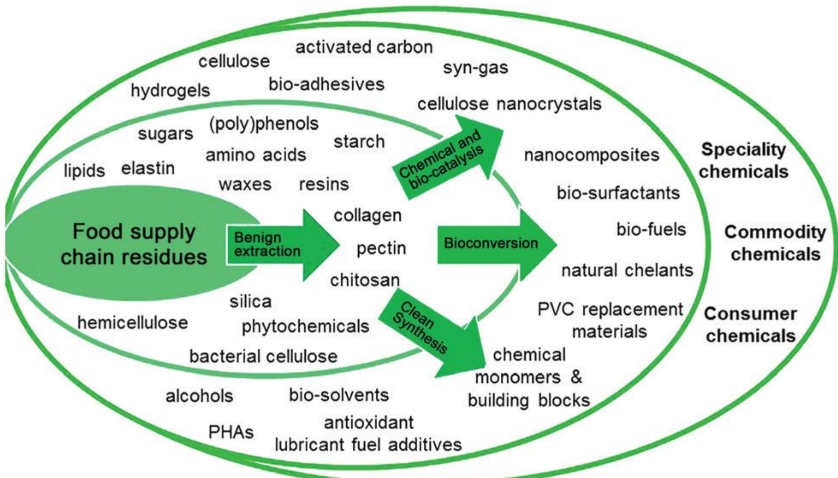

Figure 1.2 Components present in food supply chain wastes and their uses in marketable end-products(Pfaltzgraff et al., 2013)

Different kind of bioprocessing technologies could be employed for theproduction of bio-based chemicals and polymers.Microbial fermentationscould be employed for the production of various compounds, such as organic acids (lactic acid, succinic acid, fumaric acid), bioplastics/biopolymers (polyhydroxyalkanoates, PHAs), bacterial cellulose, microbial oil, biosurfactants and biocolorants (Maina et al., 2017; Esteban and Labero, 2018). The fermentation media derived from one or combined types of food wastes provide nutrients, including sources of carbon, nitrogen, minerals vitamins and trace elements, that are essential

for microbial growth and product formation. Currently, many different food industry derived wastes have been utilised as fermentation feedstock for microbial bioconversions within biorefinery concepts, including citrus wastes, wine lees, flour-rich waste streams from the confectionery industry (Maina et al., 2017).

In many cases, enzymatic hydrolysis is essential in order to produce a nutrient-rich fermentation feedstock (Figure 1.3). Enzymatic hydrolysis of polysaccharides and proteins containedin food wastescould be achieved by mixed enzyme consortia producedvia solid state fermentations (Lin et al.,2013). Crude protein-rich biomass hydrolysates have been widely utilised as substitutes for commercialnutrient supplements for the production of fermentation media. Kachrimanidou et al. (2013, 2015) demonstrated that sunflower meal hydrolysates is a nutrient-rich dermentation feedstock for the production of poly(3-hydroxybutyrate) (PHB) which could be used in biodegradable packaging applications. Dimou et al. (2015) presented an integrated biorefinery concept based on wine less valorisation for PHB production and isolation of high value-added products, such as tartratesalts, ethanol and antioxidants. Kopsahelis et al. (2018) studied the production of microbial oil through the valorisation of cheese whey and wine lees. A co-substrate was utilised in the fermentation process leading to PHB production consisting of lactose from cheese whey as carbon source and wine lees hydrolysate as nutrient source.

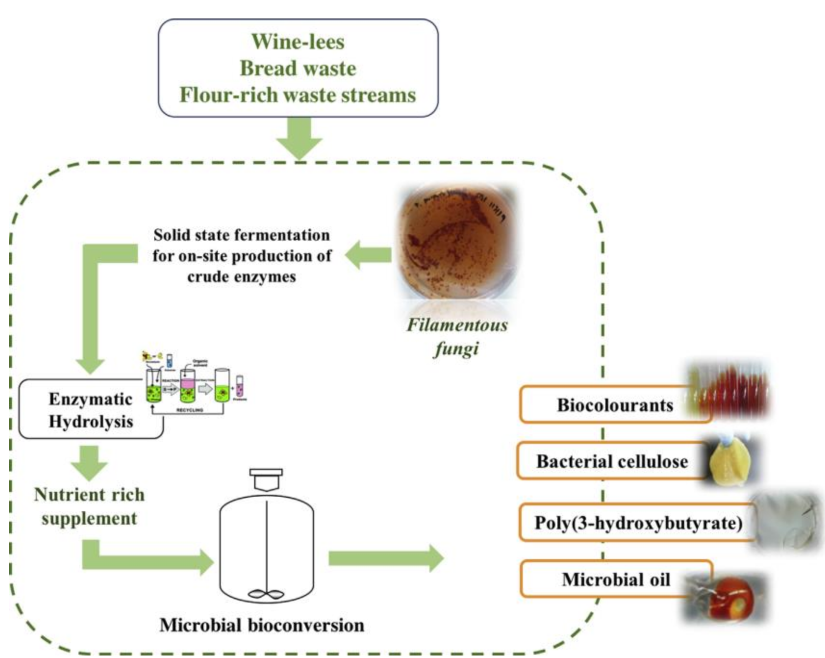

Figure 1.3 Development of a two-stage bioprocess for food waste valorization to formulate nutrient-rich supplements for microbial bioconversions (Maina et al., 2017)

Nowadays, the development of biorefineries is a necessity as fossil fuel reserves are depleted, waste recycling is becoming more expensive, global warming is a reality, and the growing population of the earth will require more energy and consumption goods. Moreover, in recent years, there is growing concern regarding the utilisation of fossil fuels for the production of fuels and chemicals mainly due to their immense environmental impact. From 2004 until 2014, in Europe, the production of electricity from renewable resources has almost doubled from almost 500 TWh to more than 900 TWh (Eurostat, 2016). However, although energy can be produced from different technologies and sources, the sustainable production of chemicals can only be achieved using renewable biomass.

Chapter 2

Valorisation of food industry side streams for the production of bio-economy products

2 Valorisation of food industry side-streams for the production of bio-economy products

2.1 The case of sugarcane processing

Sugarcane is used industrially for sugar production and is the major crop cultivated globally mainly in tropical countries, such as Brazil, China, India and Thailand. Brazil is the world's largest sugarcane producer with 39% of the world's total production with almost 740 million t. In 2014, the global sugar production was more than 170 million t (FAOSTAT 2014).

As it is shown in Figure 2.1, the first step for the production of cane sugar, is cane stalks harvesting and transportation to sugar mills for processing. The milling of sugarcane includes feeding of cane stalks into large milling units and crushing them by a series of rollers. At this point, the first by-product formation is occurred which is bagasse. This fibrous material that remains after cane stalks juice extraction is mainly used to create renewable energy and electricity. The produced sugar juice is further processed for the raw sugar production, which will be later refined to make the crystallised white sugar. This process involves the production of molasses, the main by-product derived from the sugar industry.

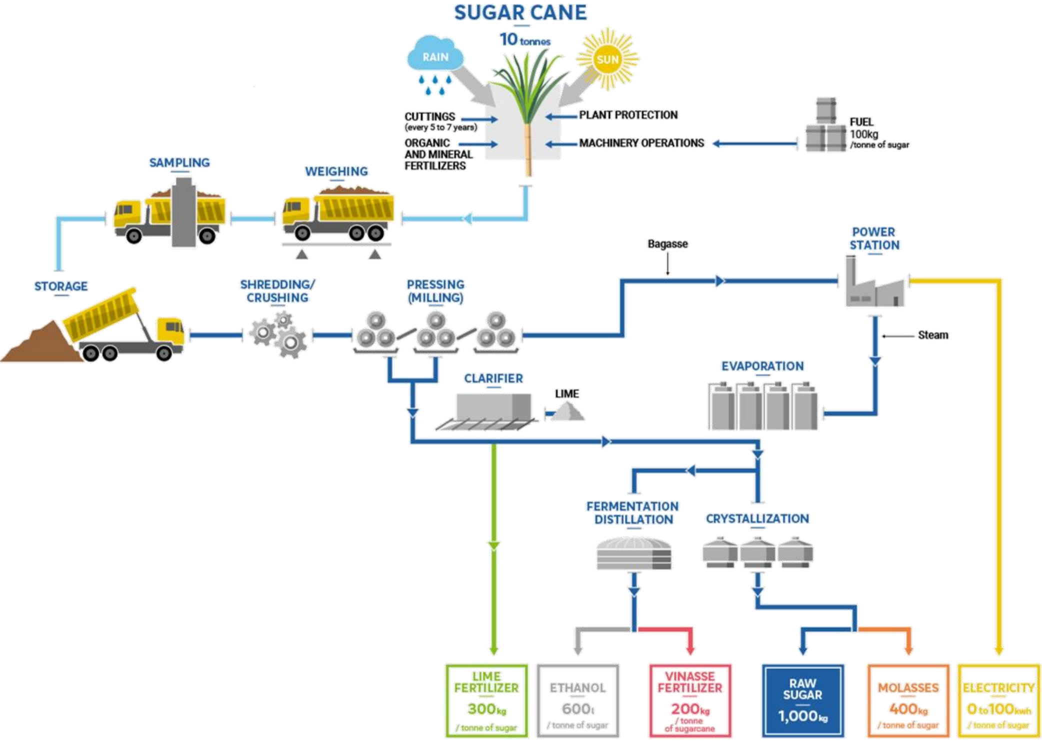

Figure 2.1 The sugarcane mill processing (source: www.sucden.com/en/products-and-services/sugar/process-flowcharts/).

2.1.1 Sugarcane processing by-products

The sugarcane process generates significant quantities of side streams, predominantly molasses and bagasse. About 3 t of bagasse are produced per 10 t of sugarcane processed, whereas it is estimated that from 100 t of sugarcane around 3 - 7 t of molasses are generated (Gharib-Bibalan, 2018).

Bagasse is a fibrous material which remains after the breaking of sugarcane to extract the juice and it mainly consists of cellulose (47 - 52%), hemicellulose (25 - 28%), and lignin (20 - 21%). The main application of bagasse is for bioenergy production. In Brazil, 19.3% of the total energy matrix from renewable resources was produced from bagasse (Hofsetz and Silva, 2012). In addition, bagasse has been employed for a variety of enzymes production, such as glucosidase, cellulose, xylanase, tannase, inulinase and invertase (Veana et al., 2014). Dotaniya et al. (2016) has proposed the utilisation of bagasse as soil fertiliser.

Molasses is considered one of the most economically important by-products of sugar industries. It is a viscous and dark sugar-rich syrup, which remains after the final crystallization stage and separation of raw sugar as a result of a circular path of washing and heating the cane sugar with hot water. Each step of the sugarcane process results to different types of molasses, the most important of which are (Clarke, 2003; Pérez, 1995):

- *High-test molasses*, produced by concentrating clarified cane juice to approximately 85 °Brix. It is a premium product, higher in sugars content with aromatic flavor, as a result of the fewer sugar decomposition products due to less heating processing than the other molasses types.
- *C molasses (final molasses or blackstrap molasses),* is the heavy, dark viscous liquid end by-product of the processing in the sugar factory. It contains considerable amounts of sucrose (approximately 32 to 42%).
- Other types are the *syrup-off*, which is the end by-product from the centrifugation of the final refined masecuite in a raw sugar refinery, and *refinery final molasses*, which is similar to C molasses.

The composition of molasses varies depending on the sugarcane variety and maturity, soil type, climate and the process employed for sugar extraction (Dotaniya et al., 2016). Molasses mainly contain sucrose, reducing sugars (glucose and fructose), proteins and various minerals. Furthermore, molasses contains various compounds, such as the B group vitamins, betaine, choline, alantoine, purine, cytosine, guanosine, cytidine, lactic acid, amino acids, and

antioxidants (Gharib-Bibalan, 2018; Veana et al., 2014). A representative composition of molasses is presented in Table 2.1.

Table 2.1 Characterization of sugarcane molasses (Veana et al., 2014; Dotaniya et al., 2016; Patil and Patil, 2017)

Parameter	Concentration range
Total solids (%)	73 - 80
Moisture (%)	23 - 23.5
Total sugars (%)	50 - 51
Reducing sugars (%)	12 - 18
Free amino nitrogen (mg/L)	4500 - 5000
Nitrogen (%)	0.3 - 0.4
Phosphorus (%)	0.14 - 0.17
Calcium and Potassium (%)	4.8 - 5
Ash (%)	16 - 16.5
Density (kg/m^3)	1350

Molasses are often used for alcohol production or the preparation of animal feedorfood(Dotaniya et al., 2016). Moreover, the recovery of polyphenols, reducing sugars, antioxidants, and colorants from sugarcane industry by-products using non-conventional techniques have gained high interest in recent years. Specifically, enzymatic saccharification and hydrolysis, microwave-assisted extraction, subcritical water, supercritical CO_2, pulsed electric field, high-voltage electrical discharges have been proposed for the “green” extraction of bioactive compounds (Gharib-Bibalan, 2018). However, the high sugar content and the relatively low market price constitutes sugarcane molasses an alternative feedstock for fermentation processes (Cazetta et al., 2007). Vieira et al., (2014) reported that since the potential cost of molasses is $180–$250 per t and the sugar is traded at around $473 per t, then molasses would not compete with human food and thus could be utilised as substrate for microbial bioconversions.

2.1.2 Current biorefineries based on molasses valorisation

A variety of sugarcane biorefinery configurations have been proposed. Molasses is currently used mostly for ethanol production. In 2013, about 60% of the total bioethanol

production was derived from molasses (Silalertruksa et al., 2015). Sugarcane molasses has also been evaluated for the production of various bio-based chemicals and polymers, including propionic acid, lactic acid, succinic acid and 2,3-butanediol (Koutinas et al., 2014a).

Organic acids including lactic acid, propionic acid, and citric acid are important chemicals for industrial use. For instance, lactic acid finds versatile applications in the traditional food, pharmaceutical, textile, leather and chemical industries. Citric and fumaric acids are valuable for food industry applications mainly as food preservatives, whereas propionic acid is useful for bactericides, fungicides, herbicides, pharmaceuticals, perfumes and emulsifying agents (Koutinas et al., 2014a). All these chemicals can be biotechnologically produced through the valorisation of renewable resources such as molasses. Dumbrepatil et al. (2008) reported the production of 166 g/L lactic acid from molasses using a mutant *Lactobacillus delbrueckii* strain. Hydrolysed molasses have been successfully employed for propionic acid production (92 g/L) by *Propionibacterium freudenreichii*through fed-batch fermentations (Feng et al., 2011). Citric acid can be manufactured via solid-state fermentation (SSF) of starch/molasses mixtures by *Aspergillus niger* (Dhillon et al., 2011). The production of succinic acid has been also reported using molasses as substrate, achieving a final concentration of 55.2 g/L by *Actinobacillus succinogenes* mutant strain (Liu et al., 2008).

Ethanol can be used as an alternative to fossil fuels and an important platform chemical for the production of ethylene and ethylene glycol (Koutinas et al., 2014a). It can be produced by fermentation of various carbon sources, including molasses. Although, several microorganisms, such as *Kluyveromyces marxianus* and *Zymomonas mobilis*, are well-known ethanol producers the yeast *Saccharomyces cerevisiae* is mostly employed for ethanol production using molasses as substrate. The ethanol concentration is usually around 10% (w/w), due to the fact that *S. cerevisiae* yeast strains cannot sustain higher alcohol concentration than 10-15% (w/w). At the end of the fermentation, the ethanol is recovered via distillation at a purity of 95.5% (w/w) (Patil and Patil, 2017).

2,3-Butanediol (BDO) is an odorless, colorless and transparent liquid useful as antifreeze agent but also in pharmaceutical, agrochemical, fine chemical and food industries. Acetoin can be produced after the dehydrogenation of BDO, which can be used as an aroma carrier in flavors and essences (Koutinas et al., 2014a). High concentrations of BDO can be produced from molasses. Specifically, *Klebsiella oxytoca* produced 118 g/L of BDO in a continuous and cell recycling culture mode (Afschar et al., 1993). A genetically engineered

Enterobacter aerogenes strain was cultivated on sugarcane molasses and achieved 98.7 g/L of BDO concentration in fed-batch mode fermentation (Jung et al., 2013).

Polyhydroxyalkanoate (PHA) is a family of biopolyesters accumulated intracellularly in bacterial strains and useful for the production of bio-plastics. In recent years, PHA production from pure carbon sources has been replaced by utilising renewable resources as fermentation feedstock. PHB is a well-studied member of the PHA family in the international literature (Koutinas et al. 2014a). A production of 30 g/L PHA with a high productivity of 1.27 g/L/h has been reported by Kulpreecha et al. (2009) using the bacteria *Bacillus megaterium* cultivated on sugarcane molasses.

Microbial oil is another value-added metabolic product, which can be utilised for biofuel and oleochemical production. Various oleaginous yeasts and fungi are able to accumulate high quantities of lipids intracellularly. The potential of lipid production from molasses by different oleaginous yeast strains including *Rhodotorula glutinis*, *Rhodosporidium toruloides*, *Rhodotorula minuta* and *Lipomyces starkey* has been evaluated showing lipid concentrations up to 15 g/L (Vieira et al., 2014). In another study, *R. glutinis* was cultivated on molasses via fed-batch fermentation leading to a biomass concentration of 62.2 g/L with a lipid content of 54% (w/w) (Vieira et al., 2016). Moreover, molasses has been utilised also by fungal strains, such as *Cunninghamella echinulata* and *Mortierella isabellina* showing lipid accumulation up to 31.4% and 53.7% (w/w), respectively (Chatzifragkou et al., 2010).

Carotenoids are bio-active compounds important for food applications with many health benefits. Biotechnological production of carotenoids has been studied using various yeasts, such as *Phaffia rhodozyma*, *Rhodotorula glutinis*, and fungal strains, such as *Blakeslea trispora*, and microalgal strains, such as *Chlorella zofingiensis* and *Haematococcus pluvialis* (Cardoso et al., 2017). Bhosale and Gadre (2001) implemented fermentations for β-carotene production by a mutant strain of *Rhodotorulaglutinis* resulting to a total carotenoid production of up to 183 mg/L.

2.2 The case of vegetable oilseed processing

Vegetable oils are employed in diverse applications in the food industry. In particular, the annual vegetable oil production is expected to increase up to 28% for the period 2012 - 2021. This increased amount of the vegetable oils will be utilised predominantly for food

consumption (64%) and to a lesser extend for biofuel production (33%) (Koutinas et al., 2014a). The major oil-rich crops are soybean, palm, rapeseed, and sunflower (Table 2.2), from which soybean and palm oils are the two most important owing to their large-scale production (Koutinas et al., 2014a) and to the wide utilisation of their corresponding oils in various food applications (Anonymous, 2018b). In addition, soybean has gained high attention due to the fact that it is an exceptional source of proteins, oils and phytochemicals (Table 2.3) (Tripathi and Shrivastana, 2017).

Crude palm oil is projected to have a compound annual growth rate of over 7.3% from 2014 to 2022, since it is mainly used as edible oil in food industries, due to its low price compared to other cooking oils. The global oil production from palm is estimated to surpass 120 million t by 2022 (Anonymous, 2015a). Furthermore, the total oilseed quantity used for oil extraction was 224 million t in 2012/2013, whereas the consumption of soybean oil was 47 million t in 2016 and a CAGR of 3% was observed during the period 2009-2016 (Anonymous, 2017a; Koutinas et al.. 2014a).

Table 2.2 The highest quantities of the major oil crops produced per region in 2012/2013 (Koutinas et al., 2014a)

Oil crop	Region of production	Quantity(million t)
Soybean	North America	87.2
Palm oil	South East Asia	49.6
Rapeseed	European Union	19.1
Sunflower	Former Soviet Union – 12	17.6
Palm kernel	South East Asia	12.7

Table 2.3 Chemical composition of soybean (Tripathi and Shrivastana, 2017)

Constituents	Content (%, w/w)
Protein	40
Carbohydrates	23
Fat	20
Moisture	8
Minerals	5
Fiber	4

The industrial processing of oilseeds for the production of refined vegetable oils includes several steps. A flow diagram of soybean processing is presented in Figure 2.2. Initially, the soybeans are dried in order to prevent microbial growth and reduce enzymatic activities, thus enhancing their storability (Tripathi and Shrivastana, 2017). Then crude oil is recovered from the soybeans by mechanical or solvent extraction (Tripathi and Shrivastana, 2017) leading to the production of soybean meal as by-product. Crude vegetable oils contain various non-edible compounds, which should be removed prior to human consumption via chemical or physical refining processes (Piloto-Rodríguez et al., 2014) as demonstrated in Figure 2.3. This process targets to improve oil properties. Such as taste, odor, color and shelf life (Anonymous, 2017b). Physical refining is generally preferred due to reduced utilisation of of chemicals and water, lower losses of triglycerides and recovery of free fatty acids. The chemical process is used due to the successful elimination of the free fatty acid fraction, phospholipids, waxes, aldehydes and ketones (Piloto-Rodríguez et al., 2014).

Degumming of the crude oil is the first step of oil refining, aiming to eliminate the impurities after oil extraction, especially the phosphatides which are commonly called as gums (Gunstone, 2003). This is a key-step as the presence of phosphatides are responsible for high refining losses, due to their emulsification properties, and for oil darkening, due to their thermal instability. The water-degumming process is carried out by hydrating the gums and separating the hydrated gums from the oil before storage. This process targets the prevention of gum precipitation during storage. The gum deposit is formed because of the insoluble nature of hydrated phosphatides that occur after the water absorption from the oil. In the case of palm oil only chemical degumming is applied, since it contains low amounts of phosphatides (Deffense, 2014; Dijkstra, 2014). The following steps include neutralisation, bleaching and deodorisation for the removal of free fatty acids, colours and undesirable odors and flavors, respectively (Gunstone, 2003).

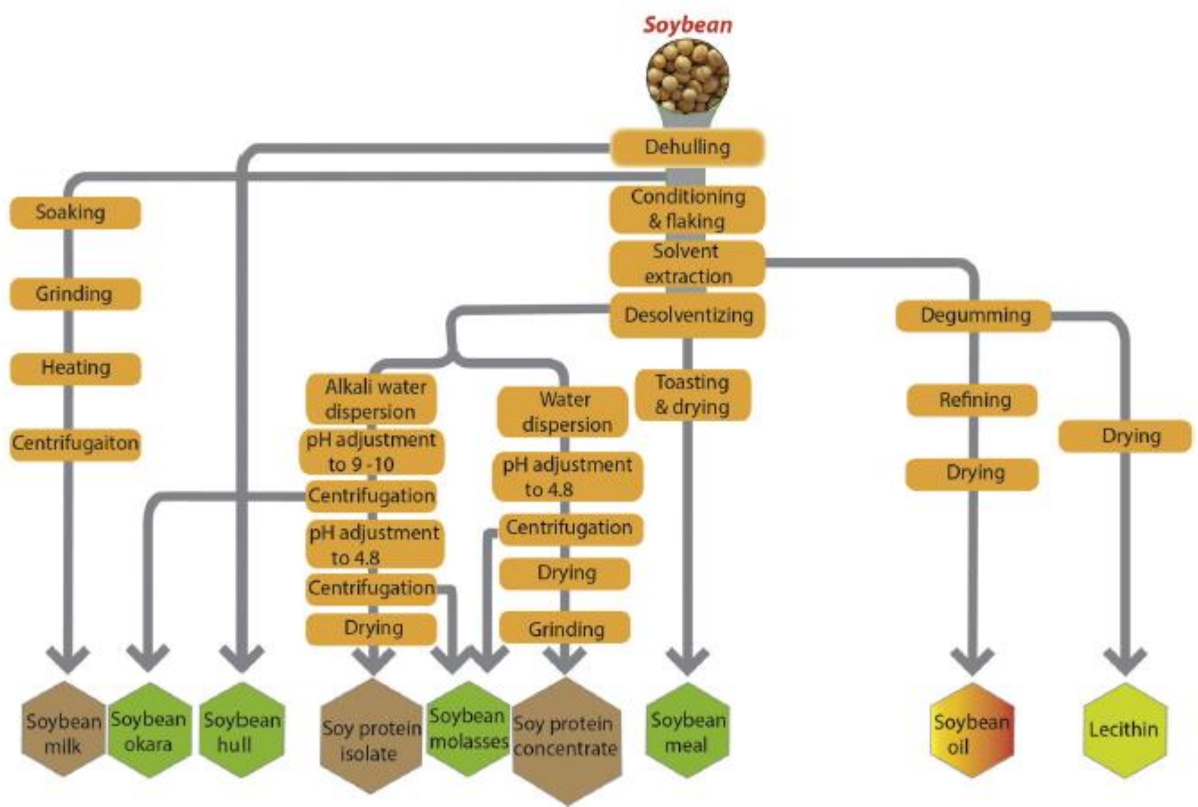

Figure 2.2 Soybean processing (Loman and Ju, 2016)

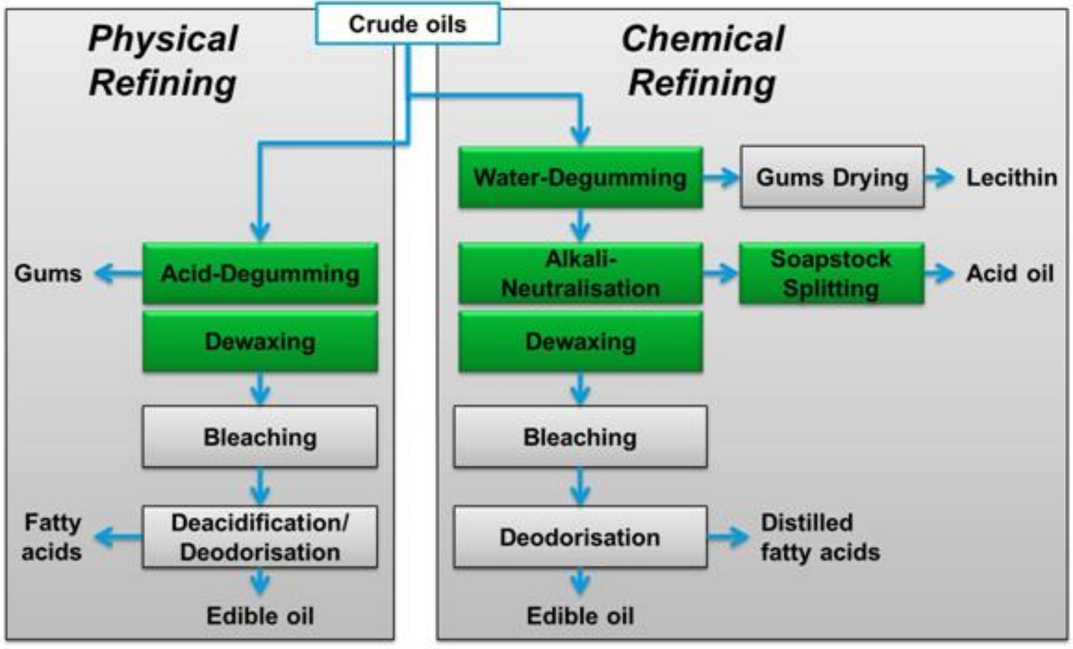

Figure 2.3 Vegetable oil refining process (Zeldenrust, 2014)

2.2.1 Vegetable oilseed processing by-products

Increasing world population has led to an increased consumption of refined vegetable oils and hence to higher production of by-products and side streams from oilseed mills (Piloto-Rodríguez et al., 2014). The main by-products derived from oilseed processing are cakes or meals, fatty acid distillates, soapstocks and gums.

A large amount (95%) of the de-oiled soybean is processed, as shown in Figure 2.2, to produce a protein-rich meal to be used as animal feed, whereas less than 5% is processed for the production of protein concentrates, protein isolates and soy flour for human and specialty animal feed applications. The resultant desolventized, toasted, dried and cooled product is commonly referred to as oilseed meal (Kemper, 2014). Oilseed meals are in the most of cases rich sources of proteins, carbohydrates, minerals and phenolic compounds. The production of soybean meal was accounted for 181 million t in 2012/2013, whereas the annual production of oilseed meal is expected to increase up to 315 million t by 2021 (Koutinas et al., 2014a). Soybean meal is a high-quality protein source presenting many nutritional and functional properties for both human and animal consumption (Gunstone, 2003; Tripathi and Shrivastana, 2017). Soybean cake or other meals can be employed as fertilisers because they improve the growth rate of plants and the quality of the agricultural products.

For the production of refined oils, the produced free fatty acid fractions should be removed from the crude oil by using molecular distillation of the oils. Fatty acid distillates and soapstocks are generated through the refining process of palm and soybean oils, respectively (Figure 2.3). The annual production of palm fatty acid distillate (PFAD) was 2.5 million t in 2016 corresponding to 3 - 10% of the crude palm oil (Anonymous, 2017b; Kapor et al., 2017). PFAD contains mainly free fatty acids (>85%), predominantly palmitic acid, triglycerides (5 – 15%) and unsaponifiable components, such as sterols, vitamin E, sterols, squalene and volatile components (Kapor et al., 2017). As in the case of crude palm oil, the alkali refining process of crude soybean oil generates the soybean soapstock as by-product, which contains entirely free fatty acids (FFA), such as linoleic, oleic and palmitic acids (Aguieiras et al., 20017a). Further acidulation of the soapstock leads to the generation of soybean fatty acid distillate (SFAD) (Haas, 2005; Haas et al., 2003). Although fatty acid distillates may have some practical applications, a high percentage of this by-product is discarded to the environment (Chongkhong et al., 2007; Chu et al., 2004; Ng and Wang, 2004). Thus, fatty acid distillates and soapstocks provide a low-cost by-product that could be used as raw material for the production of value-added oleochemicals.

2.2.2 Current biorefinery concepts

Edible oilseed meals can be utilised directly as animal feed, particularly for poultry, swine, cattle and fish (Koutinas et al., 2014a; Loman and Ju, 2016). However, the presence of specific carbohydrates, such as galactose-containing oligosaccharides (stachyose and raffinose), reduces its nutritional value as animal feed, since it is poorly digested due to the lack of the necessary endogenous enzymes (Loman and Ju, 2016). Moreover, the high fiber content of the meals and the presence of other antinutritional compounds pose many concerns for their utilisation as animal feed (Koutinas et al., 2014a). Nevertheless, the rich content of oilseed meals in proteins, carbohydrates and minerals could be valuable for the production of value-added chemicals via microbial fermentations. Research has focused on the valorisation of oilseed meals. For instance, sunflower meals have been used for the isolation of value-added products (e.g. phenolic compounds and protein isolate) and the production of nutrient-rich fermentation supplements that could replace commercial nutrient supplements in microbial fermentations (Kachrimanidou et al., 2013; Koutinas et al., 2014a). Tsouko et al. (2017) valorised palm kernel cake as raw material for the production of a generic fermentation feedstock through the utilisation of crude enzymes consortia produced via solid state fermentation of *Aspergillus oryzae*. Moreover, soybean meal has been utilised as substrate in solid state fermentations for the production of cellulases, xylanases and lipases using fungal strains of *Aspergillus niger* and *Penicillium* sp (Loman and Ju, 2016). Furthermore, an acid hydrolysate of soybean meal rich in carbohydrates has been successfully utilised as fermentation feedstock for the microbial production of succinic acid and acetoin (Loman and Ju, 2016).

Soybean molasses is a co-product generated during the production of soy protein concentrate and isolate from soybean meal (Figure 2.2). It mainly contains sucrose (28%), stachyose (18%) and raffinose (9%). Soybean molasses is considered an alternative raw material for the production of bioethanol using *Saccharomyces cerevisiae* or *Zymomonas mobilis*, butanol by *Clostridium* sp., sophorolipids by *Candida bombicola*, poly(hydroxyalkanoates) by different *Pseudomonas* and *Bacillus* strains and lactic acid by *Lactobacillus* sp. (Loman and Ju, 2016; Tripathi and Shrivastana, 2017).

Soybean lecithin could be isolated from the gums resulted after the degumming step and is considered a valuable by-product owing to its richness in phospholipids and tocopherols (Gunstone, 2013).

Currently, biodiesel is mainly produced from vegetable oils. However, low-cost feedstocks,such aswaste cooking oils, non-food grade animal fat and other waste fats and oils could be employed aiming to the development of a circular bio-economy. As palm and soybean oils are the most widely utilised oils worldwide, the by-products formed from oil processing are promising raw materials for the production of bio-based products. The Neste Company is the largest producer of biodiesel using waste and residues, such as PFAD, which account to nearly 80% of Neste's usage of renewable raw materials. The utilisation of biodiesel produced from PFAD and other waste and residues contributes to a reduction of 80-90% of greenhouse gas emissions as compared to conventional diesel. Currently, Neste has invested over 40 million € in R&D initiatives towards the development of new low-quality feedstocks (Anonymous, 2017b). Fatty acid distillates find also applications as raw material for soap, oleochemicals and animal feed production (Anonymous, 2017b; Ping and Yusof, 2009). The utilisation of PFAD for biodiesel production have been extensively studied (Aguieiras et al., 2017a; Anonymous, 2017b; Kapor et al., 2017; Piloto-Rodríguez et al., 2014). However, Fernandes et al. (2018) have recently highlighted the potential valorisation of PFAD for the production of higher value oleochemicals, such as biolubricants. Specifically, solvent-free enzymatic production of PFAD polyol esters was employed using trimethylolpropane and neopentyl glycol polyols. The produced esters exhibited good lubricant properties (Fernandes et al., 2018). Moreover, SFAD is a natural source of phytosterols, free fatty acids, tocopherols and squalene. Phytosterols play major roles in several areas, namely in pharmaceuticals (production of therapeutic steroids), nutrition (anti-cholesterol additives in functional foods, anti-cancer properties) and cosmetics (creams, lipstick) (Tripathi and Shrivastana, 2017). Although biodiesel production using the oil-based by-products from the oil refining industry is efficient, there is currently a trend towards the production of value-added oleochemical products.

2.3 Biotechnological production of value-added products from food industry side streams

2.3.1 Fumaric acid

An important field for the use of renewable resources as raw material is the biotechnological production of platform chemicals, such as succinic acid, lactic acid, fumaric acid, 1,3-propanediol and 1,4-butanediol, which are used as precursors for the production of various end-products (Jang et al., 2012). Fumaric acid is considered as one of the most

important platform chemicals with applications in medicine, food and chemical industries (Das et al., 2016). It is used mainly as a food acidulant and as chemical feedstock for the production of paper resins, unsaturated polyester resins, alkyd resins, plasticizers, and miscellaneous industrial products (Koutinas et al., 2014a; Roa Engel et al., 2008).

The global fumaric acid market demand was estimated as 225.2 thousand t in 2012 and it is expected to be over 300 thousand t in 2020 (Anonymous, 2015). Fumaric acid is currently produced via isomerization of maleic acid, which is produced from maleic anhydride (Roa Engel et al., 2008). The fermentation process yields around 85% (w/w), using glucose as carbon source, which is considered cheaper than the raw material used in the chemical process (Roa Engel et al., 2008). Since there is an increasing concern over sustainable chemical production, the utilization of microbial strains for fumaric acid production is a promising alternative to the chemical route (Roa Engel et al., 2008).

Fermentative production of fumaric acid has been investigated using various fungal strains of *Rhizopus* sp. The oxidative TCA cycle is required for fungal growth, while fumaric acid production involves the reductive TCA cycle (Koutinas et al., 2014a). According to the metabolic pathway depicted in Figure 2.4, under nitrogen-limited conditions *Rhizopus* sp. catabolise glucose via the reductive TCA cycle. In fact, pyruvate carboxylase catalyses the carboxylation of pyruvate to oxaloacetate with the participation of ATP and CO_2. Oxaloacetate is then converted to malic acid by malate dehydrogenase and then to fumaric acid by fumarase (Xu et al., 2012). The CO_2 required in the reductive TCA cycle is supplied through the reaction of $CaCO_3$ with fumaric acid leading to the production of calcium fumarate and CO_2. Hence, the theoretical yield is higher than 100% (w/w), since the production of 2 moles of fumaric acid per mole of glucose consumed is achieved upon fixation of 2 moles of CO_2 via the reductive pyruvate carboxylation (Roa Engel et al., 2008).

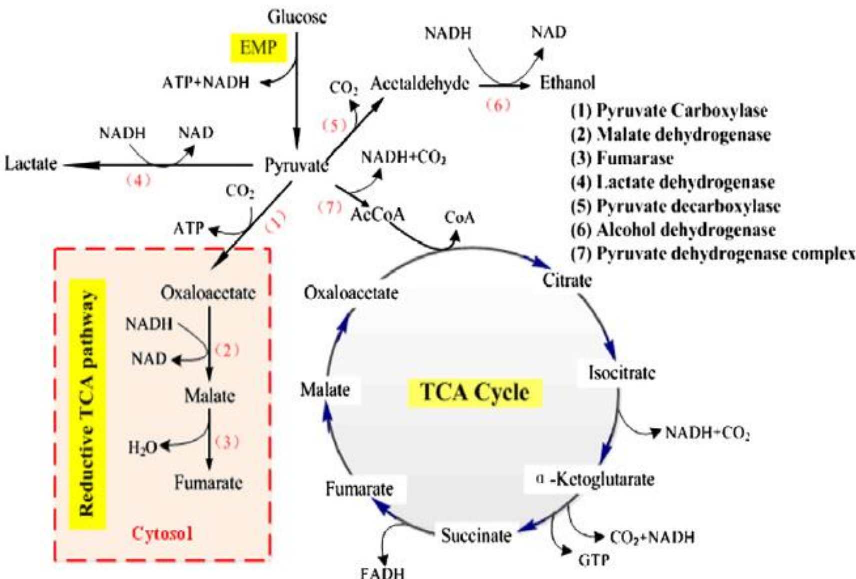

Figure 2.4 Metabolic pathway for fumaric acid production by *Rhizopus* sp. (Xu et al., 2012)

Many *Rhizopus* species have been reported to produce fumaric acid in greater or lesser extents. Some of them have shown a capability of producing significant amounts of fumaric acid at concentrations higher than 90 g/L consuming various carbon sources (Magnuson and Lasure, 2004). *R. oryzae* and *R. arrhizus* are the two most widely studied fungi for fumaric acid production. In particular, *R. arrhizus* NRRL 2582 and *R. oryzae* NRRL 1526 have presented the highest final concentrations and conversion yields (Gangl et al., 1990; Kenealy et al., 1986; Petruccioli et al., 1996; Rhodes et al., 1962). Roa Engel et al. (2008) and Xu et al. (2012) reported that the biotechnological production of fumaric acid is more efficient when mainly glucose is utilized as carbon source. Fu et al. (2010) reported that the highest fumaric acid concentration (56.2 g/L) and yield (0.56 g/g) were achieved when *R. oryzae* was cultivated on glucose using a two-stage dissolved oxygen control strategy, starting with 80% dissolved oxygen concentration until 18 h followed by 30% dissolved oxygen concentration. Most literature-cited studies are focusing on the utilisation of glucose, with only few studies reporting the use of crude renewable resources for fumaric acid production. Carta et al. (1999) reported the production of fumaric acid from cassava bagasse hydrolysate through the screening of several *Rhizopus* strains with the highest fumaric acid production (21.28 g/L) achieved by the strain *R. formosa*. Petruccioli et al. (1996) investigated the production of fumaric acid from glucose molasses using *R. arrhizus* immobilized in polyurethane sponge particles leading to the production of 17.5 g/L fumaric acid.Zhang et al. (2015) reported

thatchemically hydrolysed soybean meal can be used for the fumaric acid production by *R. oryzae* ATCC 20344.

Several studies have shown that fungal morphology is a significant factor affecting fumaric acid concentration and yield (Liao et al., 2007; Roa Engel et al., 2011; Zhang et al., 2015; Zhou et al., 2011). The formation of different fungal morphologies, such as clumps, pellets, and filaments, in submerged fermentations is affected by the growth conditions. The main disadvantage of filamentous and clump morphologies, during submerged fermentations, is the tendency to grow on bioreactor baffles, shaft and wall, which causes low oxygen transfer rates and non-homogeneous fermentation broths. Many studies have focused on the morphology of several *Rhizopus* species (Byrne and Ward 1989; Das and Brar 2014; Liao et al. 2007; Zhang et al. 2015; Zhou et al. 2000). Industrial production of fumaric acid by fungal strains is impeded due to problems associated with the low solubility of fumarate salts, fungal morphology, dissolved oxygen limitations and selection of appropriate fermentation media composition (Koutinas et al., 2014a). For instance, the low solubility of fumarate salts produced via neutralization of the broth during fermentation results in the formation of viscous broths limiting oxygen transfer rate. Fermentation efficiency could be improved via fermentation media optimization, cell immobilization and integrated fumaric acid production via fermentation with separation using adsorption on selective resins (Koutinas et al., 2014a).

2.3.2 Microbial oil production using oleaginous yeasts

Microbial oil is a secondary metabolite produced mainly by algae, yeasts and fungal strains. Oleaginous microorganisms are those that accumulate intracellularly lipids at more than 20% of their total dry weight (Athenaki et al., 2017). Among oleaginous yeasts, *Rhodosporidium toruloides* has many desirable industrial properties, such as the ability to grow and accumulate high oil concentrations and carotenoids on low-cost substrates. *R. toruloides* is able to accumulate up to 76% (w/w, db) lipids under nitrogen-limited conditions (Li et al., 2006; Park et al., 2017).

The metabolic pathway of triglycerides accumulation in the *R. toruloides* cells is presented in Figure 2.5. The key step in lipid accumulation is the exhaustion of nitrogen contained in the medium. Then, the carbon remaining after the depletion of nitrogen continues to be assimilated by the cells leading to the intracellular accumulation of microbial lipids. When the nitrogen is depleted from the medium, activation of AMP (adenosine monophosphate) deaminase occurs resulting to the reduction of AMP concentration. This

leads to the deactivation of isocitrate dehydrogenase located in mitochondrion as part of the TCA cycle. As a consequence of the isocitrate dehydrogenase deactivation, isocitrate cannot be further metabolized and both isocitrate and citrate start to accumulate in the mitochondrion. When the intra-mitochondrial citric acid concentration reaches a critical value, citrate is secreted to the cytoplasm and then it is converted by the ATP-citrate lyase into acetyl-CoA, which serves as the primer metabolite for fatty acid synthesis (Papanikolaou and Aggelis, 2011; Ratledge, 2008). Ratledge (1997) reported that the stoichiometric conversion of glucose to triacylglycerols is 0.32 g/g.

Microbial oil can be used as alternative raw materials for the production of biofuels and oleochemicals (Duarte et al., 2015; Leiva-Candia et al., 2015; Paulino et al., 2017; Soccol et al., 2017). The main problem of microbial oil production is the higher production cost than vegetable oils. Koutinas et al. (2014b) has shown that the unitary production cost of microbial oil derived from *Rhodosporidium toruloides* cultivated on glucose is around $3.4/kg at an annual production capacity of 10,000 t and zero cost of glucose. This cost could be reduced when waste streams are used as fermentation media, especially in the case that integrated biorefineries are developed for the production of various end-products. For instance, Kopsahelis et al. (2018) reported the development of a biorefinery concept using cheese whey and wine lees for the production of polyphenol-rich extracts, tartrate salts, potable ethanol, whey protein concentrate and microbial oil using the oleaginous yeast *Cryptococcus curvatus*. It should be highlighted that the development of biorefineries based on the utilisation of low-cost substrates for lipid production, aiming to reduce production cost and eliminate the disposal of agro-industrial wastes in the environment, has become a necessity towards the implementation of a circular economy (Athenaki et al., 2017). The Neste Oil Company manufactured in 2012 the first pilot plant in Europe for the production of microbial oil from waste and residues using oleaginous yeasts and fungi (Koutinas et al., 2014a).

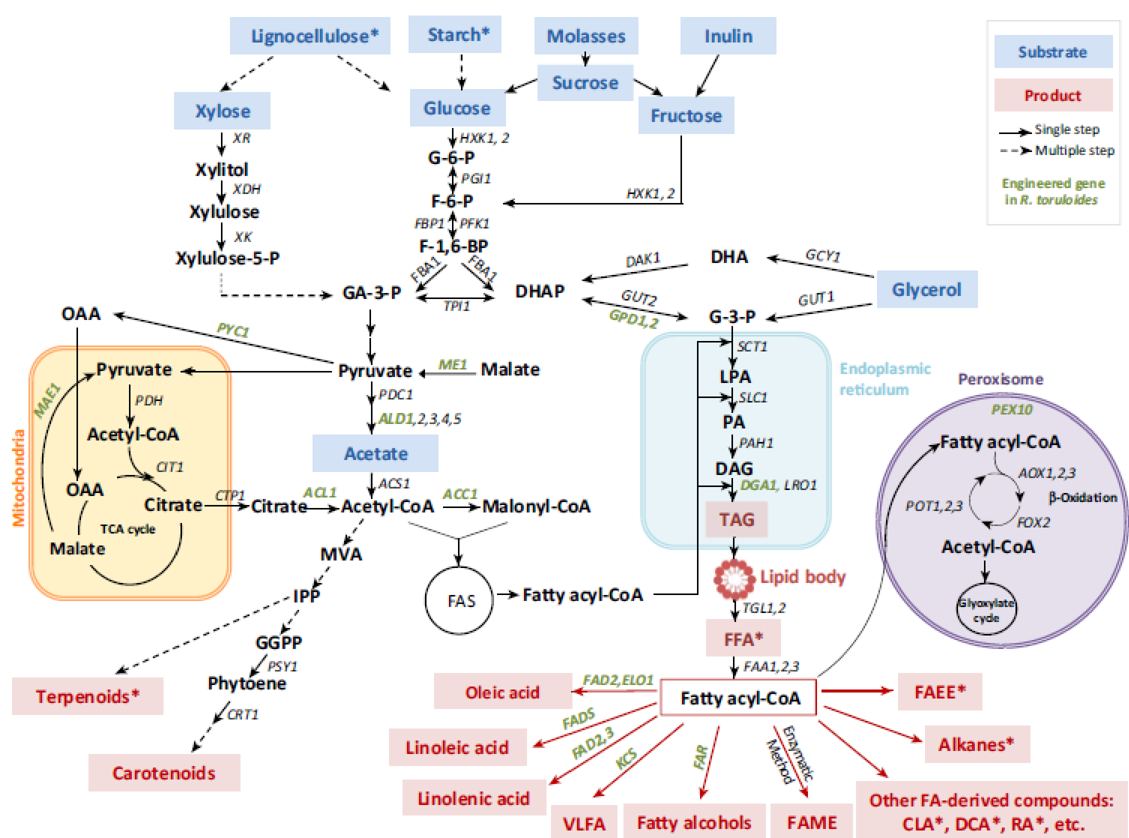

Figure 2.5 Metabolic pathway of *Rhodosporidium toruloides* for the production various biotechnological products, including microbial oil and carotenoids, from different carbon sources (Park et al., 2017)

The successful transition to the bio-economy era based on circular economy principles necessitates the development of diversified applications for microbial lipids for both fuel and oleochemical production. The most widely studies application of microbial oils is the production of biodiesel through the chemical or enzymatic transesterification of the triglycerides to fatty acid methyl esters (Park et al., 2017). The advantages of microbial lipid utilisation as industrial feedstock for oleochemicals production are their production by wild-type microbial strains at high fermentation efficiency using industrial waste and by-product streams, such as confectionery industry waste streams (Tsakona et al., 2014; 2016), crude glycerol (Leiva-Candia et al., 2015) and whey (Kopsahelis et al., 2018).

In recent years, research has focussed on the production of microbial oil rich in oleic acid. The global oleic acid market was 218.9 thousand t in 2013, with a revenue of US$ 344.6 million. In 2016, oleic acid generated more than US$ 350 million and has a significant growth prospect owing to its extensive usage in the chemical industry (Anonymous, 2017c). High-

oleic acid oils present excellent thermal and oxidative stability and these characteristics made them suitable for the production of biolubricants and hydraulic fluids (Park et al., 2017). Currently, only high-oleic vegetable oils are used for these purposes (Park et al., 2017).

2.3.3 Carotenoids

Carotenoids is a group of C40 terpenoid pigments, divided principally in carotenes containing only carbon and hydrogen (e.g. β-carotene and γ-carotene) and xanthophylls that include oxygenated derivatives (e.g. astaxanthin, canthaxanthin and zeaxanthin) (Frengova and Beshkova, 2009; Cardoso et al., 2017). The global market value of carotenoids was $1.5 billion in 2014 and is estimated to reach $1.8 billion by 2019, with a CAGR of 3.9% (Anonymous, 2015d). Lutein, astaxanthin and β-carotene comprise the principal segments in carotenoids market while β-carotene exhibits the fastest-growing market from 2016 to 2021. The synthetic β-carotene represented more than 70% of the market share in terms of volume and revenue. However, the natural derived β-carotene is projected to grow at a rate of 4.6% in terms of revenue in the period from 2015 to 2022 (Anonymous, 2016a).

Carotenoids are used in food, pharmaceutical, cosmetics and feed industries. However, around 40% of the carotenoids are applied only in the food industry (Anonymous 2016a). Carotenoids are also widely applied in the production of animal feeds, e.g. aquacultured salmons, because salmon cannot synthesise these molecules and the color of salmon is a critical parameter for the consumer (Frengova and Beshkova, 2009). The significance of carotenoids is established on their activity as vitamin A precursor along with coloring, antioxidant, anti-inflammatory, anti-cancer, cardiovascular disease and diabetes prevention (Frengova and Beshkova, 2009; Hernandez-Almanza et al., 2014; Park et al., 2017).

Commercial interest has focused on the development of food grade carotenoids from natural sources, mainly due to the reduced number of permitted synthetic colorants currently used as well as their undesirable toxic effects (Frengova and Beshkova, 2009; Cardoso et al., 2017). Natural carotenoids are usually found in plants or synthesized by algal, bacterial, fungal and yeast strains. More specifically,*Phaffia rhodozyma* represent one of the most widely evaluated pigmented yeast strain focusing on astaxanthin production. Furthermore, basidiomycetes yeasts, often designated as "red yeasts" belonging to the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Sporidiobolum* have been also reported to produce carotenoids, consisting mainly of β-carotene, γ-carotene, torulene and torularhodin. These yeast strains can effectively metabolize diversified carbon sources including glucose, xylose,

cellobiose, sucrose, glycerol and sorbitol (Mata-Gomez et al., 2014). Thereof, aiming to reduce the production cost of microbial carotenoids deriving from the cost of fermentation supplements, it is of paramount importance to valorize renewable resources, including waste and by-product streams from the agro-industrial sector and food industry. For instance, *Rhodotorula* sp. has been evaluated for carotenoids production employing various materials as fermentation feedstock including grape must, glucose syrup, maize flour, sugarcane molasses, ultra-filtrated whey and spent coffee grounds (Cheng and Yang, 2016; Frengova and Beshkova, 2009; Hernandez-Almanza et al., 2014; Mata-Gomez et al., 2014; Petrik et al., 2014). Literature-cited results report 1.20 mg/L and 135.25 mg/L total carotenoid concentrations from *R. glutinis* cultivated on brewery effluent and crude glycerol, respectively (Saenge et al., 2011; Schneider et al., 2013). Other renewable resources that have been used for natural carotenoids production are cassava residues, glycerol, potato extract and corn steep liquor (Cardoso et al., 2017).

Although the yeast species of *Rhodotorula* and *Phaffia* are the most widely investigated (Frengova and Beshkova, 2009), the yeast *Rhodosporidium* sp. is another well-known pink yeast (Park et al., 2017), which has been studied in lesser extend for carotenoids production. Dias et al. (2015) have reported the utilisation of glucose as carbon source by these yeasts for the production up to 33.4 mg/L of total carotenoids. However, in recent years some studies have focused on the valorisation of alternative sources as substrate such as molasses, carob pulp syrup, sugarcane bagasse hydrolysate and switchgrass hydrolysate (Freitas et al., 2014; Bonturi et al., 2017; Slininger et al., 2016). *Rhosporidium toruloides* was able to produce carotenoid concentrations up to 0.18 mg/L using molasses as carbon sources (Freitas et al., 2014). The carotenoids produced from *Rhodosporidium toruloides* are consisting mainly (around 90%) of torularhodin, torulene, γ-carotene, and β-carotene (Park et al., 2017).

2.4 Oleochemicals production within the bio-economy framework

The oleochemical industry is currently seeking for new, less costly and renewable raw materials in order to produce oleochemicals with high biodegradability, low toxicity and low environmental impact (McNutt and He, 2016). Oleochemicals are usually defined as the chemicals derived from plant oils and animal fats. At present, oleochemicals are derived mainly from plant oils, and are used in the production of personal-care products, cosmetics, paints, bioplastics, lubricants, surfactants, surface coatings and biofuels (Adrio, 2017; Pfleger et al., 2015). The increasing demand of oleochemicals production is expected to increase the

oilseed production by more than 20% over the next decade. Thus, research has focused on identifying alternative, sustainable and cost-effective raw materials for oleochemicals production. A potential source towards the sustainable production of fatty acid-derived oleochemicals may be the oleaginous yeasts (Adrio, 2017).

Enzymatic processes can be employed for oil and fat modification. A lipase is used to catalyse esterification and transesterification reactions for the production of esters with modified properties. The enzymatic synthesis of bio-based oleochemicals present several environmental benefits. Energy consumption is reduced because enzymatic reactions in the case of lipases are usually perform up to 70 °C, whereas the chemical reaction temperatures range between 80 to 120 °C. A life cycle assessment was employed for the enzymatic processing of palm oil-based hard stock for margarine production through interesterification reaction, taking into consideration four environmental indicators: energy consumption, global warming, acidification and smog formation. The results showed that enzymatic processing reduces the environmental impact of margarine production, contributing to a more sustainable production process (Cowan, 2014).

2.4.1 Wax esters

Waxes are long chain esters composed of fatty acids esterified to fatty alcohols with chain lengths of more than 12 carbon atoms. The worldwide demand for waxes in 2010 was around 4.35 million t including petroleum waxes (85%), synthetic waxes (11%) and animal or vegetable waxes (4%) (Anonymous, 2010). Wax esters are raw materials of high importance for various applications including candles, board sizing, lubricant, coatings, packaging, food and cosmetic industries (Doan et al., 2017; Fiume et al., 2015; Anonymous, 2010; Petersson et al., 2005). Their application and properties (e.g. melting point, oxidation stability) are dependent on the degree of unsaturation and the number of carbon atoms of both fatty alcohol and fatty acid. Although the worldwide wax demand until 2019 is expected to increase at an annual growth rate of 1.5%, the growth of wax supply is expected to increase at a lower rate of 0.4%. This is attributed to the continuous decline of worldwide petroleum wax supply that accounts for 70% of total supply. This trend has paved the way for the development of natural waxes.

Common animal waxes are beeswax and spermaceti oil. Besides wax esters (58%), beeswax contains hydrocarbons (26.8%) as well as lower quantities of free fatty acids and free fatty alcohols (Doan et al., 2017). Beeswax contains mainly saturated alkyl palmitates (C38 -

C52) and unsaturated alkyl esters of oleic acid (C46 - C54) (Tulloch, 1980). Spermaceti oil is extracted from the head cavity of sperm whale (*Physeter macrocephalus*) and constitutes a mixture of liquid wax esters and triacylglycerols. Horiguchi et al. (1999) reported that spermaceti crystalline products separated from sperm whales contained cetyl myristate, cetyl palmitate and cetyl laurate, while hydrogenated sperm oil produced from sperm whales after the separation of crystalline spermaceti contained mainly stearyl stearate, cetyl stearate, stearyl palmitate and cetyl palmitate. The wax esters contained in the spermaceti products are mainly C28 - C36. Until 1971, sulphurised spermaceti oil was employed as additive in various lubricant applications. The use of sperm whale products stopped in 1971 when the hunting of whales was banned (Nieschlag et al., 1977), since then mineral and vegetable products were used as substitutes.

The main plant derived waxes are jojoba oil and carnauba wax. Jojoba oil is produced by the desert shrub jojoba (*Simmondisia chinensis*), which is cultivated in desert areas and is not suitable for high yield cultivation in moderate climate agricultural areas (Iven et al., 2016). Jojoba oil contains predominantly wax esters (up to 97%) with C40 - C42 being the main components. Torres et al. (2006) reported jojoba oil composition of 5.4% C38, 24.1% C40, 55.1% C42, 13.1% C44 and 2% C46. Jojoba oil contains mainly C18 - C24 monounsaturated fatty alcohol and monounsaturated fatty acid components (Wenning et al., 2016). Lipase-catalysed interesterification of jojoba oil with ethyl acetate led to the production of 88.4% of ethyl cis-11-eicosenoate (C20:1) content among fatty acid derived esters and 49.7% of cis-11-eicosenyl acetate (C20:1) and 44.3% of cis-13-docosenyl acetate (C22:1) among fatty alcohol derived esters (Lei and Li, 2015). Carnauba wax contains a mixture of mainly wax esters (ca. 60%) followed by free fatty alcohols (ca. 32%) and free fatty acids. The wax esters of carnauba wax contain C16 - C24 fatty acids and C18, C30 and C32 fatty alcohols (Doan et al., 2017).

Iven et al. (2016) demonstrated that the low-input oil seed crop *Camelina sativa* could be used as biotechnological platform for the production of plant-derived sperm oil-like liquid wax esters enriched in oleyl oleate with content higher than 60%. Furthermore, jojoba oil has been used as alternative to spermaceti oil (e.g. in premium lubricants, parting agents, anti-foaming agents, cosmetics) (Shivaraju et al., 2011). However, since jojoba oil production is restricted to desert areas, there is interest towards the production of natural waxes via chemical or enzymatic catalysis. As the chemical route has many disadvantages, such as the corrosive acids required, high energy consumption and degradation of synthesized esters, the

enzymatically-catalysed process employing immobilised lipase in a solvent-free system is a promising and sustainable alternative (Serrano-Arnaldos et al., 2016).

Enzymatic production of wax esters is a sustainable process as it employs mild reaction conditions, low energy consumption and lead to high product quality due to the specificity of the enzymes used (Gunawan and Suhendra, 2008). Moreover, when the catalysis employed without the presence of solvents has the advantages of simplifying the process, reduce the cost of solvent recovery and recycling, and increase the volumetric capacity. The enzymatic synthesis of wax esters fromtriglycerides consists of a two stage reaction as depicted in Figure 2.6. The first step is the hydrolysis of triglycerides to release the free fatty acids, forming glycerol as by-product, and the second one is the esterification of the fatty acid with a fatty alcohol (Figure 2.7). The most common fatty alcohols used for wax esters synthesis are oleyl alcohol and cetyl alcohol, due to their lower cost as compared to other fatty alcohols.

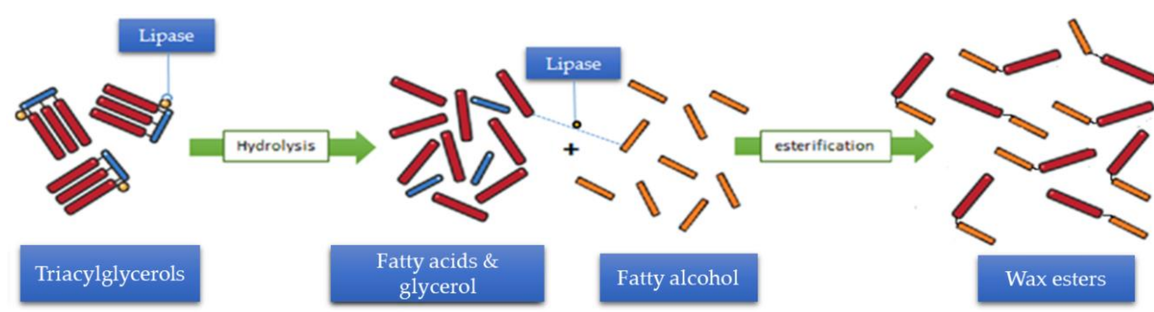

Figure 2.6 Schematic illustration of enzymatic conversion of triglycerides to wax esters

Figure 2.7 Reaction between fatty acid and fatty alcohol for wax esters synthesis

Several lipases are able to perform transesterification or esterification reaction, but in the literature the most efficient in wax esters production are the immobilised Novozyme 435 and Lipozyme. Literature-cited results concerning the enzymatic synthesis of wax esters by

these two commercial lipases using different oil sources are presented in Table 2.4. Transesterification of vegetable oils using lipases is a simple process and the raw material is inexpensive (Keng et al., 2009). Palm oil has been used in several studies for the production of wax esters using cetyl or oleyl alcohols (Basri et al., 2009; Gunawan et al., 2005). Deng et al. (2011) reported that the wax esters produced via lipase-catalysed esterification of oleic acid and cetyl alcohol could be used as substitute for jojoba oil. Also, Ungcharoenwiwat et al. (2016) reported the transesterification of jatropha oil with oleyl alcohol by Lipozyme RM IM at a conversion yield of 86% using hexane as solvent after 12 h at 45 °C. High conversion yields of wax esters (up to 95%) were also achieved via lipase-catalysed esterification of fatty acids derived from crambe and camelina oils with oleyl alcohol or alcohols derived from crambe and camelina oils using Novozyme 435 as biocatalyst (Steinke et al.. 2000).

In recent years, wax ester production has been attempted by genetically engineered microbial strains and plants. Wenning et al. (2016) has reported the production of wax esters with a chain length up to C42 resembling jojoba oil using a genetically engineered strain of *Saccharomyces cerevisiae* with a maximum yield of 12.2 ± 3.4 mg/g dry cell weight after 48 h. Alternatively to the renewable vegetable-based feedstocks, microbial lipids could be also used as industrial feedstock for the production of wax esters among other oleochemicals.

Table 2.4 Production efficiency of enzymatic synthesis of wax esters by commercial lipases using different oil sources

Enzyme	Oil source	Alcohol	Conversion yield (%)	Solvent	Conditions	Ref.
Lipozyme RM IM	Palm oil fractions	Oleyl	92	Hexane	50 ^{o}C, 5 h	Keng et al., 2009
Lipozyme RM IM	Palm oil	Oleyl	91	Hexane	50 ^{o}C, 5 h	Sin et al., 2005
Lipozyme RM IM	Palm oil	Oleyl	78-83	Hexane	50 ^{o}C, 5 h	Gunawan et al., 2004
	Palm oil (refined, bleached and deodorized)	Oleyl	85	Hexane	54 ^{o}C, 7 h	
Lipozyme RM IM	Palm kernel oil	Oleyl	84	Hexane	40 ^{o}C, 10 h	Gunawan and Suhendra, 2008
Novozyme 435	Crude fish fat	Cetyl	91	Hexane	37°C, 1 h	Ungcharoenwiwat and Kittikun, 2013
Novozyme 435	Myristic acid, Lauric acid	Cetyl	>98.5	free	70°C, 5 h	Serrano-Arnaldos et al., 2016
Novozyme 435	Palmitic acid, Stearic acid	Cetyl	>98.5	free	80°C, 5 h	Serrano-Arnaldos et al., 2016
Novozyme 435	Palmitic acid	Cetyl	99	free	65-67 ^{o}C	Petersson et al., 2005

2.4.2 Biolubricants

Lubricants are oil-based chemicals (liquid or semi-liquid oils and greases), predominantly produced from mineral oil, which are widely used for minimizing the damage due to friction, wear, and overheating in machine parts (Garcés et al., 2011). The global lubricant demand was around 41.7 million t in 2015 (Anonymous, 2015c). The major negative effect of petroleum based lubricants is associated with contamination of water and soil. Within the last decade, the production and use of conventional lubricants has been regulated by strict policies in order to minimize environmental pollution. In particular, the EU has established the "European Ecolabel for Lubricants" as an official EU mark for greener products and the Waste Framework Directive 2008/98/EC for the protection of the environment and human health from waste oils. Besides environmental pollution, the food industry demands the utilization of lubricants prepared from "generally recognized as safe" feedstocks. The USDA has categorized as H1 the lubricants used in applications where there is a possibility of incidental food contact and as H2 the lubricants used in applications where there is no possibility of food contact. Until now the formulation of food grade lubricants is based on the utilization of specific mineral oils and synthetic base oils, such as polyalphaolephins, polyalkalene glycols, and esters. The polyol and di-ester based lubricants have received H2 approval from USDA and mainly applied as oven chain and air compressor lubricants in the food and beverage industry (Rajewski et al., 2000).

Bio-based lubricants are expected to considerably increase in future years, driven by environmental and health concerns (toxicity, inadequate biodegradability, etc.) as well as by the large demand for mineral based lubricants worldwide. The low toxicity, higher bio-degrability, better lubricant properties and the lower emissions are the main advantageous characteristics of biolubricants over the lubricants produced from mineral oil. While the market for mineral-based finished lubricants has been stagnant, the market for biolubricants has shown an average growth of 10% per year over the last 10 years (Singh et al., 2015). In 2015, the global biolubricants market accounted for over $2 billion (Anonymous, 2016b).

Biolubricants are produced from vegetable oils derived from palm, soybean, sunflower, rapeseed, coconut, jatropha and castor bean (Mobarak et al., 2014). Plant-derived oils are good candidates because they are environmentally friendly and structurally similar to long-chain hydrocarbons in mineral oils (Sharma et al., 2006). However, the use of vegetable oils is limited by their high melting point and low oxidative stability, which are not desirable features for lubricants (Fox and Stachowiak, 2007). Moreover, the high demand for vegetable

oils for biolubricant production requires increased agricultural area use leading to competition with food production (Ahmad et al., 2011).

The direct use of triglycerides and free fatty acids for biolubricant production present disadvantages, such as high viscosity, low volatility and lubrication capacity, polymerization during storage, and the generation of carbon residue after combustion (Pryde, 1983). Thus, the chemical modification of triglycerides or free fatty acids is mandatory for biolubricants production. Polyol based esters pose favourable lubricant properties and they exhibit a wide range of applications (da Silva et al., 2015, 2013; Greco-Duarte et al., 2017). The production of polyol esters is based on the esterification of carboxylic acids with polyols, such as trimethylolpropane and neopentyl glycol, using an acid or enzymatic catalyst. The utilization of polyols such as trimethylolpropane and neopentyl glycol has been described for the production of lubricants (Greco-Duarte et al., 2016; Gren, 2009; Gunam Resul et al., 2012; da Silva et al., 2015). Polyol esters have good viscosity levels and a satisfactory pour point, but the final characteristics of the product depends on the polyol used, the chain sizes of the fatty acids, and the number of unsaturated bonds and their positions in the chain (Kamalakar et al., 2013).

Conventional routes using homogeneous catalysts for the synthesis of biolubricants may lead to generation of impurities derived from the acidic catalyst, difficulties in catalyst recovery, necessity to treat the generated effluents and high water consumption during product purification (Robles-Medina et al., 2009). On the other hand, enzymes can be advantageous catalysts due to their substrate selectivity and specificity, preventing undesired modifications of the substrates, and their low toxicity, environmental impact, and energy consumption (Ferreira-Leitão et al., 2017). Thus, the use of enzymes as biocatalysts represents a sustainable process eliminating the problems associated with the application of conventional homogeneous catalysts. Lipases (triacylesterol ester hydrolases, E.C. 3.1.1.3– IUPAC) catalyze the hydrolysis of triacylglycerol releasing free fatty acids, di- and mono-acylglycerols, and glycerol. This class of enzymes can also catalyze esterification and transesterification in low-water content media (Aguieiras et al., 2015; Ferreira-Leitão et al., 2017). They show high catalytic activity in esterification reactions, because they are very stable, have high activity and are suitable for a wide range of substrates (Anobom et al., 2014).

Lipases have been extensively studied as catalysts for biodiesel production using different fatty-acid sources (Aguieiras et al., 2016; Åkerman et al., 2011; da Silva et al.,

2015). The successful utilization of enzyme as biocatalysts for biolubricants synthesis has been also extensively studied using agricultural feedstocks or industrial side streams (Cavalcanti et al., 2018; da Silva et al., 2015, 2013; Greco-Duarte et al., 2017, 2016; Fernades et al., 2018). The enzymatic biolubricants production has mostly focused on utilization of crude oils (Dossat et al., 2002), or even manufactured products such as biodiesel (Greco-Duarte et al., 2017; Koh et al., 2014) as raw material. Alternatively, oil-rich fractions extracted from food waste could be used as raw materials for the production of lubricants (Lin et al., 2013). Bandhu et al. (2018) reported that the microbial oil of *Rhodotorula mucilaginosa* produced from sugar cane bagasse derived fermentation media had favorable fatty acid composition, physicochemical and tribophysical properties for good quality lubricant formulation. However, the perspective of utilizing microbial oil as a potential sustainable feedstock for polyol esters production with lubricant properties has not been studied yet.

2.5 Development of oleogels as novel food formulations

In recent years, a societal challenge related to obesity and cardiovascular diseases, as a result of high saturated and *trans* fats intake, has been appraised and poses detrimental effect not only to health but also to the healthcare system. A study in six European Union countries demonstrated that the healthcare cost due to cardiovascular diseases was over €102 billion in 2014 and it is estimated to reach up to €122.6 billion by 2020 (CEBR 2014). According to WHO/FAO (2003), the reduction of saturated fat contributes to lower risk of developing such chronic diseases.

The elimination of saturated fat from food formulations is a major food manufacture issue, since the saturated fat provides a solid texture in fat products. Oleogelation is a novel technique that entraps liquid oils in a three-dimensional fat crystal network, which leads to structured vegetable oil-based products with solid-like properties (Matheson et al., 2018; Oh and Lee, 2018). Oil structuring is widely applied in the food industry for the production of fat-based products, such as margarine, spreads and shortenings, baking products, chocolate fillings, with the aim to minimize the migration of liquid oil into food (Chaves et al., 2018). The hydrogenation process is the conventional way to modify the structure of vegetable oils, providing solid texture in food products.However this process leads to the production of saturated and *trans* fats (Hartel et al., 2018). The use of oleogels as an oil-structuring method is a healthier alternative for the production of fat-based products with improved nutritional and energy content, as a result of the saturated and *trans* fat content elimination or reduction

(Chaves et al., 2018). Oleogelation of a liquid oil is achieved with oleogelators. The most common oleogelators are lipid-based, such as waxes, fatty alcohols, mono- and di-glycerides (Chaves et al., 2018; Matheson et al., 2018) and non-lipid-based, such as methyl cellulose (Patel et al., 2014a), ethylcellulose (Gravelle et al., 2012) and hydroxypropyl methyl cellulose (Oh and Lee, 2018). The availability, low cost and gelling ability of the plant derived wax esters are considered important advantages over other oleogelators (Hwang et al., 2012). Chaves et al. (2018) reported that the production of oleogels should follow the following criteria: i) use of lipid bases with exceptional characteristics of functionality, stability and availability among commercially available oils and fats; ii) use of structuring agents from renewable materials, included in the GRAS (generally recognised as safe) category for food application; iii) formulations of lipid systems with chemical composition characteristics and crystallization properties that are compatible with the application of lipid-based foods, such as continuous or emulsified phases.

Oleogels prepared with natural wax esters have been extensively studied for the production of food products, such as cream cheese (Bemer et al., 2016), ice cream (Zulim Botega et al., 2013), chocolate spread (Fayaz et al., 2017; Patel et al., 2014b), bakery products (Jang et al., 2015; Lim et al., 2017; Mert and Demirkesen, 2016; Patel et al., 2014b), meat products (Moghtadaei et al., 2018), margarine and spreadable fat products (Hwang et al., 2013; Patel et al., 2014b; Yilmaz and Öğütcü, 2015). The properties of oleogelsdefine the final application depending on the textural and sensory requirements. The potential application of the wax-based oleogel in food products is evaluated by the firmness, which indicates the function of the wax to structure the oleogel (Imai et al., 2001). Concerning spreadable food products, firmness and stickiness are the most important parameters that define the spreadability of the product (Öğütcü and Yılmaz, 2014).

The concentration of the oleogelator is a critical parameter, which affects the properties of the oleogel and also defines the final product. For instance, the fat products are categorized according to their fat content. According to Codex Alimentarius, the “margarines” should contain at least 80% fat, whereas the “fat spreads” must contain less than 80% fat (FAO/WHO, 2007). The European Union regulations defines the “margarines” and “fat spreads” as the solid malleable emulsion, principally of the water-in-oil type, derived from solid and/or liquid vegetable and/or animal fats suitable for human consumption, and classifies them according to the fat content as follows: margarine (more than 80%, but less than 90%), three-quarter-fat margarine (more than 60%, but less than 62%) and half-fat

margarine (more than 39%, but less than 41%), and fat spread X% (i. less than 39%, ii. more than 41 and less than 60%, iii. more than 62 and less than 80%) (EC 2991/94).

In the case of the manufacture of margarines, specific physical characteristics should be met, concerning their appearance and melting behavior when stored in refrigerator (4 °C) or during consumption (mouth temperature 37 °C) as well as nutritional guidelines. Currently, mixtures of soybean oil and hydrogenated soybean oils have been utilised to fulfil these criteria. Moreover, the richness of hydrogenated oils in *trans* acids led the food industry to develop procedures of hydrogenation or blending nonhydrogenated oil with hardstock (which may be a fully hydrogenated oil or palm stearin) with the aim to decrease the *trans* acids content of the final food product (Gunstone, 2003). The oleogel technique consists of an innovative technique targeting the production of healthier food products. For this purpose, different vegetable oils can be used as oil bases for oleogels, including olive, canola, soybean, palm oil, high oleic sunflower, flaxseed, peanut, sesame, walnut, and corn oils (Chaves et al., 2018; Hwang et al., 2014). Moreover, oleogel technology has been applied recently to fish oil as an alternative method to prevent the oxidation of omega-3 fatty acids (Hwang et al., 2018a). Among these oils, olive oil presents exceptional sensory and health characteristics, whereas the importance of soybean oil is concentrated on the high content of unsaturated fatty acids (linoleic and linolenic acids) and its abundance in essential fatty acids, phospholipids, antioxidants, and sterols (Gunstone, 2003; Tripathi and Shrivastana, 2017). The utilisation of bio-based wax esters to the production of oleogels has not been investigated yet.

Chapter 3

Objectives

3 Objectives

In the bio-economy era, the valorisation of renewable raw materials is essential for the development of integrated biorefinery concepts. Circular economy will eventually replace the linear industrial production model entailing a waste-to-value strategy aiming to the elimination of the environmental impact of the utilization of fossil feedstocks. The transition to a circular economy could be achieved through the utilization of renewable resources generated by food industries and the production of valuable compounds useful in many economic sectors including the food processing industry.

This PhD thesis focuses on the development of a novel integrated biorefinery concept based on the valorisation of food industry raw materials, derived from sugarcane and vegetable oilseed processing, for the production of fumaric acid, microbial oil, carotenoids, fatty acid esters and oleogels with applications as food additives and base-chemicals in the food and chemical industry (Figure 3.1). The biorefinery scheme was based on the composition of each feedstock. In particular, enzymatic and microbial bioprocesses were developed using molasses and soybean cake for the production of crude enzymes, fumaric acid, microbial oil and carotenoids. Subsequently, microbial oil and fatty acid distillates, which are derived from the refining process of soybean oil, were enzymatically converted into fatty acid esters, such as wax esters and polyol esters. Furthermore, the potential application of wax esters was evaluated through the production of oleogels as novel food formulations. The main objectives of the experimental work are presented below:

- Chemical characterization and compositional analysis of raw materials (sugarcane molasses, soybean cake, fatty acid distillates and oilseed cakes) used for the development of bioprocesses within the biorefinery scheme.
- Bioprocess development of soybean cake hydrolysis using crude fungal enzymes in order to produce a nutrient rich hydrolysate. The effect of temperature and initial enzyme concentration during hydrolysis was evaluated.
- Bioprocess development of cane sugar, molasses and soybean cake hydrolysate as fermentation feedstocks for the biotechnological production of intermediate value-added products, such as fumaric acid, microbial oil and carotenoids.
- Evaluation of fermentation conditions for fumaric acid, microbial oil and carotenoids production through submerged fermentations in shake flasks and bioreactors using yeast and fungal strains.

- Bioprocess development using microbial oil and fatty acid distillates as raw materials for the production of wax esters and polyol esters in a lipase-catalysed solvent-free system. The enzymatic reaction conditions (temperature, substrate molar ratio and initial enzyme concentration) were optimized and the physicochemical properties of the esters were determined in order to evaluate their potential use as food additives and biolubricants.
- Oleogelation process development for the production of novel oleogels using the produced bio-based wax esters with olive oil, soybean oil and microbial oil as base oils. Oleogelation process and potential applications of the oleogels were evaluated through the analysis of crystal morphology, color, texture, rheology and thermal behavior.

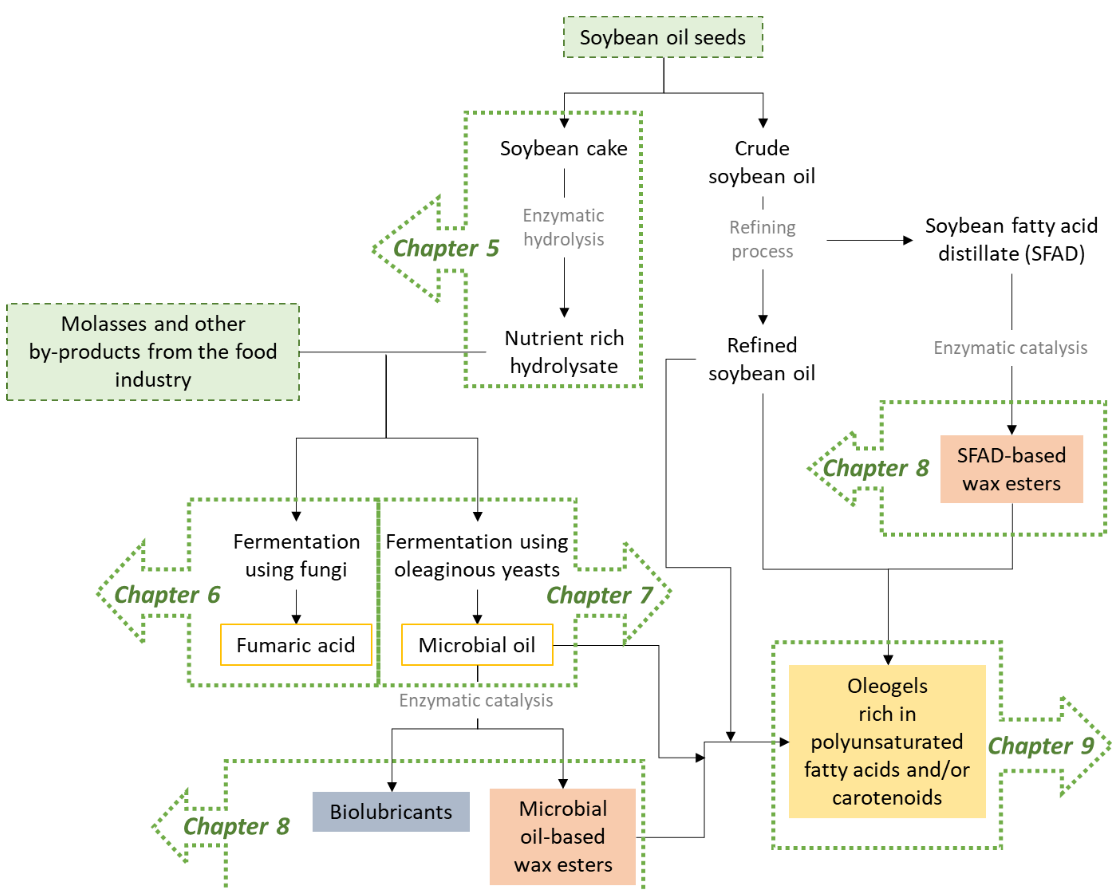

Figure 3.1 Schematic diagram presenting the main objectives of the PhD thesis based on the bioprocesses development using raw materials derived from the sugarcane and oilseed processing industries for the production of bio-economy products

Chapter 4

Materials and methods

4 Materials and methods

4.1 Fungal strains: preservation and pre-culture conditions

The fungal strain *Aspergillus oryzae*, isolated by from a soy sauce industry (Amoy Food Ltd., Hong Kong, China) and kindly provided by Professor Colin Webb (University of Manchester, Manchester, UK), was utilised for the production of crude proteolytic enzymes in order to carried out the hydrolysis process of soybean cake. The conditions of storage, maintenance, sporulation and inoculation of the fungal strain *A. oryzae* have been described in previous studies (Kachrimanidou et al. 2013; Wang et al. 2005). Specifically, spores were preserved in silica sand and reconditioning was performed with PBS solution (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4). Thefungus was maintained at 4 °C on agar slopes containing 30 g/L sunflower meal, 20 g/L wheat bran and 20 g/L agar. The fungal strain *Rhizopus arrhizus* NRRL 2582 was purchased from the ARS Culture Collection (NRRL) (Peoria, Illinois, USA) and utilised in: a) solid state fermentations (SSF) for the production of crude proteolytic enzymes and b) submerged fermentations (SmF) for fumaric acid production. The storage and maintenance conditions were the same as described above for *A. oryzae* with the difference that agar slopes contained 50 g/L soybean cake and 20 g/L agar.

The slopes of *A. oryzae* and *R. arrhizus* were used as inoculum for the SSF aiming to increase the spore concentration. In particular, spores from slants were washed with 5 mL of sterilised water supplemented with 0.01% (v/v) of Tween 80 (Sigma-Aldrich) and then 2 mL were aseptically transferred to Erlenmeyer flasks of 250 mL, containing the same growth medium as in slopes for each fungi, following by incubation at 30 °C for 3 - 4 days. Fungal spores were liberated by adding 50 mL sterilised water supplemented with 0.01% (v/v) Tween 80 and glass beads (4 mm diameter) following vigorous shaking of the flasks. Spore suspensions of *R. arrhizus* used for the SmF for fumaric acid production were also collected and stored in cryovials filled with pure glycerol, at -80 °C. The vials containing spore suspensions were used to inoculate pre-culture medium, which is described in the section 4.6. All media were autoclaved at 121°C for 20 min.

4.2 Yeast strains: preservation and pre-culture conditions

The oleaginous yeast *Rhodosporidium toruloides* DSMZ 4444 (purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH) was utilized for microbial oil and carotenoids production through SmF. Also, the yeasts

Cryptococcus curvatus ATCC 20509 (purchased from the American Type Culture Collection, USA) and *Lipomyces strakeyi* DSMZ 70296 (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH) were employed in SmF for microbial oil production, with the aim to utilise it for oleochemicals production. The yeast was maintained on agar slopes containing glucose (10 g/L), yeast extract (10 g/L), peptone (10 g/L) and agar (2%, w/v) and stored at 4 °C. The inoculum was produced at 28 °C for 24 h using the same medium besides agar. The inoculum was cultivated in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at an agitation rate of 180 rpm. These inoculums was used in batch and fed-batch fermentations. All media were autoclaved at 121°C for 20 min.

4.3 Renewable resources as raw materials

4.3.1 Very high polarity cane sugar and molasses

Very high polarity (VHP) sugar and molasses were provided by the sugarcane industry Cruz Alta (Guarani, São Paulo, Brazil). VHP sugar contained predominantly sucrose at more than 99%(w/w). Molasses had a purity of 47% (w/w) and was mainly consisted of sucrose, glucose and fructose. Detailed composition of these raw materials is presented in chapters 5 and 6.

4.3.2 Soybean cake

Soybean cake was utilised for the production of a nutrient rich feedstock through enzymatic hydrolysis and was kindly provided by the biodiesel production industry BSBios (Passo Fundo, Rio Grande do Sul, Brazil).

4.3.3 Fatty acid distillates from oil mills

Palm fatty acid distillate (PFAD) originated from palm oil refining process was provided by Companhia Refinadora da Amazônia (Grupo Agropalma, Brazil). Soybean fatty acid distillate (SFAD) obtained by acidulation of soap stock from soybean oil refining process was provided by Miracema-Nuodex (Brazil). The fatty acid composition was analysed by gas chromatography (GC) and the results are presented in chapter 8.

4.3.4 Vegetable oils

Palm mesocarp oil was purchased from a local supplier of raw materials for cosmetics and was used for the optimisation of transesterification conditions for wax esters production. Extra virgin olive oil and soybean oil were also purchased from a local market and was utilised as base oils for the preparation of oleogels. The fatty acid composition of the oils was analysed by GC and the results are presented in chapters 8 and 9.

4.3.5 Others

Babassu cake, a solid residue from the babassu oil industry was kindly provided by Tobasa S.A. (Tocantinópolis, Brazil) and was utilised for the immobilisation of a crude fungal lipase derived from *Rhizomucor miehei*. For the preparation of a home-made lipase, castor bean (*Ricinnus communis* L.) seeds were supplied by Embrapa Cotton Research Center (Campina Grande, Brazil) and stored at 4 °C until use.

4.4 Crude enzyme production by solid state fermentation using soybean cake

The crude enzyme consortia production were conducted through SSF in 250 mL Erlenmeyer flasks, which contained 5 g (dry basis, db) of soybean cake. In the case of SSF using *A. oryzae*, the moisture content of the substrate was adjusted to 65% (w/w, db) by inoculating with the fungal spore suspension (2×10^6 spores/mL) as described previously in section 4.1 (Kachrimanidou et al. 2013; Dimou et al. 2015). The SSF of *R. arrhizus* was carried out using a fungal spore suspension of 1.5×10^8 spores/mL under the same conditions as in the SSF of *A. oryzae*. The solid substrates used in all SSF were sterilized at 121 °C for 20 min and after inoculation all cultures were incubated at 30 °C. At the end of fermentation, the fermented solids were suspended in sterile water and macerated using a blender under aseptic conditions. The suspended macerated solids were mixed with untreated soybean cake in order to promote the enzymatic hydrolysis of soybean cake.This experimental setup evaluated the effect of fermentation time on the enzyme activity of proteases. The data represent the mean values of duplicate experiments.

4.5 Enzymatic hydrolysis process of soybean cake

The enzymatic hydrolysis of soybean cake using the crude enzymes of *A. oryzae* and *R. arrhizus* was carried out in 1 L Duran bottles containing 50 g/L total solids, which was consisted of untreated soybean cake (90%) and fermented soybean cake derived from the SSF of each fungi (10%). The SSF derived fermented solids provided the crude enzymes required for the production of the nutrient-rich hydrolysate. The Duran bottles containing the untreated soybean cake were sterilized at 121 °C for 20 min. Then the macerated and suspended fermented solids from SSF cultures were added in the bottles under aseptic conditions.

The hydrolysis process of soybean cake using the crude enzymes of *A. oryzae* was previously optimised, regarding the temperature by Papadaki et al. (2017). Thus, the present thesis focused on the evaluation of temperature (40 °C, 45 °C and 50 °C), initial enzyme activity (2.7 U/mL, 5.4 U/mL and 6.7 U/mL) and fermentation time on soybean cake hydrolysis using the crude enzymes of *R. arrhizus*. All hydrolysis experiments were agitated using magnetic stirrers, while the pH was not controlled. The time course of free amino nitrogen (FAN) and inorganic phosphorus (IP) production was studied during hydrolysis. The samples were collected at random intervals and the enzymatic reaction was terminated by adding trichloroacetic acid (5 %, w/v). The solids were separated via centrifugation (3000 × g, 5 °C, 10 min) and the supernatant was used for the analysis of FAN and IP. Hydrolysis yield was expressed as the percentage of total Kjeldahl nitrogen (TKN) to FAN conversion. The experiments were carried out in duplicates and the data represent the mean values.

After the hydrolysis, the remaining solids were removed by centrifugation (9000 × g, 5 °C, 15 min) and vacuum filtration. The pH of the hydrolysate was adjusted to 6 with 5 M NaOH and then was filter-sterilized using a 0.2 μm filter unit (Polycap TM AS, Whatman Ltd., Maidstone, UK) or autoclaved at 121 °C for 15 min. The hydrolysate was further utilised as substitute of the commercial nitrogen sources for the SmF for fumaric acid, microbial oil and carotenoids production.

4.6 Fungal fermentations for fumaric acid production

The fumaric acid production was investigated in batch and fed-batch fermentations using pure sugars, VHP sugar, molasses and pre-treated molasses as carbon sources. Moreover, the soybean cake hydrolysate, derived from either *A. oryzae* or *R. arrhizus* crude proteases, was evaluated as a nutrient and nitrogen source in the pre-culture medium at various concentrations.

In all fermentations $CaCO_3$ was added as neutralising agent and additional source of CO_2. The initial $CaCO_3$ concentration used in each fermentation was 80% of the initial carbon source concentration, because higher $CaCO_3$ concentrations caused problems in the regulation of the pH value.

The inoculum used in all cases was consisted of uniformly dispersed mycelia, which were produced in Erlenmeyer flasks of 250 mL filled with 50 mL of the pre-culture medium (Table 4.1). The composition of the pre-culture medium was optimised on previous study (Papadaki et al. 2017), which showed that commercial nitrogen sources could efficiently substituted by soybean cake hydrolysate. The pH of the pre-culture medium was controlled at 6.0 with 5 M NaOH or 5 M HCl. The pre-culture was incubated at 30 °C for 24 h in a rotary shaker with an agitation speed of 180 rpm and dispersed mycelia was inoculated (10%, v/v) in

Table 4.1 Composition of the pre-culture and the fermentation medium for fumaric acid production

Constituents	Pre-culture medium (g/L)	Fermentation medium (g/L)
Carbon sources	Glucose, 25	Glucose:Fructose, 50 VHP sugar, 25 Molasses, 25 Pre-treated molasses, 25
Soybean cake hydrolysate	Various concentrations	-
$CaCO_3$(%)	-	80 [a]
$(NH_4)_2SO_4$	-	0.2
KH_2PO_4	0.6	0.6
$MgSO_4 \cdot 7H_2O$	0.4	0.4
$ZnSO_4 \cdot 7H_2O$	0.044	0.044
$FeCl_3 \cdot 6H_2O$	0.016	0.016
Tartaric acid	-	0.0075
Methanol (mL/L)	-	15
Corn steep liquor (mL/L)	3	0.5
Corn starch	30	-
Agar	1	-

[a] $CaCO_3$was used as a percentage of the initial total sugar concentration

the fermentation medium. The pre-culture medium without the nitrogen source was autoclaved at 121 °C for 20 min and then the filter-sterilized soybean cake hydrolysate was aseptically added.

Initially, preliminary experiments were conducted in shake flask fermentations in order to evaluate the ability of *R. arrhizus* to produce fumaric acid using a co-substrate consisting of pure glucose and fructose at a ratio of 1:1 at an initial total sugar concentration of 50 g/L. Batch fermentations were conducted in Erlenmeyer flasks of 250 mL containing 50 mL of fermentation media. The composition of the fermentation medium is presented in Table 4.1.

The following experiments investigated the fumaric acid production using VHP sugar in fed-batch fermentations, which were conducted in Erlenmeyer flasks of 250 mL containing 50 mL of fermentation media, but using VHP sugar as carbon source at an initial concentration of 25 g/L (Table 4.1). A concentrated VHP sugar solution (400 g/L) was added to the medium at random intervals in order to maintain the total sugar concentration above 5-10 g/L. When feeding with the concentrated solution of VHP sugar was employed an additional stoichiometric quantity of $CaCO_3$ was added aseptically. At this point of the study, the effect of different initial FAN concentrations (100, 200 and 400 mg/L)of soybean cake hydrolysate on fumaric acid production was also evaluated.

The next experiments were conducted using molasses as carbon source in batch fermentations (Table 4.1). The effect of the presence of the trace elements (KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$) and the other supplements ($(NH_4)_2SO_4$, corn steep liquor, methanol, tartaric acid) was evaluated. Fumaric acid production was also studied using clarified molasses as substrate. Specifically, a molasses solution containing 30% (w/v) total sugar concentration was acid-treated using 5 M H_2SO_4 in order to obtain a final pH of 3.5. Subsequently, the molasses temperature was increased to 100 °C for 1 h. The treated molasses was centrifuged (9000 × g, 5 °C, 15 min) (Xiao et al. 2007; Liu et al. 2008) and the supernatant was used as a substrate after adjusting the pH at 6.5. Also, cationic resin (Amberlite IR 120H, Sigma-Aldrich) of sulfonic (SO_3H) type based on a polystyrene-divinylbenzene copolymer was utilised for further treatment of the acid clarified molasses. Specifically, after the acid pretreatment, the pH of molasses solution was adjusted at 2 and then was passed through a column of resins at room temperature. The pH of the clarified molasses solution was adjusted at 5.5 and utilized in SmF.

In all fermentations, the medium was autoclaved at 121 °C for 20 min, whereas corn steep liquor and $(NH_4)_2SO_4$, were autoclaved separately, and methanol was filter sterilized.

Then, all components were added to the sterilized medium prior to inoculation under aseptic conditions. The pH of the culture was manually controlled during fermentation at the optimum pH value of 5.5 using 5 M NaOH or 5 M HCl. For all fermentations the data represent the mean values of duplicate experiments.

4.7 Yeast fermentations for microbial oil and carotenoids production

4.7.1 Batch fermentations

Shake flask fermentations were carried out in 250 mL Erlenmeyer flasks using 50 mL of the medium. VHP sugar and molasses were utilised as carbon sources, at an initial total sugar concentration of around 70 g/L, whereas the nitrogen source was a mixture of yeast extract 0.5g/L and $(NH_4)_2SO_4$ 0.5 g/L. The medium was also consisted of phosphate buffersand various trace elements as it is shown in Table 4.2. The inoculation was performed using 10% (v/v) of a 24 h YPD pre-culture, following incubation at 28 °C in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at an agitation rate of 180 rpm. The pH was maintained in the range of 5.5 - 6.2, by adding aseptically an appropriate volume of sterilized 5 M NaOH or 5 M HCl when needed. The data represent the mean values of duplicate experiments.

Table 4.2 Composition of supplements of the fermentation medium for the yeast *R. toruloides*

Type of supplement	Supplements	Concentration (g/L)
Phosphate buffers	KH_2PO_4	7.0
	Na_2HPO_4	2.5
Trace elements	$MgSO_4 \cdot 7H_2O$	1.5
	$CaCl_2 \cdot 2H_2O$	0.15
	$FeCl_3 \cdot 6H_2O$	0.15
	$MnSO_4 \cdot H_2O$	0.06
	$ZnSO_4 \cdot 7H_2O$	0.02

4.7.2 Fed-batch fermentations

Fed-batch fermentations were conducted in a 2 Lbench top bioreactor (Labfors, Infors4) with 1.0 L working volume equipped with pH probe (Mettler Toledo, USA), dissolved

oxygen probe, temperature detector, agitation, gas inlet and outlet, pumps and sampling system. The temperature was controlled at 28 °C and the pH was automatically regulated in the range of 5.8 - 6.2 using 5 M NaOH and 5 M HCl. An agitation cascade (200 - 500 rpm) was employed in order to maintain the dissolved oxygen level at 20% of saturation with an aeration rate of 1 vvm.The pH probe was calibrated before autoclaving, whereas the dissolved oxygen probe calibration was employed prior to inoculation. A sterilised Tween-80 solution (1 %, v/v) was used as antifoam agent during the fermentation.Inoculation was performed using 10 % (v/v) of a 24 h YPD pre-culture.

The medium was consisted of molasses and the supplements as mentioned in Table 4.2. Molasses was used at an initial total sugar concentration of around 70 g/L. A concentrated solution of VHP sugar (500 g/L) was used as feeding solution and was automated added into the bioreactor using a peristaltic pump in order to maintain the concentration of total sugars at 20 g/L during the fermentation.

Two set of experiments were employed to evaluate the effect of different nitrogen sources which was either the mixture yeast extract 0.5g/L and $(NH_4)_2SO_4$ 0.5 g/L or soybean cake hydrolysate at initial FAN concentration of 300 mg/L. Carotenoids production was also studied during fed-batch fermentations.

4.8 Lipases for the enzymatic synthesis of oleochemicals

The commercial lipases Novozyme 435 (lipase B from *Candida antarctica*) immobilised on a macroporous acrylic resin and Lipozyme® (lipase from *Mucor miehei*) immobilized on a macroporous ion-exchange resin (purchased from Sigma-Aldrich) were used for wax ester synthesis from palm oil and microbial oil.

Also, non-commercial lipases were evaluated for the production of microbial oil wax esters. Home-made lipases were prepared in the Biochemistry Department and Polymer Engineering Laboratory / Engepol of the Federal University of Rio de Janeiro (Brazil). Particularly, a two-step enzymatic process was developed, which include i) the hydrolysis of the microbial oil and ii) the esterification of the free fatty acid fraction derived from the hydrolysed microbial oil (HMO). The two-step process was considered essential since the home-made enzymes could not performed direct transesterification catalysis. Initially, the lipase for the microbial oil hydrolysis was extracted from the dormant castor bean seeds by triturating and washing the seeds in acetone, according to the methodology described by

Cavalcanti et al. (2007). The acetone powder obtained was stored at 4°C until use and named as Vegetable Enzyme (VEG). Thereafter, the production of lipase B from *Candida antarctica* (LipB) in *Pichia pastoris* was conducted using glycerol according to Manoel et al. (2016). The supports were prepared by core shell technology using polymethylmethacrylate (PMMA). The preparation of the polymer particles used as immobilisation support and the immobilization technique employed have been described by Cipolatti et al. (2018). The LipB enzyme was utilized for the esterification reactions between the HMO and fatty alcohols.

In the case of PFAD and SFAD derived wax esters, the esterification reactions was performed using a home-made lipase which was produced through SSF by the fungal strain *Rhizomucor miehei* (IDAC accession number 071113-01) utilising babassu cake as substrate. The production method of this lipase have been described by Aguieiras et al. (2014). Specifically, SSF were carried out for 72 h and then the fermented solids were lyophilized until the moisture content was less than 3% (w/w) and stored at 4 ºC. The dry fermented solid (DFS) that contained the biocatalyst were then used in the synthesis of wax esters.

The biolubricants production from microbial oil was carried out through the catalysis of the lipase Lipomod 34MDP (derived from *Candida rugosa*), which was purchased from Biocatalysts Ltd., and kindly provided by Biochemistry Department of the Federal University of Rio de Janeiro (Brazil).

4.9 Development of enzymatic processes for wax esters production

The enzymatic reactions were carried out in 50 mL round bottom flasks containing the corresponding oil and fatty alcohol at 1:3 molar ratio in the case of transesterifications (using microbial oil and palm oil as raw material) and 1:1 molar ratio for the esterifications (using HMO, PFAD and SFAD as raw materials). The fatty alcohols cetyl (C16:0) (100%), oleyl (C18:2) (85%) and behenyl (C22:0) (98%) alcohols (Sigma-Aldrich) were used in the lipase-catalysed transesterification and esterification reactions. After mixing and temperature equilibration of substrates (oil and alcohol), using a temperature controlled water bath and magnetic stirrer, the reaction was initiated by adding the enzyme. At regular intervals, samples were taken and diluted in n-heptane for the quantitative analysis.

The microbial oils used in this study were produced by three different yeast strains *R. toruloides*, *L. starkeyi* and *C. curvatus* using fermentation media derived from: i) molasses, ii) confectionery industry waste streams and iii) a co-substrate of cheese whey and wine lees.

The production of microbial oil using molasses as substrate by the yeast *R. toruloides* is described in details in chapter 7, whereas the cultivation of the oleaginous yeasts *R. toruloides* and *L. starkeyi* have been previously evaluated using crude hydrolysates derived from confectionery industry waste streams as the sole fermentation media (Tsakona et al. 2014, 2016). Specifically, fed-batch fermentation of *L. starkeyi* led to a microbial oil concentration of 64.5 g/L with a microbial oil content of 57.8% (w/w) (Tsakona et al. 2014). In the case of *R. toruloides* fed-batch cultures, the total dry weight was 61.2 g/L lipid concentration with a lipid content of 61.8% (w/w) (Tsakona et al. 2016). Moreover, the oleaginous yeast *C. curvatus* was cultivated on deproteinised and concentrated cheese whey mixed with hydrolysates derived from fractionated wine lees as described by Kopsahelis et al. (2018). Fed-batch cultures of *C. curvatus* resulted in a microbial oil concentration of 33.1 g/L with a microbial oil content of 49.6% (w/w) (Kopsahelis et al. 2018). The produced microbial oils were extracted and utilised as raw material for the enzymatic reactions.

The average molecular weight of palm and microbial oils used was calculated from the respective fatty acid profile of each oil (Shrestha and Gerpen 2010). Specifically, the molecular weights of the raw materials used for the enzymatic catalysis are presented in table 4.3.

Table 4.3 Average molecular weights of the raw materials used in the enzymatic synthesis of wax esters

Type of reaction	Source of oil	Molecular weight (g/mol)
Transesterification	Palm oil	836.5
	Cryptococcus curvatus	862.6
	Rhodosporidium toruloides	850.8
	Lipomyces starkeyi	838.7
Esterification	Palm fatty acid distillate	271.0
	Soybean fatty acid distillate	276.0
	Hydrolysed microbial oil from *Rhodosporidium toruloides*	270.9

Initially, experimental trials were carried out using the commercial enzymes Novozyme 435 and Lipozyme for the production palm oil derived wax esters in order to optimise the

reaction conditions. The parameters of temperature, enzyme concentration, reaction time and the reuse of the enzyme were evaluated in a solvent-free system as described below:

Effect of temperature

The conversion of palm oil to wax esters was investigated at four temperatures (40, 50, 60 and 70 °C). As the melting point of the different fatty alcohols used is different (19 °C for oleyl alcohol, 50 °C for cetyl alcohol, 69 °C for behenyl alcohol) the experiments at 70 °C were performed using all alcohols. The experiments at 60 °C were carried out with oleyl and cetyl alcohols, while the experiments at 50 °C and 40 °C were conducted using only oleyl alcohol.

Effect of enzyme concentration

The enzyme concentration required in order to maximise the transesterification yield was also studied during palm wax ester synthesis. Three different initial enzyme activities (2, 3 and 4 Units per g of substrate) were used for each enzyme for all the alcohols evaluated.

Effect of reaction time

The optimised reaction conditions concerning the temperature and the enzyme concentration were employed for the production of microbial oil derived wax esters. The kinetic profile of palm oil and microbial oils derived wax esters was evaluated by taking samples at different reaction times (0 - 12 h).

Effect of enzyme reuse

Sequential batch reactions were carried out in order to assess the stability of the lipase used at the optimised conditions established from the aforementioned experiments. The reaction period was 7 h and at the end of each batch 20 mL of n-heptane were added to solubilize the formed products. Lipase was separated by filtration, followed by double washing with 25 mL petroleum ether, drying at room temperature and then used in the next batch reaction (Deng et al. 2011).

The following experiments were focused on the wax esters production using non-commercial enzymes aiming to the cost reduction of the process. The hydrolysis of microbial oil was essential for producing a free fatty acid fraction, since the non-commercial enzymes present either hydrolytic or esterification activity. The details concerning the hydrolysis of microbial oil is described in a following section (4.12.14). The production of wax esters was

performed through esterification of the HMO with oleyl and cetyl alcohols. The reactions were carried out using a stoichiometric molar ratio (1:1) of HMO:alcohol and 2.5% (w/w) of LipB enzyme. The esterifications were evaluated at different temperatures, from 35 °C to 50 °C. The effect of the addition of limonene, as green solvent, was also evaluated. All reactions were stopped at 24 h and samples were taken for quantitative analysis of wax ester content.

The final experiments focused on the valorisation of PFAD and SFAD for wax esters production. The solvent-free esterification reactions were catalysed by the addition of DFS at 8.8 U per g of substrate at 50 °C. Samples were taken at specific intervals and the reactions stopped at 24 h. The efficiency of the esterification process using DFS was evaluated through five repeated batch reactions. At the end of each batch reaction, the DFS biocatalyst was washed with 20 mL of hexane, following vacuum filtration. The DFS was then dried in a desiccator for 24 h, before reused in a new batch reaction. In all experimental cases, the data represent the mean values of triplicate experiments.

4.10 Development of enzymatic processes for biolubricants production

The production of biolubricants was monitored during the enzymatic reaction by recording the reduction of the acidity caused by the esterification of free fatty acids because analytical standards for the esters formed are not commercially available. Therefore, the microbial oil was enzymatically hydrolysed into free fatty acids as described in the section 4.12.14.

The esterification reactions between the HMO and the trimethylolpropane (TMP) and neopentyl glycol (NPG) (Sigma-Aldrich) polyols were carried out in 50 mL round bottom flasks, which were temperature controlled in a water bath and agitated using a magnetic stirrer. The esterification conditions (i.e. substrate to molar ratio, enzyme concentration and temperature) were selected based on previous literature-cited publications (Greco-Duarte et al. 2017; Fernandes et al. 2018). In the case of HMO:TMP the molar ratio used was 3:1, whereas in the case of HMO:NPG the molar ratio used was 2:1. All reactions were carried out after the addition of 1 % distilled water. The temperature was set at 45 ºC and the enzyme used was added at 4% (w/w, based on the reaction quantity). Samples of 100 μl were taken at specific intervals (from 5 min to 72 h). The reaction mixture were centrifuged (8000 × g, 5 min) in order to separate the enzyme from the product containing the polyol esters and some non-reacted polyols and fatty acids, which was collected for further analysis. The reactions were conducted in duplicate and data represent the mean values.

4.11 Oleogels production

Olive oil, soybean oil and microbial oil were utilized as the base oils for the production of oleogels. Microbial oiland SFAD derived cetyl wax esters were evaluated as potential oleogelator agents at different concentrations (7%, 10% and 20% w/w). The oils and wax esters were precisely weighted and the mixture was heated at 90 ºC under agitation for 10 min. Screw capped glass vials were filled with approximately 15 mL of the oleogel and cooled at room temperature for 24 h to allow gel formation. The samples were then stored at 4 ºC for a period of 30 days for further analysis of the physical properties. The data represent the mean values of triplicates.

4.12 Analytical methods

4.12.1 Determination of sugar content and composition of raw materials by HPLC

The sugar content and the composition analysis of VHP sugar and molasseswereperformed using High Performance Liquid Chromatography (HPLC) (Shimadzu) equipped with a Bio-rad Aminex HPX-87H column (300 mm length x 7.8 mm internal diameter) coupled to a differential refractometer. The mobile phase was a solution of 10 mM H_2SO_4 with 0.6 mL/min flow rate and 65 °C column temperature. Monosaccharides were also determined with a Shodex SP0810 column at 60 °C with 1 mL/min flow rate of pure water as the mobile phase.

Calibration curves of the sugars were as follows:

Sucrose: y = 6.50143e-006x, (R^2= 0.9994)

Glucose: y=7.2726e-006x, (R^2=0.9996)

Frucrose: y = 6.90429e-006x, (R^2 = 0.9999)

4.12.2 Determination of total Kjeldahl nitrogen content of raw materials

The total Kjeldahl Nitrogen (TKN) content of soybean cake and molasses was determined using a KjeltekTM 8100 distillation Unit (Foss, Denmark). The method is based on the total conversion of the initial forms of nitrogen in ammonium salts. In the case of soybean cake dried and fine milled samples of 0.5 g were accurately weighted into a digestion

tube, following addition of 25 ml of $H_2SO_4$and 1 kjeltab tabletcatalyst containing 3.5 g K_2SO_4 and Cu_2SO_4. Also, a blank sample was prepared and followed the same procedure. The digestion was carried out at 420 ^{0} C for 1h and after the tubes were cooled down the distillation was followed by the automatic addition of 30 ml H_2O and 100 ml NaOH (40%, w/v). In the case of liquid samples, such as molasses, samples of 5 g are introduced into the digestion tubes,following by the addition of 10 ml of $H_2SO_4$and 1 kjeltab tabletcatalyst containing 3.5 g K_2SO_4 and Cu_2SO_4. The distillation was carried out by the automatic addition of 80 ml H_2O and 50 ml NaOH (40%, w/v).

According to the principles of the method the samples were acid digested in order to convert the nitrogen of protein to ammonium salts ($(NH_4)_2SO_4$) at a boiling point elevated by the addition of K_2SO_4 with a Cu catalyst to enhance the reaction time. During the distillation process the ammonium salts are converted to ammonia (NH_3), which is subsequently distilled into a boric acid solution by alkaline steam distillation and quantified titrimetrically with a standard acid solution (0.1 N HCl). Total nitrogen was calculated according to the equation:

$$\mathrm{TKN}\ (\%) = \frac{\left((\mathrm{Vs} - \mathrm{Vb}) \times \mathrm{N} \times 14.007\right) \times 100}{\mathrm{w}}$$

where, *Vs = volume of 0.1N HCl consumed for the sample in mL,*

Vb = volume of 0.1N HCl consumed for the blank in mL,

N = normality of HCl, and

w = mg of the sample

In order to estimate the protein content of soybean cake and molasses, TKN was multiplied by 6.25 given the fact that proteins contain 16% nitrogen (100/16=6.25).

4.12.3 Determination of fibers, ash and lipid content of raw materials

The acid detergent fiber (ADF), acid detergent lignin (ADL), and neutral detergent fiber (NDF) of soybean cake were determined according to AOAC Official Method 973.18. The results of ADL, ADF, and NDF were expressed as cellulose (ADF-ADL), hemicellulose (NDF-ADF), and lignin (ADL) content. Theash and lipid content of soybean cake wasanalysedaccording to AACC Approved Methods 08-01 and 30-25, respectively.

4.12.4 Determination of phenolic compounds and antioxidant activity of raw materials

The phenolic compounds in molasses were extracted following the methodology presented by Alexandri et al. (2016). Specifically, a molasses aqueous solution (~280 g/L) was prepared regulating the pH at 2 with concentrated HCl. The molasses was subsequently treated with ethyl acetate for 30 min, using a 1:3 (v/v) of molasses to solvent ratio at room temperature. After phase separation, the ethyl acetate extract was collected and the solvent was vacuum evaporated. The extract was weighed and 5 mL of methanol was added. The methanolic solution was stored at -20 °C for further analysis. The determination of total phenolic content (TPC) was carried out using the Folin-Ciocalteu colourimetric method as described by Faustino et al. (2010). The TPC was expressed as g of gallic acid equivalent (GAE) per g of molasses using the following equation: $y = 1.3768x$ ($R^2 = 0.9978$).

The antioxidant activity of the molasses extract was determined according to the DPPH• (2,2-diphenyl-1-picrylhydrazyl) scavenging radical method (Scherer and Godoy, 2009). The sample (0.1 mL) was mixed with 3.9 mL of DPPH• solution (31.6 μg/mL) and the mixture was agitated before incubation at room temperature in the dark for 90 min. Aqueous methanol (0.1 mL, 70:30 v/v) mixed with 3.9 mL DPPH• was used as control. A double-beam UV-Vis spectrophotometer (Jasco V-530, Tokyo, Japan) was used to measure the decrease in absorbance at 517 nm.

The individual phenolic compounds in the extract were determined by High Pressure Liquid Chromatography coupled with a diode array detector (HPLC-DAD) analysis (Alexandri et al. 2016).

4.12.5 Elemental analysis of raw materials by ICP-OES

An inductively coupled plasma optical emission spectrometer (ICP-OES, Ultima 2, Horiba Jobin Yvon) was used for elemental determination of the molasses and VHP sugar, with slight adaptation of the ASTM-D1976 method. Samples were diluted in water when it was necessary. The analysis was performed by Petrobras in Brazil.

4.12.6 Fatty acid composition analysis of raw materials by GC-FID

The fatty acid composition of oils was determined by converting triacylglycerols into fatty acid methyl esters (FAMEs) through a two-step derivatization reaction with methanol using sodium methoxide (MeONa) and HCl as catalysts. FAMEs were analysed on a Fisons GC-8060 gas chromatography equipped with a CPWAX 52CB column (30 m × 0.32 mm i.d., 0.25 μm film thickness, Chrompack), a split/splitless injector and a flame ionization detector (FID). Helium was used as carrier gas (2 mL/min). The oven temperature was held initially at 100 °C for 1 min. It was programmed at a ratio of 25 °C/min to 200 °C, maintained for 1 min, then ramped at a ratio of 3 °C /min to 230 °C, for 6 min, and then raised at a ratio of 30 °C /min to 250 °C, for 2.5 min. The temperature of the injector and the detector were set at 250 °C and 270 °C, respectively. The split ratio was 1:50 and the sample injection volume was 1 μL. Peak identification was accomplished by comparison of retention times with those of a certified reference FAME mixture (Supelco 37 Component FAME mix). Fatty acid data were expressed as area percentage of FAME.

4.12.7 Determination of proteolytic activity of crude enzymes production

The enzymatic activity of proteases produced during SSF of *A. oryzae* and *R. arrhizus* on soybean cake was evaluated following the method described by Kachrimanidou et al. (2013). Briefly, fermented solids obtained from SSF were mixed with citric acid-Na_2HPO_4 (0.2 M, pH 6) buffer solution for the extraction of proteases. After centrifugation (3000× g, 4 °C, 15 min) extracts were used for the determination of enzymatic activities. Protease activity was quantified by the FAN production during hydrolysis of 7.5 g/L of casein at 55 °C. One unit of protease activity (U) was defined as the protease required for the production of 1 μg FAN in one minute at 55 °C and pH 6.0. The results were expressed as U/g of fermented solids.

4.12.8 Determination of lipases activity

Enzymatic assay of the commercial Novozyme 435 and Lipozyme RM IM was performed as described in ACS Specifications (1993). One unit of lipase activity was defined as the amount of enzyme that catalyses the hydrolysis of 1.0 μmole of fatty acid from a triglyceride per minute at pH 7.7 and at 37 °C using olive oil as substrate. The enzyme activity was expressed as Unit of lipase activity per g of the reaction mixture (U/g).

The activity of the following lipases was performed in the Biochemistry Department of the Federal University of Rio de Janeiro (Brazil):

The activity of the Lipomod 34MDP was measured using 25 mM p-nitrophenyl laureate in acetonitrile/DMSO (1:1 v/v) as substrate dissolved in 25 mM phosphate buffer at pH 7.0 (Gutarra et al., 2009). One unit (U) of hydrolytic activity was defined as the amount of enzyme that releases 1 mmol of p-nitrophenol per minute under the assay conditions. The hydrolytic activity for the Lipomod 34MDP used in this study was 10679 U/g.

The hydrolytic activity of the VEG enzyme was determined using tributyrin (5% w/v) as substrate in Triton X-100 (25% w/v) and 0.1 M sodium acetate buffer pH 4.0. Hydrolysis was performed at 30 ºC under agitation according to Cavalcanti et al. (2007). Lipase activity was determined by titration of free fatty acids released by enzyme action according to Freire et al. (1997). One unit (U) of hydrolytic activity was defined as the amount of enzyme that releases 1 μmol of butyric acid per minute under the assay conditions.

For LipB activity, hydrolysis was carried out under mild stirring at 30 ºC, using p-nitrophenyl laurate (p-NPL) as substrate. The hydrolytic activity procedure was detailed in previous studies (Robert et al., 2017; de Castro et al., 2011). One unit of enzyme activity (U) corresponds to the amount of enzyme required to produce 1 μmoL of p-nitrophenol per minute under the assay conditions.

The enzymatic assay for the determination of the DFS lipase activity was carried out using p-nitrophenyl-laurate (pNP-laurate) as substrate according to Gutarra et al. (2009). Lipases were extracted from the fermented solid with phosphate buffer (0.1 M, pH 7.0) as described by Gombert et al. (1999) and the supernatant was used for hydrolytic activity determination at 412 nm. One unit (U) of hydrolytic activity was defined as the amount of enzyme that releases 1 μmol of p-nitrophenol per minute under the assay conditions. The hydrolytic activity of the DFS was 43 U/g.

4.12.9 Determination of free amino nitrogen and inorganic phosphorus

The FAN and IP concentrations in the hydrolysates and in the fermentations were measured according to the ninhydrin colorimetric method and the ammonium molybdate spectrophotometric method, respectively (Lie 1973; Harland and Harland 1980). The concentration of FAN refers to the nitrogen contained in free amino groups of amino acids and peptides. For the determination of FAN the following reagents were regularly prepared:

Color reagent: a mixture of around 49.7 g $Na_2HPO_4.2H_2O$, 5 g ninhydrin, 3 g fructose and approximately 60 g KH_2PO_4 was prepared to a final volume of 1L until the pH of the reagent was in the range of 6.6 - 6.8. Stored at 4 °C.

Dilution reagent: 2 g KIO_3 were dissolved in 616 mL of deionised water and subsequently 384 mL of absolute ethanol (99%, v/v) was added to make 1L final volume. Stored at 4 °C.

Glycine stock solution: 0.1072 g of glycine was dissolved in deionised water to a final volume of 100 mL.Stored at 4 °C.

Glycine standard solution: 1 mL of the glycine stock solution was transferred and diluted in deionized water until 100 mL final volume in order to obtain a final concentration of FAN 2 mg/L. Stored at 4 °C.

The methodology for FAN determination was as follows: 1 mL of a properly diluted sample was added into glass test tubes, followed by the addition of 0.5 mL of color reagent. A blank sample was also prepared with water instead of diluted sample. The samples were boiled for exactly 16 min and then were cooled down for 20 min, at 20 °C. Subsequently, 2.5 mL of dilution reagent were added, following vigorously shaking with vortex. The absorbance was read in spectrophotometer at 570 nm (U-2000, Spectrophotometer, Hitachi) against blank sample. All the samples were carried out in duplicates.

The calibration curve was carried out by diluting glycine standard solution to achieve different concentrations of FAN in the range of 0.5 - 2 mg/L). The calibration curve was the following: $y = 6.4941x + 0.0762$ ($R^2 = 0.9995$).

The IP concentration was determined using the following reagents: perchloric acid (60 %, v/v), ascorbic acid (1 %, w/v), ammonium molybdate (4 %, w/v) and a K_2HPO_4 standard solution. Specifically, the standard solution was prepared by dissolving 0.0561 g K_2HPO_4 in 1 L of deionised water to obtain a final concentration of 10 mg/L IP.

According to the methodology samples were appropriately diluted to 5 mL final volume into glass test tubes, 0.4 mL of perchloric acid was added under the fuming cabinet and the samples were vortexed for 10 sec. Then, 0.3 mL of freshly prepared ascorbic acid was added and the samples were vortexed again for 10 sec. Finally, 0.4 mL of ammonium molybdate was added and the samples were vigorously shaken. The tubes were left for 10 min, allowing for the colour to develop and then absorbance was read at 730 nm using a spectrophotometer

(U-2000, Spectrophotometer, Hitachi) against water as a blank sample. All the samples were carried out in duplicates.

The standard solution was diluted in order to obtain different IP concentrations in the range of 2 - 10 mg/L for the preparation of the calibration curve: y = 12.156x +0.1666 (R^2 = 0.9989).

4.12.10 Monitoring of fungal fermentations: determination of metabolic products

Fumaric acid determination in culture broths was carried out via dilution with deionized water and 3 M H_2SO_4 to dissolve the residual $CaCO_3$ and reduce the pH to less than 1.0, followed by treatment at 80 °C until the broth became clear (Goldberg et al. 1983). Suspensions were filtered to separate the biomass and the solids due to $CaSO_4$ formation. Samples from the clear filtrate were obtained and sugar consumption, fumaric acid and other by-products were quantified using High Performance Liquid Chromatography (HPLC) analysis (Shimadzu) equipped with a Bio-rad Aminex HPX-87H column (300 mm length x 7.8 mm internal diameter) coupled to a differential refractometer. The mobile phase was a solution of 10 mM H_2SO_4 with 0.6 mL/min flow rate and 65 °C column temperature.

The calibration curves of the sugars were as follows aforementioned in the section 4.12.1, whereas the calibration curves of the other metabolic products were as follows:

Fumaric acid: y = 7.20454e-006, (R^2 = 0.9997)

Succinic acid: y = 9.30553e-006x, (R^2= 0.9999)

Ethanol: y = 1.68071e-005x, (R^2 = 0.9999)

The sugar consumption and fumaric acid production were expressedg per L of fermentation. The sugar to fumaric acid conversion yield was expressed as g of fumaric acid produced per g of consumed sugars.

4.12.11 Monitoring of yeast fermentations: determination of total dry weight and metabolic products

The fermentation of *R. toruloides* was monitored by taking samples at random intervals, following centrifugation (9000 x g, 4 ºC, 10 min). For the determination of total dry weight (TDW) the precipitated biomass was washed twice with deionized water and then was oven

dried at 70 °C until constant weight. The supernatant was used for the analysis of sugar consumption and other metabolic products using High Performance Liquid Chromatography (HPLC) analysis (Shimadzu) equipped with a Rezex ROA-organic acid H+ column (300 mm length x 7.8 mm internal diameter, Phenomenex) coupled to a differential refractometer. The mobile phase was a solution of 10 mM H_2SO_4 with 0.6 mL/min flow rate and 65 °C column temperature. The calibration curves of the sugars were as follows aforementioned in the section 4.12.1. Thesugar consumption and the TDW were expressed as g per L of fermentation.

For the carotenoids determination samples of 5 mL were collected, centrifuged (9000 x g, 4 ºC, 10 min), washed twice with deionized water and lyophilized. All the samples were manipulated under mild light conditions.

4.12.12 Extraction and determination of microbial oil and carotenoids

The disruption of the dried cells was performed as described by Tsakona et al. (2014). A chloroform:methanol solution at 2:1 (v/v) ratio was employed for the extraction of microbial oil. The solvent from the extracted microbial oil was removed by vacuum evaporation and the microbial oil was gravimetrically determined in a 4-digit analytical balance. The oil production was expressed as g per L of fermentation and the intracellular content as the percentage of g of lipid produced per 100 g of TDW.

Carotenoids extraction was employed by adding 20 mL chloroform:methanol solution containing 20 mg/L BHT (Butylated hydroxytoluene, Sigma-Aldrich), as antioxidant agent, to the lyophilised samples. The samples was left in dark and dry place for exactly 3 days. Then the solvent was removed by vacuum evaporation under mild light and temperature conditions. The carotenoids extraction was collected by adding specific quantity of light petroleum ether (boiling point 40-60 ºC). The total carotenoids extract was filtered using 0.22 μm membrane and the absorbance was read in spectrophotometer at 444 nm (U-2000, Spectrophotometer, Hitachi) against blank sample.The total carotenoids concentration (expressed as μg of β-carotene equivalents per g of TDW) was determined using the following equation (Lopes et al. 2017):

$$\text{Total carotenoids } (\mu g/g) = \frac{A \times V \times 10000}{E \times w}$$

where, A = *absorbance at 444 nm*

V = *volume of petroleum ether (ml)*

E = *1% extinction coefficient (2592 for petroleum ether)*

w = *TDW of samples (g)*

4.12.13 Fatty acid composition analysis of microbial oil

The determination of fatty acid composition of microbial oil was performed by GC-FID, as described previously in the section 4.12.6.

4.12.14 Production of free fatty acid fraction from microbial oil through enzymatic hydrolysis

In the experiments carried out for wax esters production, the hydrolysis of microbial oil was performed by mixing it with 0.1 M of acetate buffer pH 4.0 at a ratio of 1:1 (v/v) in a flat bottom flask of 250 mL. The mixture was equilibrated at 30 ºC using a temperature controlled water bath and agitated with a magnetic stirrer. Hydrolysis was carried out by adding 5% (w/v) of VEG enzyme at 30 ºC for 2 h.

In the case of biolubricants production, the hydrolysis of microbial oil was performed using the same protocol as mentioned above, but using 0.1 M of Tris-HCl buffer pH 8.0 and 2% (w/w, by oil) of the commercial lipase Lipomod 34MDP. The reaction was terminated after 24 h by adding hexane.

In both cases, the reaction mixture was transferred in a separating funnel in order to obtain the free fatty acid fraction. The lower layer that contained the buffer, glycerol and biocatalyst was discarded and the upper layer containing the solubilised free fatty acids in hexane was collected. The solvent was removed by vacuum evaporation. The acidity of the HMO was measured by titration against 0.04 M NaOH using an autotitrator.

4.12.15 Fractionation of microbial oil

Microbial oils were also fractionated into neutral lipids (NL), sphingolipids & glycolipids (S & G) and phospholipids (P), in order to determine the percentage (w/w) of each

fraction and their free fatty acid composition (Fakas et al. 2006). Around 100 mg of microbial oils were weighted and subsequently dissolved in chloroform following fractionation using a column (25 × 100 mm) of silicic acid, which was activated overnight at 110 °C. Successive applications of chloroform, acetone and methanol produced fractions containing NL, S & G and P lipid fractions, respectively. The weight of each fraction was determined after evaporation of the respective solvent. The fatty acid profile of the fractions were analysed by GC-FID, as described previously in the section 4.12.6.

4.12.16 Qualitative determination of wax esters by TLC

Thin Layer Chromatography (TLC) was employed for monitoring the components of reaction mixture during the wax ester synthesis. The chromatoplates (silica gel 60, Merck) were developed in a petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v) solvent system. Subsequently, the plates were dried at room temperature in a desiccator and the separated components were visualised as brown spots after staining in an iodine chamber.

4.12.17 Quantitative determination of wax esters by GC-FID

The determination of wax esters in the reaction mixture was carried out by GC using a Fisons GC-8130 unit equipped with FID and a cold on-column injection system. Separation of the compounds was performed on a ZB-5HT inferno capillary column (15 m x 0.25 mm i.d., 0.25 μm film thickness, Phenomenex). The oven program was started at 80 °C for 1 min, heated to 340 °C at a ratio of 20 °C/min, increased at a ratio of 5 °C/min up to 350 °C and then ramped to 400 °C at a ratio of 20 °C/min and maintained for 10 min. Detector temperature was set at 410 °C. Helium was used as carrier gas at a flow rate of 1 mL/min.

The quantification of wax esters was based on calibration curves prepared with reference standards of cetyl, oleyl and behenyl esters of palmitic, oleic, linoleic and stearic acids (purchased from Sigma-Aldrich and LGC standards), as these were the predominant fatty acids of the oils used in this study. Methyl palmitate was used as the internal standard. The conversion yield of esters was calculated by considering that 1 mol of palm or microbial oil produces 3 moles of ester according to the following equation (Keng et al. 2009):

$$\text{Conversion yield (\%)} = \frac{\text{mmol ester produced}}{3 \times \text{mmol oil used}} \times 100$$

4.12.18 Quantitative determination of biolubricants

The determination of the conversion yield (%) of fatty acids into polyol esters was based on the difference of acidity between the substrate and the product. Specifically, a precisely weighted sample was diluted in 40 mL of acetone:ethanol solution (1:1 v/v) followed by titration with 0.04 M NaOH using an autotitrator. The acidity was calculated using the equation:

$$\text{Acidity } (\%) = \frac{\text{V} \times \text{ M} \times \text{ FA}}{\text{w}}$$

where, *V = the volume of NaOH used for titration in ml,*

M = the NaOH molarity,

FA = the molecular weight of the fatty acidsand

w = the weight of the sample used for titration in g

The conversion of the free fatty acids into the non-acidic product was determined using the equation:

$$\text{Conversion yield } (\%) = \frac{(\text{Ai} - \text{Af})}{\text{Ai}} \times 100$$

where, *Ai = the initial acidity and*

Af = the final acidity

The calculation of conversion yields were given using the estimated modification of hydroxyl groups, calculated from the decrease in acidity.

4.12.19 Determination of physichochemical properties of wax esters

The melting points of the end products of transesterification reactions were determined with a differential scanning calorimeter Q100 (TA Instruments, DE, U.S.A.). About 2 - 3 mg

of each sample was weighed in a hermetically sealed aluminium pan. An empty pan was used as reference. After cooling with nitrogen, the sample was heated from 0 °C to 100 °C and then was cooled to 0 °C (Ruguo et al., 2011). A temperature ramp of 10 °C/min was used by flushing with nitrogen.

The rheological properties of the samples were performed using a Discovery HR 3 Hybrid Rheometer (TA Instruments, New Castle, DE, USA) equipped with a plate-plate geometry. Temperature was kept constant (52.0 ± 0.1 °C) by a constant temperature circulator. For the steady flow curves, the shear rate ranged from 0.01 to 100 sec-1 in 736 sec. For the temperature ramp, the shear rate was kept constant at 0.1 sec^{-1}, while the temperature varied from 40 °C to 65 °C with a rate of 2 °C/min. Viscosities were recorded after equilibrating at the desired temperature for 5 min.

Acid, iodine and saponification values were determined for the end products of transesterifications according to U.S. Pharmacopeia methods for carnauba wax. The acid value was expressed as mg of potassium hydroxide required to neutralise the free acids per g of sample. The saponification value was expressed as mg of potassium hydroxide required to neutralise the free acids and saponify the esters contained per g of sample. The iodine value was expressed as g of iodine absorbed per 100 g of sample.

4.12.20 Determination of physichochemical properties of biolubricants

The analysis of the structure and purity of the synthesized products was carried out by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) using an Agilent INOVA-300 (7.05T) spectrometer. Samples were prepared in CDCl3 at 25 °C, at 5% (1H) and 20% 13C concentration and compared with the spectra of the pure chemical compounds.

The viscosity was measured using a calibrated viscometer tube (Cannon-Fenske) under two different temperatures, 40 ºC, and 100 ºC. The viscosity index (Vi) was calculated according to the ASTM D 2270 international viscosity index table.

The pour point was determined by the following procedure: after heating the sample was cooled at a specific speed and the characteristics of the flow were observed every 3ºC. The lowest temperature in which movement was observed was determined as the pour point.

Differential scanning calorimetry (DSC) measurements were carried out in a DSC Q2000 (TA-Instruments) calorimeter calibrated for temperature and enthalpy by melting high-purity indium. The evaluated temperature range was -60 ºC to 110 ºC.

The oxidative stability of HMP-TMP esters and HMO-NPG esters was studied using a Metrohm 679 Rancimat apparatus (Herisau, Switzerland). The analysis was carried out using 3 g of each sample at 110 °C and an air flow rate of 10 L/h. Measurements were carried out in triplicate and the oxidative stability was expressed as induction time (h).

All analyses, except for oxidative stability, were performed by Petrobras in Brazil.

4.12.21 Determination of physical properties of oleogels

The crystal morphology of cetyl wax esters and oleogels was studied using a polarized light microscope (Axiolab, Zeiss) equipped with a digital camera (DSC-575 model, Sony, Japan) at the magnification of ×10. The microstructural analysis was employed using 24 h prepared oleogels stored at 4 °C.

The color determination of the oleogels was measured using a colorimeter (ChromaMeter CR-400/410, Konica Minolta, Japan)after standardization with a white and a black calibration plate. The samples were poured in cylindrical tubes with 2 cm diameter and 1 cmheight. The colorimeter standardization was performed using a white and black calibration plate. The color was recorded using CIE-$L^*a^*b^*$ uniformcolorspace (CIE-Lab), where L^* indicates lightness, a^* indicateshue on a green (−) to red (+) axis, and b^* indicates hueon a blue (−) to yellow (+) axis. The analysis was performed in duplicates.

The rheological properties of the oleogels were performed using a Discovery HR 3 Hybrid Rheometer (TA Instruments, New Castle, DE, U.S.A.) equipped with a plate-plate geometry system and a measuring gap of 700 µm. The viscositywas evaluated at a steady shear rate of 100/s in the temperature range from 25 °Cto 80 °Cwith a heating rate of 2 °C/min. The viscositywas recorded after equilibrating at the desired temperature for 5 min. The parameters storage modulus (G'), loss modulus (G'') and loss tangent ($\tan \delta = G''/G'$) versus temperature were also determined.

Textural properties were studied using a texture analyser (Instron 1011, Massachusetts, U.S.A.). The texture of the oleogels was correlated to the storage time by carrying out analysis at specific intervals (1, 10, 20 and 30 days). Around 15 ml of oleogels were poured intoglass tubes with internal diameter of 2 cm and maintained at 4 °C for 30 days. The penetration force was measured at 2 cm depth of samples after plunging a cylindrical probe into samples with a penetration speed of 100 mm/min. The maximum force was reported as a penetration force (N).

DSC analysis (Q100 model, TA Instruments, DE, U.S.A.) was also performed to evaluate the thermal properties of oleogels. Thesamples (approximately 5 - 10 mg) were accurately weighed into a hermetically sealed aluminum pan. After the samples were heated to 140 °C at a 10 °C/min heating rate, followed by cooling at -20 °C at a 10 °C/min heating rate and then reheated to 140 °C at a rate of 5 °C/min (Yilmaz and Öğütcü 2015). An empty pan was used as reference.

4.13 Statistical analysis

The statistical differences among treatments were estimated by analysis of variance (ANOVA). Whenever ANOVA indicated a significant difference between variables at a significance level of 5% ($P < 0.05$), the Tukey's HSD (honest significant difference) test was carried out using the Excel software.

Chapter 5

Development of enzymatic process for soybean cake hydrolysis

5 Development of enzymatic process for soybean cake hydrolysis

5.1 Introduction

The first aim of this study was the development of a two-stage bioprocess for soybean cake hydrolysis in order to obtain a nutrient-rich feedstock suitable for microbial fermentations. The soybean cake was initially utilised as a substrate for the production of crude enzyme consortia by *Aspergillus oryzae* and *Rhizopus arrhizus* via solid state fermentation (SSF). The crude enzymes were then used for the hydrolysis of untreated soybean cake. Soybean cake is a renewable resource rich in protein, and the enzymatic hydrolysis could provide an alternative exploitation of this cake as nitrogen-rich source suitable for microbial fermentations.

5.2 Composition of soybean cake

As depicted in Table 5.1, soybean cake contained high amounts of protein (47%), thus it could be utilised as an alternative raw material for producing a nutrient-rich feedstock for microbial fermentations. The most common use of soybean cake is as animal feed, while smaller amount is further processed into food ingredients. Soy protein isolate, which is the precipitation of the alkaline protein extract derived from soybean cake, is a popular soy ingredient with many functional properties and useful to the food production (Chen et al., 2013; Rickert et al., 2004). Soy protein is considered a good source of protein owing to the nutritional profile. Chen et al. (2013) have reported that employing bioprocessing methods to soybean cake could lead to the production of smaller peptidesand amino acids.

Table 5.1 Composition of soybean cake

Composition	% (dry basis)
Moisture (%, wet basis)	13.0 ± 0.4
Protein (TKN × 6.25)	47.0 ± 1.2
Ash	6.5 ± 0.09
Lipids	2.2 ± 0.2
Cellulose	24.1 ± 0.9
Hemicellulose	18.1 ± 1.6
Lignin	2.1 ± 0.08

5.3 Crude enzyme production via solid state fermentation

In this chapter, the protein fraction of soybean cake was hydrolyzed using crude enzymes produced via SSF of two fungal strains, namely *A. oryzae* and *R. arrhizus*. The fungal strain *A. oryzae* was selected due to the efficient production of proteolytic enzymes on various substrates (Dimou et al., 2015; Kachrimanidou et al., 2013; Tsouko et al., 2017). The initial moisture content and pH value have been optimised in previous studies (Dimou et al., 2015; Kachrimanidou et al., 2013), thus the SSF on soybean cake was employed at an initial moisture content of 65% and uncontrolled pH. The time course profile of proteolytic activity during the SSF on soybean cake has been evaluated by previous study (Papadaki et al., 2017), showing a highest proteolytic activity of 205 U/g at 70 h.

The time course profile of the proteolytic activity produced by *R. arrhizus* during SSF is presented in Figure 5.1. The highest proteolytic activity (155.4 U/g) was achieved at 73 h. The proteolytic activity produced by *R. arrhizus* was lower than the corresponding activity achieved by *A. oryzae* (205 U/g at 70 h) using soybean cake under the same SSF conditions. *A. oryzae* has been extensively studied for protease production (de Souza et al., 2015). Ito et al. (2017) showed that soybean meal led to the highest proteolytic activity of *R. microsporusvar.oligosporus* (50 U/g at 72 h). Furthermore, *R. oligosporus* presented proteolytic activity in the range of 162 - 195 PU/g during SSF in different amounts of sunflower meal (Rauf et al., 2010).The SSF of both fungi were utilised for the hydrolysis process of soybean cake, as described in the next section, when the proteolytic activity had reached the maximum value.

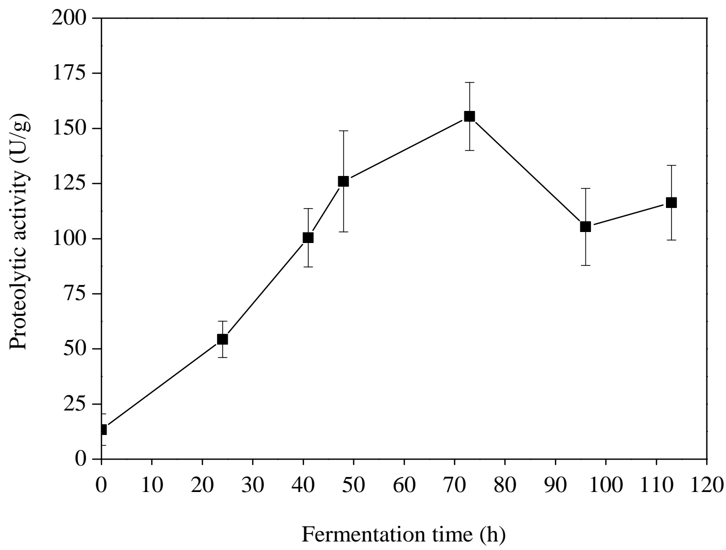

Figure 5.1 Proteolytic activity (U/g fermented solids) produced by *R. arrhizus* during solid state fermentation carried out on soybean cake

5.4 Soybean cake hydrolysis

An enzymatic hydrolysis process was employed to generate a nutrient-rich feedstock from soybean cake using the fermented solids of *R. arrhizus*. The temperature (40 °C, 45 °C and 50 °C) and the initial enzyme activity (2.7 U/mL, 5.4 U/mL and 6.7 U/mL) employed during hydrolysis were evaluated by comparing the production of FAN and IP.

The hydrolysis of soybean cake using the crude enzymes derived from the SSF of *A. oryzae* has been previously optimised (Papadaki et al., 2017). Specifically, the activity of proteases was enhanced at 45 °C, producing about 1.4 g/L FAN at 28 h of hydrolysis. The results showed that soybean cake was effectively hydrolysed by the proteases produced by *A. oryzae*. In particular, a TKN to FAN conversion yield of 43% was achieved.

Concerning the hydrolysis performed by the crude proteolytic enzymes of *R. arrhizus*, Figure 5.2a shows that the optimum temperature of protein hydrolysis was 45 °C with the highest FAN production (0.78 g/L) achieved at 46 h. The highest IP production (260 mg/L at 48 h) was observed at 50 °C (Figure 5.2b). Figure 5.3a shows that increasing initial proteolytic activity led to increasing FAN concentration. The highest FAN concentration (0.99 g/L) was achieved, when 6.7 U/mL of initial proteolytic activity was utilized. However, proteolytic activities of 5.4 U/mL and 2.7 U/mL led to similar FAN production. IP production was not improved with increasing initial crude enzyme activity. The highest IP concentration in the hydrolysate was around 200 mg/L (Figure 5.3b).

Previous studies have focused on the optimization of oilseed cake hydrolysis using the crude enzymes produced by *A. oryzae* showing that the optimum hydrolysis conditions were 40 °C - 50 °C and approximately 6 U/mL of initial proteolytic activity (Dimou et al., 2015; Kachrimanidou et al., 2013; Papadaki et al., 2017; Tsouko et al., 2017; Wang et al., 2010). Kachrimanidou et al. (2013) reported FAN production of around 0.6 g/L and 1.5 g/L at 45 °C when crude proteolytic enzymes of *A. oryzae* were employed for the hydrolysis of 45 g/L and 90 g/L of sunflower meal, respectively, using an initial proteolytic activity of 6.4 U/mL. Higher FAN production of 2.3 g/L was achieved when the initial proteolytic activity increased at 16 U/mL (Kachrimanidou et al., 2013). Tsouko et al. (2017) achieved around 0.45 g/L FAN production concentration and 160 mg/L IP production concentration during hydrolysis of 98.7 g/L of palm kernel cake at 50 °C, using 6 U/mL initial proteolytic activity derived from crude enzyme consortia produced by *A. oryzae*.

Although chemical hydrolysis of soybean cake has been studied for fumaric acid production (Zhang et al., 2015), the present study proposes the development of a soybean cake hydrolysis process utilizing crude enzymes produced by *R. arrhizus*, the same strain that is subsequently used for fumaric acid production.

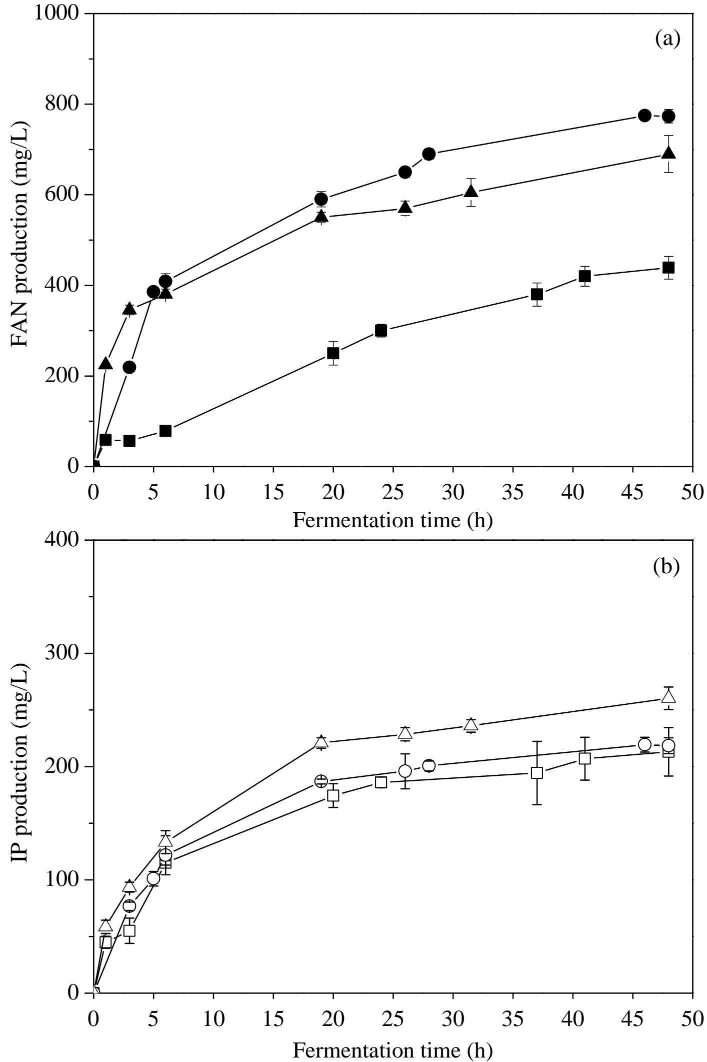

Figure 5.2 Effect of temperature, 40 °C (■,□), 45 °C (●,○) and 50 °C (▲,△), on (a) FAN and (b) IP production during hydrolysis of 50 g/L of soybean cake using proteases produced by *R. arrhizus* with initial proteolytic activity of 2.7 U/mL

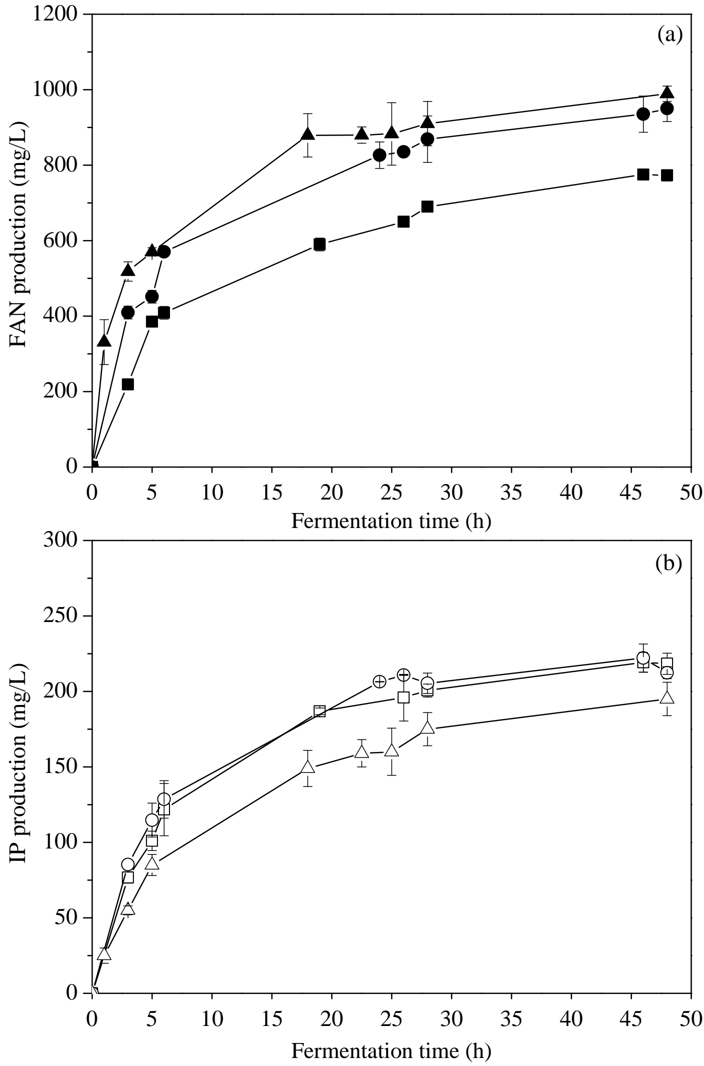

Figure 5.3 Effect of initial proteolytic activity, 2.7 U/mL (■,□), 5.4 U/mL (●,○) and 6.7 U/mL (▲,△), on (a) FAN and (b) IP production during hydrolysis of 50 g/L of soybean cake at 45 °C using proteases produced by *R. arrhizus*

Chapter 6

Fumaric acid production

6 Fumaric acid production

6.1 Introduction

The main objective of this chapter was the evaluation of VHP (Very High Polarity) sugar, molasses and soybean cake as fermentation feedstocks for fumaric acid production using the fungal strain *Rhizopus arrhizus* NRRL 2582. Submerged fermentations were carried out using a nutrient-rich feedstock derived from soybean cake in order to replace commercial nitrogen sources. The soybean cake hydrolysate was obtained via enzymatic hydrolysis as described in Chapter 5.

6.2 Composition of molasses

The composition of sugarcane molasses was determined and was found that the main sugar is sucrose (>70%), whereas glucose and fructose represented around 28% of the total sugar composition (Table 6.1). The total phenolic content (TPC) was determined as 0.065 g GAE per 100 g of molasses. The antioxidant activity index (AAI) of the phenolic extract was 0.1, which is characterized as poor antioxidant activity as indicated by the scale proposed by Scherer and Godoy (2009), where poor antioxidants are considered those with AAI lower than 0.5.

The phenolic compounds in the extract derived from molasses were determined by HPLC-DAD analysis. The main phenolic compounds identified were 3,4-dihydroxybenzoic acid (2.9 mg/100 g molasses), p-coumaric acid (1.0 mg/100 g molasses) and 4-hydroxybenzaldehyde (0.9 mg/100 g molasses). Lower concentrations of vanillin, 2-hydroxycinnamic acid, syringic acid, 4-hydroxybenzoic acid, vanillic acid and ferulic acid were also detected. The phenolic compounds of syringic acid, vanillic acid, vanillin, p-coumaric acid have been determined in sugarcane molasses (Hashizume et al., 1967). Obata et al. (1963) identified 7 phenolic compounds in beet molasses, including catechol, 2-hydroxydihydrocinnamic acid, 4-hydroxybenzoic acid, syringic acid, vanillin and vanillic acid. Chen et al. (2015) reported that gallic acid, vanillin, hydroxybenzoic acid, syringic acid and ferulic acid were identified in sugar beet molasses.

ICP-IOS analysis (Table 6.2) showed that molasses contained mainly 6.67 mg/g Ca, 5.23 mg/g S, 4.7 mg/g Mg and 1.75 mg/g K. Lower quantities of P, Fe Mn, Zn and Co were also detected. Teclu et al. (2009) reported that molasses contained Al, As, Cu, Fe, Mn and Zn in concentrations of 0.54, 0.24, 8.7, 0.35, 11.1 and 19.7 μg/g, respectively. The elemental

analysis carried out in the case of VHP cane sugar showed that the major elements were K, Ca, S and Mg, but in significant lower concentrations than in molasses.

Table 6.1 Composition of sugarcanemolasses

Component	Quantity (w/w, %)
Moisture	29.7
Solids	70.4
Sugars (of which):	46.9
Sucrose	33.6
Glucose	6.1
Fructose	7.2
TKN	0.5
Protein (TKN ×6.25)	3.2
Free Amino Nitrogen	1.1
TPC [a]	0.065

[a] Total Phenolic Content is expressed as g gallic acid equivalents per 100 g molasses. The determination of TPC was carried out using the method described by Faustino et al. (2010).

Table 6.2 Elemental analysis of VHP sugar and sugarcane molasses

Elements	VHP sugar [a] (mg/g)	Molasses [a] (mg/g)
Ca	0.034	6.67
S	0.027	5.23
Mg	0.014	4.70
K	0.052	1.75
P	0.002	0.21
Fe	0.001	0.12
Mn	<0.0005	0.03
Zn	0.0002	0.003
Co	<0.0005	<0.0005

[a] Expressed as mg per g of VHP sugar or sugarcane molasses

6.3 Fermentations carried out with VHP cane sugar

High concentrations of fumaric acid have been achieved using various *Rhizopus* species. The fungal strain *R. arrhizus* NRRL 2582 was selected in this study, since it can consume sucrose for fumaric acid production (Rhodes et al., 1959). The fungal morphology has a significant effect on fumaric acid production (Liao et al., 2007; Roa Engel et al., 2011; Zhang et al., 2015; Zhou et al., 2011). Therefore, regular monitoring of the fungal morphology was conducted throughout fermentation (Papadaki et al., 2017).

Since molasses contain sucrose, glucose and fructose, the ability of *R. arrhizus* to metabolise a co-substrate containing pure glucose and fructose was studied in batch fermentation (Figure 6.1). Based on the results presented by Papadaki et al. (2017), the highest fumaric acid production was achieved with a soybean cake hydrolysate of 200 mg/L initial FAN concentration. Thus, the pre-culture medium used in the fermentation, presented in Figure 6.1, was carried out with a FAN concentration of 200 mg/L. Glucose was depleted at around 180 h, whereas the consumption rate of fructose was slower (Figure 6.1). The highest fumaric acid concentration (30.8 g/L) was achieved at 140 h and the yield was 0.98 g/g. In the case of *Rhizopus* sp., a conversion yield of more than 1.0 g/g could be achieved, since the theoretical stoichiometry shows that 1 mole of glucose requires 2 moles of CO_2 to produce 2 moles of fumaric acid (Roa Engel et al., 2008). Under nitrogen-limited conditions, *Rhizopus* sp. catabolise glucose via the reductive TCA cycle. The CO_2 required in the reductive TCA cycle is supplied through the reaction of $CaCO_3$ with fumaric acid leading to the production of calcium fumarate and CO_2 (Roa Engel et al., 2008).

Subsequently, VHP cane sugar was used in fed-batch fermentation (Figure 6.2). The pre-culture medium used in this fermentation contained 200 mg/L of FAN concentration provided by a soybean cake hydrolysate produced with crude enzymes derived from *A. oryzae* SSF cultures. The highest fumaric acid concentration (40 g/L) with a yield of 0.86 g fumaric acid per g consumed sugars was achieved at 219 h. The main by-product at 219 h was succinic acid (2.8 g/L), whereas negligible ethanol was determined. Zhang et al. (2015) reported the production of 50 g/L fumaric acid with a yield of 0.72 g/g by *R. oryzae* ATCC 20344 using a chemically-derived soybean meal hydrolysate in the pre-culture medium and commercial glucose as carbon source.

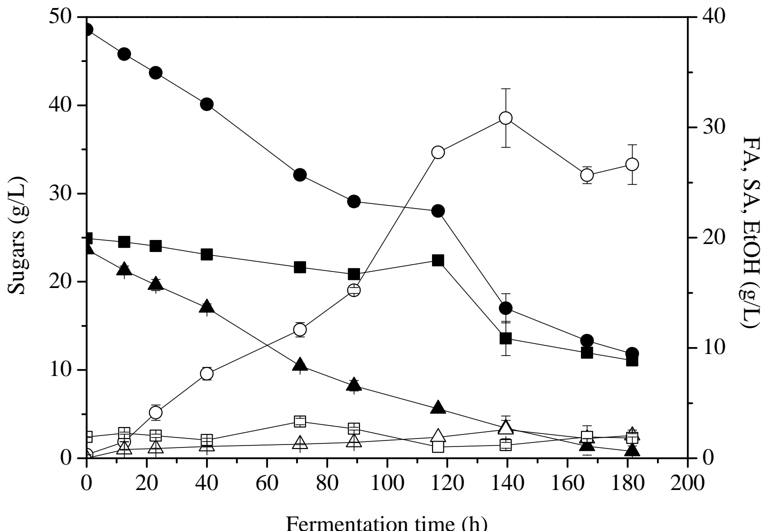

Figure 6.1 Time change of glucose (▲), fructose (■), total sugars (●), fumaric acid (FA) (○), succinic acid (SA) (△) and ethanol (EtOH) (□) during batch fermentation of *R. arrhizus* in synthetic medium containing 50 g/L of glucose-fructose (1:1). The pre-culture medium was produced with soybean cake hydrolysate (200 mg/L FAN concentration) that was produced using proteases from *A. oryzae* SSF cultures

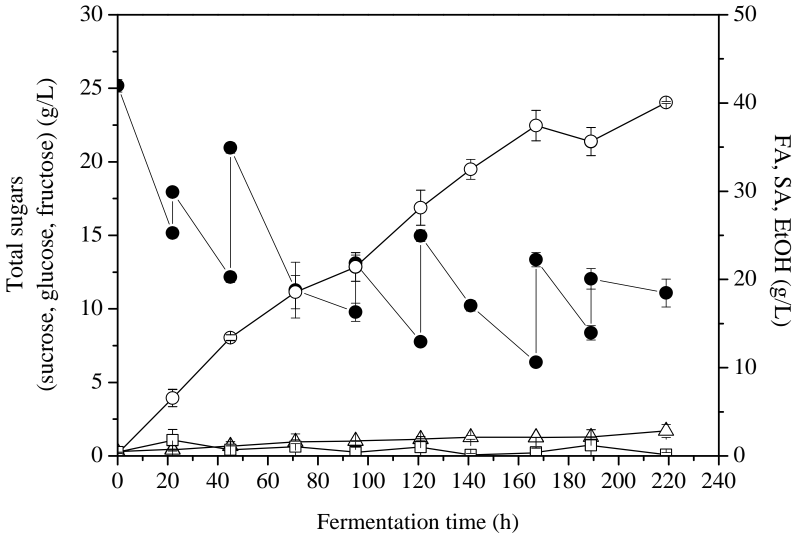

Figure 6.2 Time change of total sugars (●), fumaric acid (FA) (○), succinic acid (SA) (△) and ethanol (EtOH) (□) during fed-batch fermentation of *R. arrhizus* in VHP sugar. The pre-culture medium was produced with soybean cake hydrolysate (200 mg/L FAN concentration) that was produced using proteases from *A. oryzae* SSF cultures

Figure 6.3 present three fed-batch fermentations carried out with *R. arrhizus* cultivated on VHP cane sugar using three pre-culture media that were produced with three different initial FAN concentrations (100 mg/L, 200 mg/L and 400 mg/L) provided by soybean cake hydrolysate produced with crude enzymes derived from *R. arrhizus* SSF cultures. Figure 6.3 shows that the highest initial FAN concentration used (400 mg/L) led to the highest fumaric acid concentration (30.8 g/L). At 100 mg/L and 200 mg/L initial FAN concentration in the pre-culture media used, the fumaric acid production reached 19.3 g/L and 24.5 g/L, respectively. The total consumed sugar to fumaric acid conversion yield (ca. 0.7 g/g) observed in the fermentations carried out with 100 mg/L and 200 mg/L initial FAN concentration in the pre-culture medium was higher than the respective yield (0.50 g/g) achieved at 400 mg/L FAN.

The results presented in Figures 6.2 and 6.3b show that higher fumaric acid concentration and yield are achieved when the pre-culture medium is prepared with soybean cake hydrolysate produced using crude enzymes derived from *A. oryzae* SSF cultures. It should be stressed that the same initial FAN concentration (200 mg/L) was used in both cases for the production of pre-culture media. The pre-culture medium produced using soybean cake hydrolysate derived from *R. arrhizus* SSF cultures is apparently not as efficient to stimulate fumaric acid production by *R. arrhizus*. These results indicate that the enzymes produced by the two fungal strains are different leading to the production of soybean cake hydrolysates with different nutrient composition. Although the same FAN concentration was initially used for the production of the two pre-culture media, the presence of specific peptides or other nutrients were not quantified in the two soybean cake hydrolysates. However, using *R. arrhizus* for soybean cake hydrolysis has the processing advantage that the same fungal strain is used for both soybean cake hydrolysate and fumaric acid production.

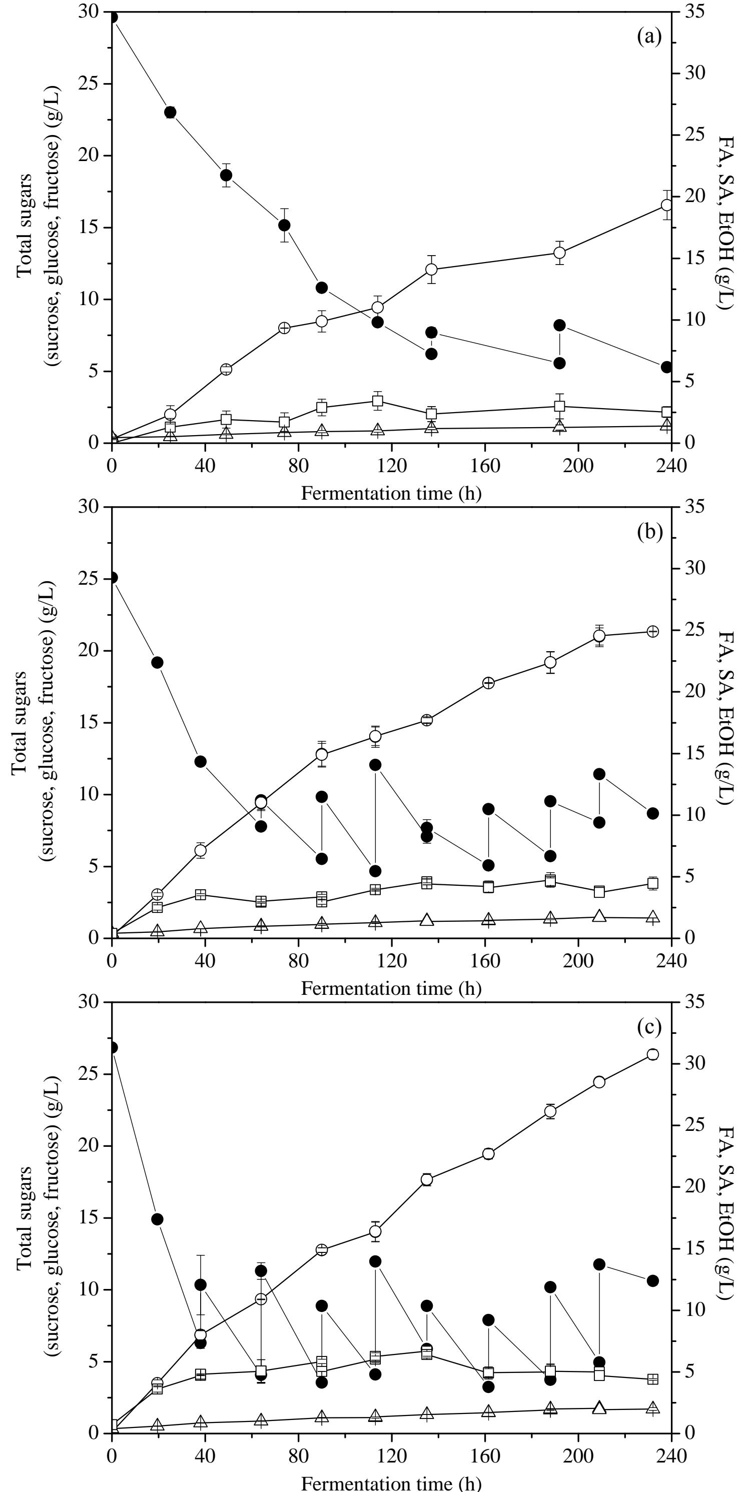

Figure 6.3 Time change of total sugar (●), fumaric acid (FA) (○), succinic acid (SA) (△) andethanol (EtOH) (□) during fed batch fermentations of *R. arrhizus* in VHP sugar. The pre-culture medium was produced with soybean cake hydrolysate containing FAN concentrations of (a) 100 mg/L, (b) 200 mg/L and (c) 400 mg/L FAN. The soybean cake hydrolysate was produced using crude enzymes produced by *R. arrhizus* SSF cultures

6.4 Fermentations carried out with sugarcane molasses

Table 6.3 presents the final fumaric acid and ethanol concentrations and the consumed sugar to fumaric acid conversion yield in batch fermentations conducted using molasses or pretreated molasses supplemented with two different soybean cake hydrolysates produced by crude enzymes derived from either *A. oryzae* or *R. arrhizus*. A significant reduction in fumaric acid concentration and yield was observed when either untreated or pretreated molasses was used as fermentation feedstock compared to the respective fumaric acid concentration and yield achieved when VHP sugar was used as carbon source. In particular, when untreated molasses was used the final fumaric acid concentration was 4.04 g/L, whereas the ethanol concentration was 6.2 g/L, when *A. oryzae* derived enzymes were used for the production of soybean cake hydrolysates. The fumaric acid production was slightly enhanced when molasses was pretreated by acid or combined acid and resin treatment and the fermentation medium was not supplemented with any minerals. Acid treatment of molasses that was combined with mineral supplementation led to the lowest fumaric acid production (2.35 g/L). The highest fumaric acid concentration (7.9 g/L) and yield (0.32 g/g) were achieved when the acid and resin pretreated molasses was used and the fermentation medium was not supplemented with minerals. The acid and resin treatment of molasses have been successfully studied in other microbial fermentations. The acid treatment of sugarcane molasses increased the citric acid production by *A. niger* (Asraf et al., 2015; Kundu et al., 1984), mainly because of the reduction of mineral concentration (e.g. Cu, Fe, Mn, Zn) as these are involved in the activation of enzymes associated with the TCA cycle (Kundu et al., 1984). Acid and cation exchange resins treatment of cane molasses led to higher succinic acid production by *Actinobacillus succinogenes* (Liu et al., 2008).

Table 6.3 shows also that higher ethanol production was observed when molasses was used than in the case that VHP sugar was used as carbon source. This could be attributed to the formation of fungal biomass agglomerates during fermentation that led to the conversion of carbon sources to ethanol that is observed under micro-aerobic or anaerobic conditions (Meussen et al., 2012). It has been reported that the concentrations of metal ions have a key role in the metabolism of *Rhizopus* species and affect fungal morphology. Zhou et al. (2000) underlined the significant effect of four metal ions on the morphology of *R. oryzae*. The optimum metal ion concentration in the medium was 50 ppm of Mg, 4 ppm of Zn, and 100 ppb of Fe and no addition of Mn, while other concentrations led to different cell morphologies and lower fumaric acid concentrations. Das et al. (2015) stated that these metal ions act as

cofactors or activators for many cellular enzymes involved in catabolism and biosynthesis of macromolecules.

Table 6.3 Fumaric acid and ethanol production in batch fermentations carried out with molasses or pretreated molasses with an initial total sugar concentration of around 25 g/L and two different soybean cake hydrolysates (SBC) using 200 mg/L FAN concentration for the production of pre-culture medium

Carbon source	Nitrogen source	Time (h)	FA (g/L)	$Y_{FA/S}$ [a] (g/g)	EtOH (g/L)
Molasses	SBC hydrolysate produced by *A. oryzae* enzymes	48	4.04	0.19	6.2
Acid treated molasses - NM [b,c]		38	6.37	0.29	4.9
Acid & resins treated molasses - NM [b,c,d]		40	7.60	0.31	5.8
Molasses	SBC hydrolysate produced by *R. arrhizus* enzymes	44	3.60	0.18	5.9
Acid treated molasses [c]		48	2.35	0.18	4.9
Acid treated molasses - NM [b,c]		52	7.06	0.32	3.7
Acid & resins treated molasses - NM [b,c,d]		44	7.90	0.32	6.0

[a] The yield was expressed as g of fumaric acid (FA) produced per g of consumed sugars
[b] NM: no mineral supplements were added in the fermentation medium
[c] Molasses were treated with H_2SO_4
[d] The H_2SO_4 treated molasses was subsequently treated with resins

The phenolic compounds present in molasses may have also caused the reduction in fumaric acid production by *R. arrhizus*. Zhang et al. (2016) studied the individual effect of many phenolic compounds on the activity of the enzymes of *R. oryzae*, which are involved in lactic acid production, using corn cob and corn stover hydrolysates. It was observed that the activity of the lactate dehydrogenase, which is responsible for lactic acid production, was strongly inhibited by phenolic compounds whereas the activity of alcohol dehydrogenase, involved in ethanol production, was enhanced. The lactic acid production was significantly inhibited (over 90%) in the presence of trans-cinnamic acid or syringaldehyde in the medium.

Moreover, lactic acid production was reduced up to 71% when 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, 4-hydroxybenzaldehyde and vanillin were used. Zhang et al. (2016) also stated that syringic, ferulic and p-coumaric acids presented the lowest toxicity, reducing lactic acid production by less than 10%. The effect of phenolic compounds varies with the type of compound, their combination and concentration (Zhang et al., 2016; Hu et al., 2009; Kumar et al., 2015) and it is dependent on the microbial strain used (Delgenes et al., 1996; Martín and Jönsson, 2003). Other studies have highlighted that the combination of different phenolic compounds in the medium influenced negatively the performance of microorganisms during fermentation (Alexandri et al., 2016; Hu et al., 2009; Zhang et al., 2012).

This chapter shows that VHP sugar and an enzymatic soybean cake hydrolysate using the crude enzymes consortia of *A. oryzae* could be efficiently employed for the fumaric acid production. Figure 6.4 presents a mass balance of fumaric acid production (0.86 kg) from 1 kg VHP sugars and 6.25 g soybean cake when *A. oryzae* crude enzymes were used for the production of soybean cake hydrolysates. The use of VHP sugar as carbon source led to low succinic acid and ethanol production as by-products, which is essential in order to enhance sucrose to fumaric acid conversion yield.

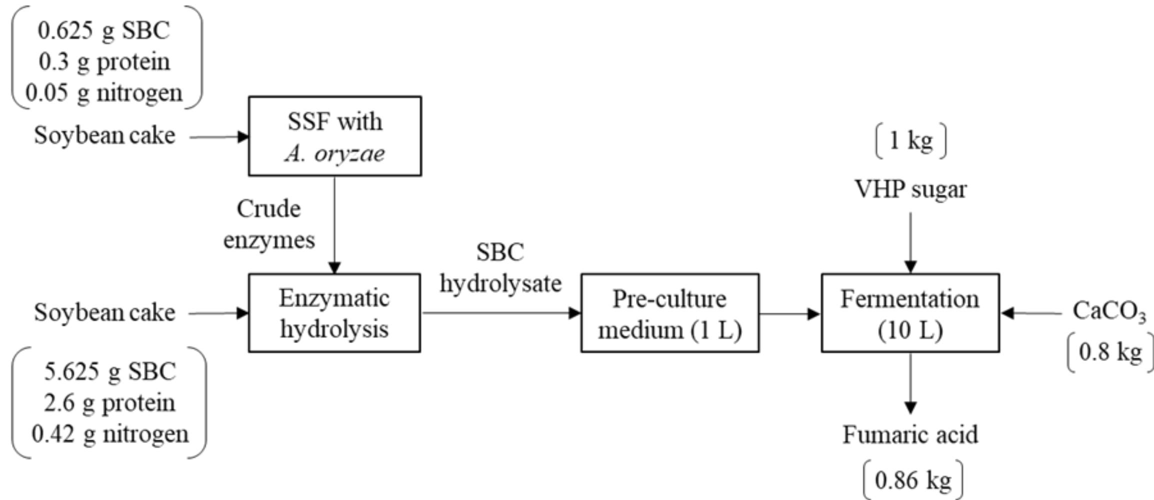

Figure 6.4 Mass balance of the proposed bioprocess using VHP cane sugar and soybean cake hydrolysate produced with crude enzymes derived by *A. oryzae* SSF cultures

Chapter 7

Microbial oil and carotenoids production

7 Microbial oil and carotenoids production

7.1 Introduction

The objective of this chapter is the development of a biorefinery concept based on the valorisation of side-streams derived from the processing of sugarcane and soybeans. Molasses, the main by-product of the sugarcane industry, and soybean cake, which is generated after oil extraction from soybeans, were utilised as carbon and nitrogen sources for the production of microbial oil rich in carotenoids by the oleaginous yeast strain*Rhodosporidium toruloides*. The efficiency of microbial lipid accumulation was initially assessed using very high polarity cane sugar as the sole carbon source. The effect of medium composition, concerning the mineral and phosphate supplements, on lipid production and fatty acid composition was evaluated in batch fermentations using a molasses-based medium. Microbial oil and carotenoid production were subsequently evaluated in bioreactor fed-batch fermentations.

7.2 Shake flask batch fermentations using cane sugar

The results presented in Figure 7.1, concerning the fermentation by *R. toruloides* in shake flask cultures, showed that the initial sucrose concentration of around 75 g/L was totally consumed at 110 h. The initial FAN (92 mg/L) and IP (4 g/L) concentrations were consumed until 24 h. Although IP was still present in the fermentation broth, the concentration of FAN was very low and the remaining peptides could not be further assimilated by the oleaginous yeast. Thus, after the depletion of assimilable nitrogen source, lipid synthesis was initiated leading to the highest lipid concentration of 8.1 g/L at 89 h with a TDW of 23.8 g/L and anintracellular lipid content of 34%(w/w). The microbial oil contained mainly oleic acid (51%), palmitic acid (29%) and stearic acid (13%) (Table 7.1). The results showed that *R. toruloides* is a promising oleaginous yeast able to metabolise sucrose, which suggests that sugarcane molasses could be a potential fermentation substrate for efficient lipid production.

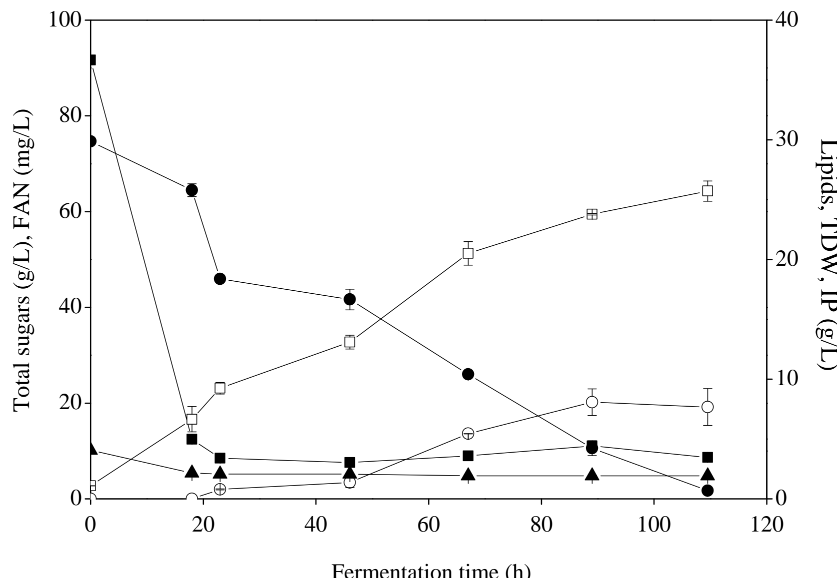

Figure 7.1. Time course of total sugar (●), free amino nitrogen (FAN) (■) and inorganic phosphorus (IP) (▲) consumption and production of total dry weight (TDW) (○) and lipids (□) in batch shake flask fermentation of *R. toruloides* cultivated on VHP cane sugar-based medium

Table 7.1 Profile change of fatty acid composition of microbial oil produced by *R. toruloides* cultivated on VHP cane sugar medium

Time (h)	Fatty acids (%)					
	C 14:0	C 16:0	C 18:0	C 18:1	C 18:2	Others
46	1.9	31.2	6.5	49.8	7.6	3.1
89	1.7	29.9	8.2	51.0	7.5	1.8

7.3 Shake flask batch fermentations using molasses

The oil accumulation of the oleaginous yeast *R. toruloides* was initially evaluated in shake flask batch fermentations using molasses as the sole carbon source at an initial total sugar concentration of 60 – 70 g/L. The sugars contained in molasses were predominantly sucrose with lower amounts of glucose and fructose. Microbial oil production and fatty acid composition was evaluated via different molasses treatments targeting the reduction of the content of phosphates and trace elements. The initial carbon to FAN ratio (C/FAN) was around 230 g/g in all cases, except for the fermentation conducted without any additional

nitrogen source, neither yeast extract nor $(NH_4)_2SO_4$, in which the C/FAN ratio was around 400 g/g.

The results presented in Table 7.2 show that the individual addition of trace elements or phosphate salts did not significantly affect lipid accumulation. The fermentation carried out without any addition of trace elements or phosphate salts led to a final lipid concentration and TDW of 4 g/L and 18.3 g/L, respectively, with an intracellular lipid content of 22.1%. Similar results were obtained in the fermentation carried out withsupplementation of either trace elements or phosphate salts achieving lipid production of up to 4.7 g/L, with an intracellular lipid yield of up to 26.4%. When only trace elements were added in the fermentation medium, the yeast cells were able to consume around 68% of the initial sugar concentration, whereas in all fermentations where phosphate salt supplementation was carried out the sugar consumption ranged from 80 to 90%. This suggests that phosphorus is an essential compound for the growth and metabolism of *R. toruloides* (Gientka et al., 2017; Wu et al., 2010). Cortez and Roberto (2006) reported that different concentrations of phosphate buffers in the medium affected cell growth and xylose assimilation in fermentations conducted by *Candida guilliermondii* for xylitol production. However, it has been reported that phosphorus did not affect lipid production, but the fatty acid profile of the lipids (Gientka et al., 2017). Interestingly, the fermentations carried out with simultaneous addition of trace elements and phosphate salts showed an increased lipid concentration (9.8 g/L) and lipid content (38.9%). The trace element mixture contained $MgSO_4$, which may induce key enzymes for lipid synthesis, since ATP-citrate lyase is strongly dependent on the presence of cations such as Mg^{2+} (Gientka et al., 2017). Subsequently, shake flasks fermentation were performed without any additional nitrogen source, since molasses contains around 0.5% (w/w) total Kjeldahl nitrogen. In this case, lipid concentration increased to 12.7 g/L with a lipid accumulation of 59% (w/w) and lipid yield of 0.21 g/g based on the consumed sugars.

The fatty acid composition in the fermentations presented in Table 7.2 was evaluated and the results are presented in Table 7.3. The main fatty acids in the microbial oil were oleic acid, palmitic acid, stearic acid and linoleic acid. The content of oleic acid ranged from 51 to 57% with the highest content observed in the fermentation media that contained trace elements and phosphate supplements. High oleic acid oils are utilised in the food industry to replace *trans*-containing frying fats in foods as they present significant health benefits in reducing cardiovascular risk (DeBonte et al., 2012; Lopez-Huertas, 2010).

Table 7.2 Effect of trace elements (TE) and phosphate salts (P) supplements on lipid production by*R. toruloides*in shake flask fermentations using sugarcane molasses

Supplement addition	Time (h)	S_o [a] (g/L)	S_r [b] (g/L)	TDW [c] (g/L)	L [d] (g/L)	$Y_{L/TDW}$[e] (%)
No supplements [f]	90	60.0±0.0	14.0±1.1	18.3±0.3	4.0±0.5	22.1±3.1
TE [f]	73	69.4±0.2	22.1±4.7	14.0±1.1	3.7±0.4	26.4±0.5
P supplements [f]	97	64.0±0.3	12.2±1.4	19.9±0.1	4.7±0.3	23.4±1.6
TE& P supplements [f]	100	69.0±1.8	6.0±0.2	25.3±0.3	9.8±0.1	38.9±1.0
TE& P supplements, without nitrogen sources	90	69.4±3.0	13.7±0.3	21.5±1.0	12.7±1.8	59.0±5.7

[a] S_o: initial total sugar concentration, [b] S_r: residual total sugar concentration, [c] TDW: total dry weight, [d] L: lipids, [e] $Y_{L/TDW}$: intracellular lipid content, [f]thefermentation medium contained 0.5 g/L yeast extract and 0.5 g/L$(NH_4)_2SO_4$

Table 7.3 Effect of trace elements (TE) and phosphate salts (P) supplements on fatty acid composition of *R. toruloides* microbial oil in shake flask fermentations using sugarcane molasses

Supplement addition	Time (h)	Fatty acids (%)				
		C 16:0	C 18:0	C 18:1	C 18:2	Others
No supplements [a]	90	32.2	11.5	51.2	2.8	2.4
TE [a]	73	35.0	7.2	52.6	2.3	2.9
P [a]	97	29.2	13.2	50.6	4.5	2.2
TE& P [a]	100	28.2	9.8	56.6	3.8	1.5
TE& P, without nitrogen sources	90	29.8	6.6	54.8	6.7	2.1

[a]the fermentation medium contained 0.5 g/L yeast extract and 0.5 g/L $(NH_4)_2SO_4$

7.4 Bioreactor fed-batch fermentations using sugarcane molasses

7.4.1 Microbial oil production

Fed-batch fermentations were implemented using a molasses-based medium, which was supplemented with trace elements, phosphate salts and commercial nitrogen sources with an initial C/FAN ratio of 59 g/g. The results presented in Figure 7.2, show that FAN concentration was sharply decreased until 15 h. The initial total sugar concentration of 70 g/L was reduced to 27 g/L at 47 h and continuous feeding of a concentrate very high polarity cane sugar solution was initiated at this point. The concentration of microbial lipids reached 13.5 g/L at 121 h with an intracellular content of 54.6% (w/w). It should be stressed that the yeast hydrolysed the sucrose into glucose and fructose during fermentation, but the consumption rate of glucose was much higher than fructose. Thus, fructose was the main remaining sugar towards the end of the fermentation (data not shown). The lipid yield based on the consumed sugars was 0.13 g/g.

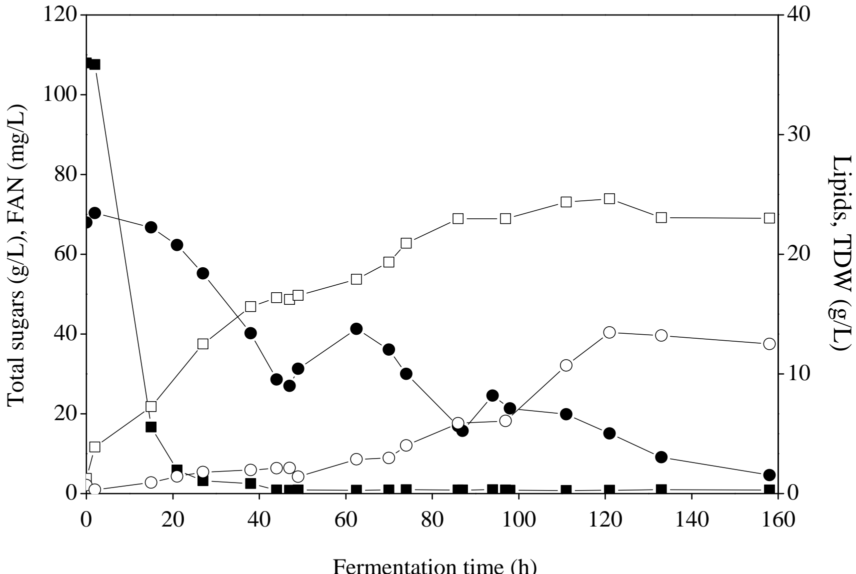

Figure 7.2 Time course of total sugars (●) and free amino nitrogen (FAN) (■) consumption and production of total dry weight (TDW) (□) and lipids (○) in fed-batch bioreactor fermentation of *R. toruloides* cultivated on molasses-based medium supplemented with commercial nitrogen sources, trace elements and phosphate salts

The following fed-batch fermentation was aimed to the valorization of soybean cake by utilizing its hydrolysate as nutrient-rich fermentation supplement. In this case, the initial C/FAN ratio was 40 g/g. The initial FAN concentration was 300 mg/L. As shown in Figure 7.3, the FAN and sugar concentrations were consumed at higher rate than in the fermentation with the commercial nitrogen source. Continuous feeding was initiated at 39.5 h when the total sugar concentration was reduced to 17 g/L. The highest oil production (18.4 g/L) was observed at 91.5 h showing an intracellular oil accumulation of 49.8 % (w/w). It is evident that during fermentation, sucrose was rapidly hydrolysed by the yeast cells into glucose and fructose. Glucose was completed consumed, while fructose was less preferred by the yeast cells (data not shown). The lipid yield based on the consumed sugars was 0.15 g/g.

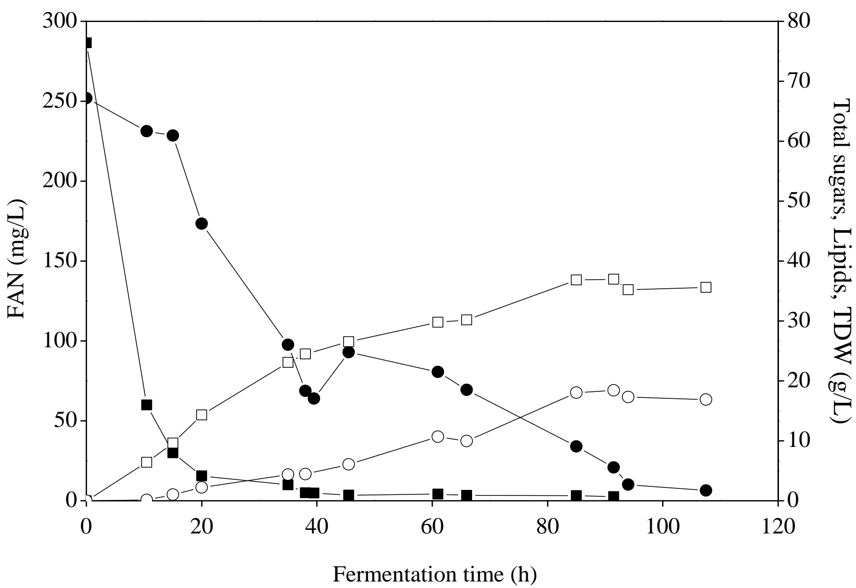

Figure 7.3 Time course of total sugars (●) and free amino nitrogen (FAN) (■) consumption and the production of total dry weight (TDW) (□) and lipids (○) in fed-batch bioreactor fermentation of *R. toruloides* cultivated on molasses-based medium with soybean cake hydrolysate at around 300 mg/L initial FAN concentration

Microbial lipids production by *Rhodosporidium* sp. have been studied using various carbon sources such as glucose, glycerol, sucrose, fructose, inulin and xylose among others with lipid yields up to 70% (w/w) (Xu and Liu, 2017). Tchakouteu et al. (2017) reported the production of 24 g/L lipids by the same yeast strain used in this study, when cultivated on a glucose-based medium. Furthermore, hydrolysates from corn stover, sorghum stalks and

bagasse, crude glycerol, pinewood pyrolysate, aqueous extracts from fruit pulps and confectionery food by-products have been utilised as renewable carbon sources, achieving up to 37.8 g/L lipids and 61.8% (w/w) lipid content (Tsakona et al., 2016; Xu and Liu, 2017). Papanikolaou et al. (2017) reported a lipid concentration of 13.7 g/L using the same strain of *R. toruloides* with an intracellular lipid yield of 37% when it was cultivated in crude glycerol. Although molasses is considered a valuable feedstock for microbial lipid production and in many cases could enhance the microbial lipid yields (Cho and Park, 2018), literature-cited results concerning its utilisation by the promising oleaginous yeasts *Rhodosporidium* sp. are limited. Fed-batch fermentation on molasses using *R. toruloides* CCT 0783 resulted in the production of a microbial lipid concentration of 16 g/L with a lipid content of 44% (w/w) (Vieira et al., 2014).

The results presented in Figure 7.3 also showed that soybean cake hydrolysate was efficiently utilized as nitrogen source for the production of microbial lipids. The highest microbial lipid production achieved in the fermentation enriched with the hydrolysate could be attributed to the presence of various minerals, such as Mg, Ca, Fe and Zn (Papadaki et al., 2017). It has been also reported that the substitution of commercial nitrogen sources by soybean cake hydrolysate enhanced fumaric acid production by *Rhizopus arrhizus* cultivated on a glucose-based medium (Papadaki et al., 2017). Tsouko et al. (2017) reported that palm kernel cake hydrolysate was successfully applied for microbial lipids production by *Lipomyces starkeyi* resulting in lipid concentration of 8.2 g/L and lipid content of 36.8% (w/w) in shake flask fermentations. Oilseed cake hydrolysates could be used for the production of various metabolic products, including microbial lipids from rapeseed meal and sunflower meal (Xu and Liu, 2017), fumaric acid from soybean cake (Zhang et al., 2015), and poly(3-hydroxybutyrate) from sunflower meal (Kachrimanidou et al., 2014).

The fatty acid profiles of the microbial lipids produced in the two bioreactor fed-batch fermentationsare presented in Table 7.4. The main fatty acids present in both microbial lipids were palmitic acid and oleic acid. The palmitic acid content was 35.2% and 32.5% at the end of fermentations carried out with commercial nitrogen sources and soybean cake hydrolysate, respectively. The oleic acid content was significantly higher than palmitic acid reaching 51.4% and 54.8% at the end of fermentationscarried out with commercial nitrogen sources and soybean cake hydrolysate, respectively. The fatty acid composition was similar in both cases.

Table 7.4 Fatty acid composition of *R. toruloides* microbial oils produced in bioreactor fed-batch fermentations using sugarcane molasses as carbon source and different nitrogen sources

Nitrogen source	Fatty acids (%)					
	Time (h)	**C 16:0**	**C 18:0**	**C 18:1**	**C 18:2**	**Others**
Commercial nitrogen sources [a]	86	28.6	8.0	54.6	6.2	2.5
	111	28.2	5.6	58.7	5.4	2.1
	133	35.2	5.1	51.4	5.2	3.1
Soybean cake hydrolysate [b]	20	30.6	4.9	56.0	5.8	2.6
	61	33.9	4.6	55.8	5.3	1.4
	91.5	32.5	4.3	54.8	5.6	2.8

[a] Yeast extract (0.5 g/L) and $(NH_4)_2SO_4$ (0.5 g/L); [b] Initial FAN concentration of 300 mg/L

7.4.2 Carotenoids production

Total carotenoids production was also determined during fed-batch fermentations. The results presented in Figure 7.4 demonstrate that the highest concentration was 83.1 μg/g and 89.4 μg/g in the fermentation carried out with commercial nitrogen sources and soybean cake hydrolysate, respectively. It is evident that the highest total carotenoids production coincided with the highest lipid concentration. This allowed to recover microbial lipids rich in carotenoids making them more valuable for food applications.

The most widely studied yeast strains for carotenoids production is *Rhodotorula* sp., while few studies have been published for *Rhodosporidium* sp. The yeasts *Rhodosporidium diobovatum* and *Rhodosporidium toruloides* have been reported to produce carotenoids up to 69.0 and 122.6 μg/g of dried biomass using glucose as carbon source. The produced carotenoids was consisted at more than 60% of torularhodin and torulene (Buzzini et al., 2007). Dias et al. (2015) investigated the effect of different pH strategies during fermentation of *Rhodosporidium toruloides* on glucose aiming at enhancing carotenoid production. Moreover, carob pulp syrup and sugarcane molasses have been utilised as low-cost carbon sources for carotenoids production by *Rhodosporidium toruloides*. The highest carotenoid content of around 90 μg/g and 35 μg/g were achieved using 100 g/L initial sugar concentration of carob pulp syrup and 75 g/L of sugarcane molasses, respectively (Freitas et

al., 2014). Moreover, Bonturi et al. (2017) reported the production of 1.2 mg/L carotenoids of *Rhodosporidium toruloides* cultivated on sugarcane bagasse hydrolysate. The carotenoids production and composition is strongly depended on the microbial strain, the substrate and the cultivation conditions employed (Xu and Liu, 2017).

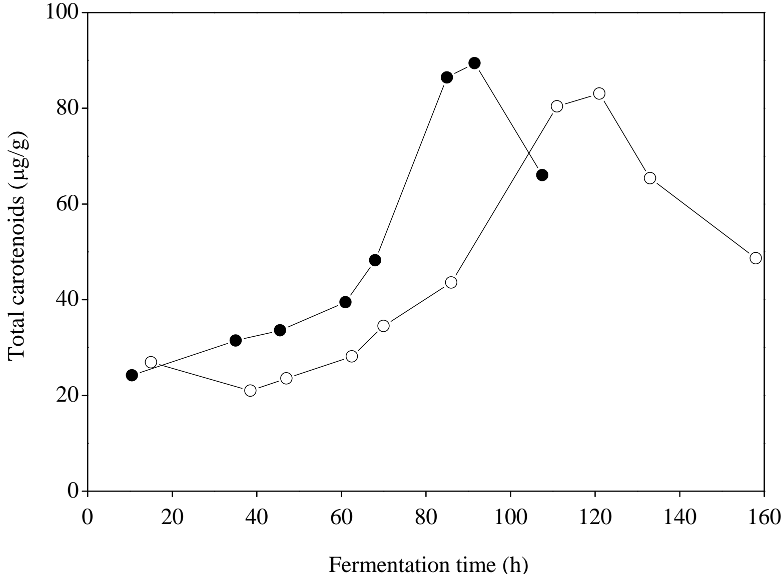

Figure 7.4 Time course of total carotenoids production in fed-batch bioreactor fermentations of *R. toruloides* carried out with either commercial nitrogen sources (○) or soybean cake hydrolysate (●) supplementation in the molasses-based medium

Chapter 8

Development of bio-economy products through enzymatic bioprocesses

8 Development of bio-economy products through enzymatic bioprocesses

8.1 Introduction

The development of value-added bio-economy products, such as wax esters and biolubricants, using various oils and fat-based by-products as raw materials was the aim of this chapter. The production of these chemicals was performed through enzymatic catalysis as an environmentally friendly process. Different lipases, commercial and crude produced on-site via fermentation, were employed for esterification and transesterification reactions. Increasing the use oflipases in industrial applications demands the reduction of their production cost. This could be achieved by using industrial solid residues as substrate in solid state fermentations (SSF) for the production of lipases. For instance, oilseed cakes that remain after the extraction of vegetable oils could be employed in SSF for lipase production. Furthermore, the naturally immobilized biocatalyst on agro-residues could be used directly in esterification reactions without further purification of the lipase (Soares et al., 2013). For instance, the use of fermented solids of babassu cake as biocatalyst has been successfully used for biodiesel synthesis (Aguieiras et al., 2017a; 2017b; 2014).

In the case of commercial lipases utilisation, this study focused on wax esters production from palm oil aiming to the evaluation of the reaction conditions, which thereafter were employed for the production of wax esters from microbial oil. Wax esters production using either microbial oil or fatty acid distillates was also studied using non-commercial lipases. A simplified schematic diagram describing the bioprocess for wax esters production is presented in Figure 8.1.

This chapter also presents the production of bio-based lubricants from microbial oil using commercial lipases. Bio-based lubricants production is expected to considerably increase in the future, driven by environmental and health concerns (toxicity, inadequate biodegradability etc.). Figure 8.2 represents the bioprocess followed for biolubricants production from microbial oil using food industry by-products as renewable feedstock for fermentative production of microbial oil.

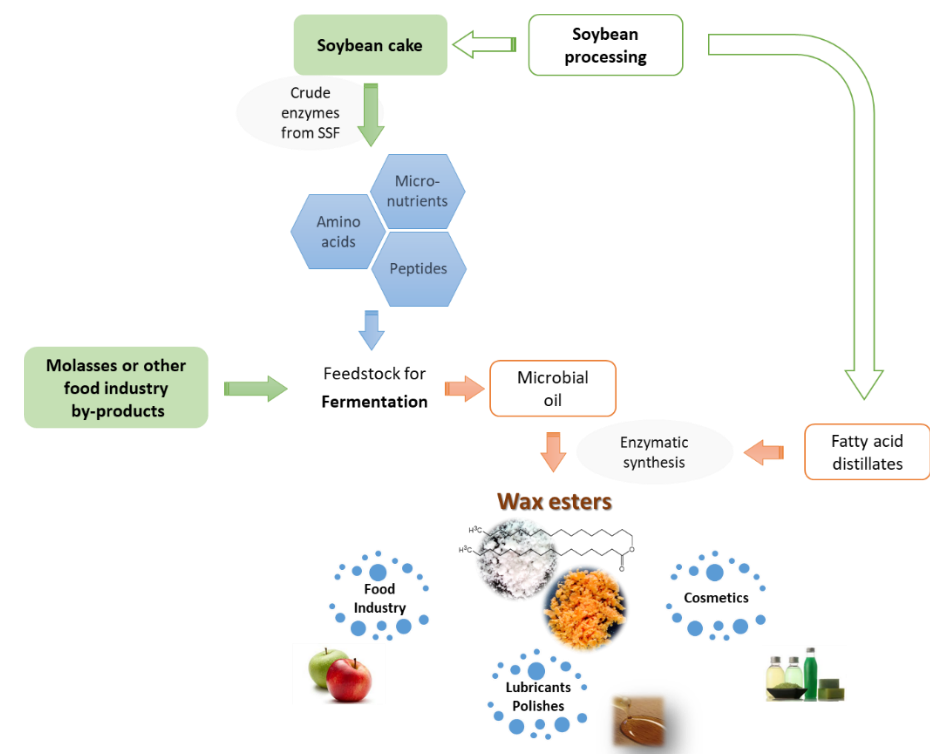

Figure 8.1 Schematic diagram of bio-based wax esters production from food industry by-products

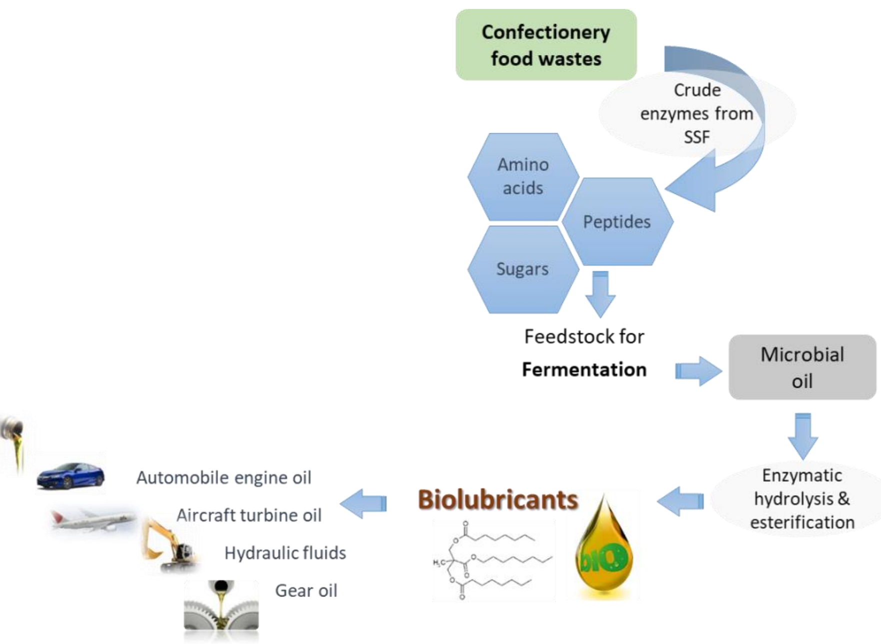

Figure 8.2 Schematic diagram of bio-based lubricants production from food industry by-products

8.2 Wax esters production from oil mills' by-products

8.2.1 Analysis of fatty acid composition

The acidities of SFAD and PFAD weredetermined 100% and 97%, respectively. The fatty acid composition of the PFAD and SFAD feedstocks were analysed by GC. Specifically, PFAD was mainly consisted of palmitic acid (47.4%), oleic acid (36.5%) and linoleic acid (9.6%), whereas the major fatty acids contained in SFAD were linoleic (42.6%), oleic (28.4%) and palmitic (18.7%) acids.

8.2.2 Wax esters production using fatty acid distillates

The experiments described in this section were carried out using dry fermented solid (DFS) as biocatalyst that was obtained through the SSF of the fungal strain *Rhizomucor miehei*on babassu cake.

PFAD was esterified with oleyl and cetyl alcohols for the respective wax esters production in a solvent-free system. Figure 8.3 presents the conversion yield obtained after 24 h reaction, which demonstrates that DFS was able to produce both oleyl and cetyl wax esters from PFAD. The reactions were performed using a stoichiometric molar ratio at 50 °C. The temperature of 50 °C was chosen because this is lowest possible temperature in which the fatty acid distillates (PFAD and SFAD) are soluble in the solvent-free esterification process employed. A higher temperature was not used in order to avoid denaturation of enzymes. The conversion yield of 80%, obtained at 24 h for PFAD-oleyl wax esters, was significantly different ($P< 0.05$) than the conversion yield of PFAD-cetyl wax esters (73%). The same biocatalyst had been previously used in PFAD esterification reactions with ethanol and methanol, showing conversion yields above 80% (Aguieiras et al., 2017a). Despite the fact that temperature, molar ratio of the reactants and the type of alcohol were different than this study, similar conversion yields were achieved. Thus, the lipases of the DFS can use both short (e.g. methanol, ethanol) and long (e.g. cetyl and oleyl) chain alcohols as acyl acceptors. The PFAD-oleyl esters contained mainly 37.3% oleyl palmitate and 42.9% oleyl oleate and linoleate, whereas the PFAD-cetyl esters contained 33.5% cetyl palmitate and 39.3% cetyl oleate and linoleate.

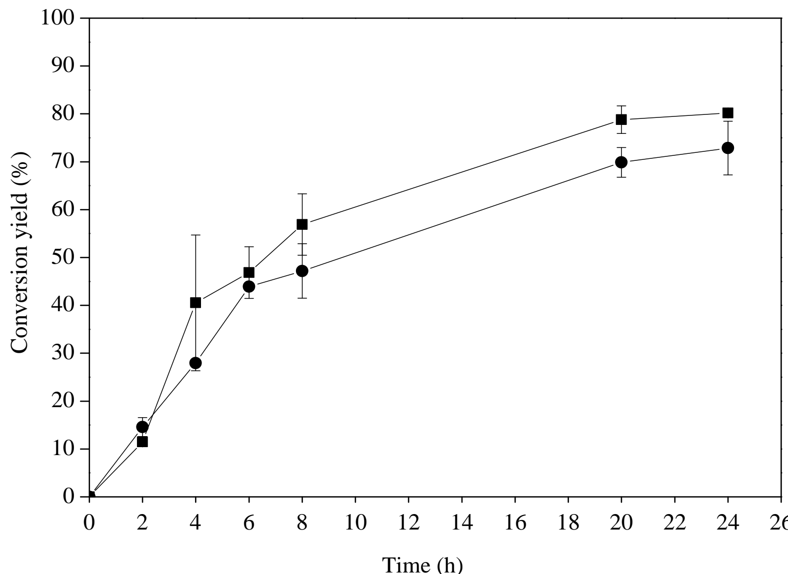

Figure 8.3 Wax esters production catalysed by dry fermented solids (DFS) using PFAD with (●) cetyl and (■) oleyl alcohols. Reaction conditions: 8.8 U of fermented solid per g of PFAD, alcohol:oil molar ratio of 1:1 and 50 °C

Figure 8.4 presents the conversion yield of wax esters production in a solvent-free system using SFAD with oleyl and cetyl alcohols leading to conversion yields of 81% and 79%, respectively, after 24 h. The utilization of different alcohols was not influenced significantly ($P < 0.05$) the final conversion yield. The kinetic profile of the conversion yields observed in Figures 8.3 and 8.4 for the esters produced using either PFAD or SFAD were similar, showing that the free fatty acids composition of the two industrial side streams did not significantly affect the conversion yields ($P < 0.05$). The oleyl and cetyl esters derived from SFAD contained mainly around 62% of oleate and linoleate esters and 16.6 - 18.7% palmitate esters.

Cetyl and oleyl alcohols have been used in previous studies for the production of wax esters using various vegetable oils or free fatty acids. Immobilized lipase from *Candida rugosa* was used by Bi et al. (2016) for oleyl oleate production with 92.6% conversion yield at 12 h. Immobilized *Candida rugosa* lipase was also applied in a non-solvent esterification reaction to produce twelve types of wax esters (Guncheva and Zhiryakova, 2008). Conversion yields up to 95% were achieved via esterification of fatty acids derived from crambe and camelina oils with oleyl alcohol or alcohols from crambe and camelina oils, using the commercial lipase Novozyme 435 (Steinke et al., 2000). Kuo et al. (2012) used high concentrations of the commercial lipases Lipozyme RM IM (46%) and Novozyme 435 (34%)

in a medium with n-hexane for cetyl octanoate synthesis, reaching a conversion yield up to 90%. Maximum conversion of cetyl decanoate (85.4%) was achieved by Alves et al. (2016) after 50 min using 10% (w/w) of immobilized lipase from *Thermomyces lanuginosus* in a solvent-free system. Immobilized lipase from *Candida* sp. 99-125 was used as biocatalyst of esterification between oleic acid and cetyl alcohol with a conversion of 98% after 8 h (Deng et al., 2011). Previous studies used pure fatty acids (e.g. oleic acid) or vegetable oils for wax esters production. This study employed for the first time crude industrial side streams that could be used as renewable feedstocks for wax ester production using DFS as natural biocatalyst in a solvent-free system.

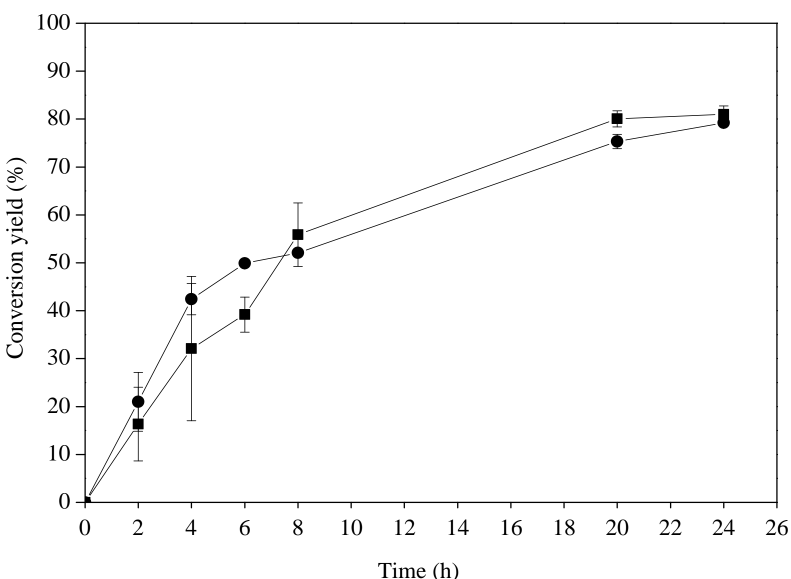

Figure 8.4 Wax esters production catalysed by dry fermented solids (DFS) using SFAD with (●) cetyl and (■) oleyl alcohols. Reaction conditions: 8.8 U of fermented solid per g of SFAD, alcohol:oil molar ratio of 1:1 and 50 °C

8.2.3 Evaluation of enzyme stability

The stability of the biocatalyst was evaluated in five successive batch reactions with reaction duration of 24 h. After each batch, the DFS was washed with hexane to remove any substrate or product that could be adsorbed on the biocatalyst and dried by vacuum filtration. This methodology is widely used for both immobilized enzymes and DFS used as crude biocatalyst (Aguieiras et al., 2017a; Alves et al., 2016; Bi et al., 2016; Radzi, 2005).

The results illustrated in Figure 8.5 show that after five repeated cycles of reaction, the relative conversion obtained in the reactions carried out with SFAD and cetyl alcohol was 100% even after five consecutive batch esterification reactions. However, in the case of SFAD-oleyl esters, the conversion yield was reduced (40.9%) to almost half the value achieved the previous four batch esterification reactions. When PFAD was used as raw material, relative conversions of 87% were obtained in the 4th cycle for both alcohols used, while the conversion yield was reduced further in the 5th cycle. The conversion yield of PFAD-oleyl esters was reduced to 54.4%, while the conversion yield of PFAD-cetyl esters was reduced to 72.5%. The results showed also that the conversion yield was significantly ($P < 0.05$) reduced in the fifth batch reaction for PFAD-cetyl esters and for PFAD- and SFAD-oleyl esters.

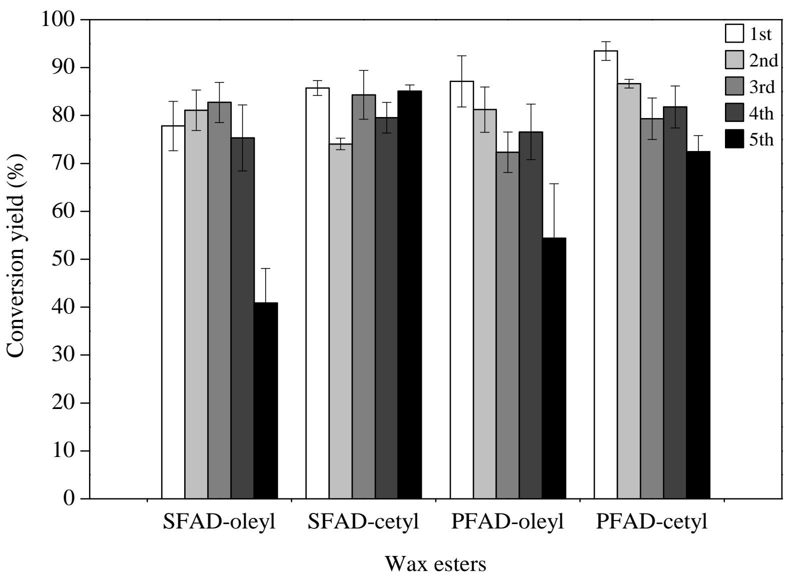

Figure 8.5 Conversion yields observed in repeated batch esterification reactions of PFAD and SFAD with oleyl and cetyl alcohols. Reaction conditions: 8.8 U of fermented solid per g of PFAD or SFAD, alcohol:oil molar ratio of 1:1 and 50 °C

Although the biocatalyst used in this study has not been used in the synthesis of wax esters, it has been used in other esterification reactions. The solid enzyme preparation of *R. miehei* has been used for 10 cycles in esterification of the free fatty acids from macaúba oil with ethanol and the conversion yields achieved were > 95% of the initial conversion yield after 8 cycles (Aguieiras et al., 2014). The same biocatalyst was used in esterification reactions of PFAD and SFAD with ethanol demonstrating good stability in 6 consecutive

batch reactions, carried out at 45 °C for 6 h, after washing the biocatalyst with hexane (Aguieiras et al., 2017a). It should be noted that in this study the esterification of PFAD or SFAD with oleyl or cetyl alcohols was carried out at higher temperature (50 °C) than the esterifications with ethanol presented by Aguieiras et al. (2017a) and the biocatalyst was maintained at this temperature for a longer duration (24 h). The higher temperature was used in order to decrease the viscosity of the reaction mixture and increase the solubility of reactants, especially in the case of cetyl alcohol, which has a melting point of 49 °C. However, high temperatures may cause enzyme denaturation which may led to lower conversion yuelds. The decrease in the conversion yield in the 5th batch cycle for cetyl-PFAD, oleyl-PFAD and oleyl-SFAD synthesis could be related to the combined effect of denaturation phenomena and/or accumulation of components of the reaction medium in the biocatalyst. Soares et al. (2013; 2015) observed that some components of the reaction medium were adsorbed on the fermented solids during esterification reactions between free fatty acids and ethanol. The deposition of some free fatty acids from the raw materials as well as oleyl alcohol in biocatalyst pores may hinder the interaction between substrate and lipases (Claon and Akoh, 1994; Krishna and Karanth, 2001; Radzi, 2005).

Literature cited results have shown great stability of immobilized lipases in wax ester synthesis (Alves et al., 2016; Bi et al., 2016; Deng et al., 2011; Kuo et al., 2012; Radzi, 2005). The immobilised lipases used in the literature-cited studies were either immobilised commercial lipases or crude lipase formulations using different fungal strains than the one used in this study. Sellami et al. (2011) reported high conversion yield (98.5%) of palm stearin derived cetyl wax esters using a non-commercial immobilised lipase from *Rhizopus oryzae*. Chen and Wang (2001) utilised a fungal lipase from *Rhizopus niveous*, immobilised on cellulose biomass support particles, for the synthesis of cetyl oleate achieving a conversion yield of 76%. The results of the present work showed that the DFS produced by *R. miehei* immobilised on babassu cake presented good thermal stability at high temperature in wax ester synthesis.

8.2.4 Physicochemical characterisation of wax esters

Table 8.1 presents the iodine values, acid values and saponification values of the PFAD and SFAD derived wax esters. The iodine value characterizes the degree of unsaturation of the substance and is expressed as g of iodine consumed by 100 g of a chemical substance. The double bonds of the substance react with iodine and the higher the iodine values the higher the

double bonds in the substance. The iodine values of PFAD and SFAD cetyl esters were 30.4 and 54.8 g I_2 per 100 g, respectively. This difference could be attributed to the higher amount of unsaturated fatty acids contained in SFAD than PFAD. The iodine value of oleyl esters derived from PFAD and SFAD was around 57 g I_2 per 100 g. Despite the fact that SFAD has higher unsaturation degree than PFAD, it should be stressed that oleyl alcohol is unsaturated (contains one double bond) and this contributes to higher iodine value of PFAD-oleyl esters than the iodine values of the PFAD-cetyl esters. Keng et al. (2009) reported an iodine value of 69.7 g I_2 per 100 g for oleyl esters produced from palm oil that is close to the iodine value of PFAD-oleyl esters produced in this study. The iodine value of PFAD-cetyl esters was found similar to lanolin (up to 36 g I_2 per 100 g), candelilla wax (up to 21 g I_2 per 100 g) and cotton wax (24.5 g I_2 per 100 g), whereas the iodine values of PFAD-oleyl, SFAD-oleyl and SFAD-cetyl esters produced in this study are higher than the iodine values of common commercial and vegetable waxes (Anonymous, 2016c; Mark and Kroschwitz, 1989). Generally, the application of wax esters with high iodine value in cosmetics improve the moisturizing properties (Keng et al., 2009).

Table 8.1 Iodine, acid and saponification values for wax esters derived from PFAD and SFAD with oleyl and cetyl alcohols

Wax ester	Iodine value ($g\ I_2$/100 g)	Saponification value (mg KOH/g)	Acid value (mg KOH/g)
PFAD-oleyl	57.3 ± 1.2	117.7 ± 5.3	7.9 ± 0.0
PFAD-cetyl	30.4 ± 0.5	107.0 ± 15.2	5.5 ± 0.1
SFAD-oleyl	57.5 ± 0.3	113.5 ± 4.0	6.8 ± 0.0
SFAD-cetyl	54.8 ± 0.1	116.8 ± 3.0	5.0 ± 0.9

The presence of free fatty acids in the wax esters was determined by the acid value. The acid values of the PFAD and SFAD esters were in the range of 5 to 7.9 mg KOH per g (Table 8.1). Carnauba wax (2.5 - 5 mg KOH per g) and sugarcane wax have similar acid value, while other common vegetable waxes present higher acid values (Anonymous, 2016c; Davidsohn and Milwidsky, 1968). Generally, low acid content is preferable since higher values promotes the oxidation of the wax esters (Keng et al., 2009).

DSC analysis was carried out to determine the thermal behaviour of the produced wax esters. The onset (T_{onset}), peak (T_p) and completion (T_{com}) temperatures of the wax esters are

shown in Table 8.2. According to Nassu and Gonçalves (1999), the melting point of a substance corresponds to the T_{com}, since a complete melt of the compound occurs at this point. The T_{com} of the produced waxes ranged from 43.8 to 47.6 °C for the cetyl esters derived either from PFAD or SFAD. The T_{com} of PFAD-oleyl esters (30.6 °C) and SFAD-oleyl esters (11 °C) was lower than the respective cetyl esters. The studied wax esters presented a wide range of melting temperatures (T_{onset}, T_p and T_{com}) indicating the presence of different compounds. This is also observed in natural fats which consist of various components and exhibit a melting range of temperatures rather than a specific melting point (Hartel et al., 2018). The completion of melt temperature is also affected by the melting point of the corresponding oil (Dun, 2002). The differences found between oleyl and cetyl esters could be attributed to the chain length of each alcohol. Yao et al. (2008) reported that the utilisation of longer chain alcohol led to increased melting temperatures. Moreover, the straight chain esters, such as cetyl esters, showed increased melting point when the number of carbon atoms in the alcohol moiety was more than four (Knothe and Dunn, 2009).

Table 8.2 Melting temperatures of wax esters derived from PFAD and SFAD as determined by differential scanning calorimetry (DSC)

Wax ester	T_{onset} [a] (°C)	T_{peak} [b] (°C)	T_{com} [c] (°C)
PFAD-oleyl	-20.40 ± 0.3	17.55 ± 0.3	30.6 ± 0.3
PFAD-cetyl	8.05 ± 0.4	13.67 ± 0.1	47.6 ± 0.8
SFAD-oleyl	-32.80 ± 3.1	-24.35 ± 0.9	11.07 ± 1.3
SFAD-cetyl	3.36 ± 0.2	12.02 ± 1.9	43.8 ± 1.5

[a] T_{onset}: onset melting temperature; [b] T_{peak}: maximum peak temperature; [c] T_{com}: completion of melting

8.3 Wax esters production from palm oil and microbial oil using commercial enzymes

The study of the reaction conditions for wax ester production using two commercial lipases, is described in the following sections, which was carried out with palm oil. The optimum conditions were subsequently used for the production of wax esters using three different microbial oils. These microbial oils was produced from confectionery wastes in the case of *R. toruloides* and *L. starkeyi*, and from a mixture of cheese whey and wine lees hydrolysate in the case of *C. curvatus*.

8.3.1 Analysis of fatty acid composition

The fatty acid composition of palm oil as well as the lipid classes of the three microbial oils used is presented in Table 8.3. The palm oil derived from the mesocarp of the palm fruit contains mainly palmitic (48.1%) and oleic (33.5%) acids with a degree of saturation of 53.7%. Oleic (47 - 52%) and palmitic (26 - 38.1%) acids were the predominant fatty acids in all microbial oils with lower degree of saturation (36.7 - 44.8%) than palm oil. Neutral lipis (NL) was the predominant lipid class (83.9 - 87.2%) in all microbial oils followed by sphingolipids and glucopholipids (S & G) and phospholipids (P). The results presented in Table 8.3 demonstrate that the main fatty acids in all oils were palmitic acid and oleic acid, whereas palm oil presented a higher saturation degree than all microbial oils.

Table 8.3 Fatty acid composition and lipid classes of palm oil and microbial oils produced by *Cryptococcus curvatus*, *Lipomyces starkeyi* and *Rhodosporidium toruloides*

Oil source	Fractions	% (w/w)	Fatty acids (%)					Saturation Degree
			C16:0	C18:0	C18:1	C18:2	Others	
C. curvatus	TL[a]	-	26.0	10.7	52.1	7.3	3.9	36.7
	NL[b]	83.9	28.9	9.7	53.4	8.0	0.0	
	S & G [c]	11.9	26.4	11.7	51.3	10.6	0.0	
	P [d]	4.2	26.1	10.2	52.9	6.1	4.7	
L. starkeyi	TL	-	38.1	6.7	47.1	2.0	6.1	44.8
	NL	84.1	39.1	7.1	48.6	1.9	3.3	
	S & G	11.5	37.2	6.3	49.6	4.1	2.8	
	P	4.4	26.6	6.6	45.2	1.1	0.1	
R. toruloides	TL	-	30.4	7.7	52.0	5.5	4.4	38.1
	NL	87.2	35.3	7.8	51.8	5.1	0.0	
	S & G	9.7	25.5	13.0	53.9	5.8	1.8	
	P	3.1	15.2	3.0	62.6	4.9	0.0	
Palm oil	TL	-	48.1	5.6	33.5	6.7	4.0	53.7

[a] TL, total lipids; [b] NL, neutral lipids; [c] S & G, sphingolipids & glycolipids; [d] P, phospholipids

8.3.2 Wax esters production using palm oil as raw material

Transesterification reactions were carried out with cetyl, oleyl and behenyl alcohols. The cetyl and oleyl alcohols were chosen as it has been reported that the wax esters produced with these alcohols and vegetable oils or oleic acid have similar properties to spermaceti oil or

jojoba oil (Basri et al., 2009; Deng et al., 2011; Gunawan et al., 2005; Steinke et al., 2000; Ungcharoenwiwat et al., 2016;). Behenyl alcohol was chosen because behenyl esters can be used in cosmetic applications, such as behenyl beeswax and behenyl olivate (Fiume et al., 2015), wood coatings (Petersson et al., 2005) and as fat substitute in various food products (Hepburn et al., 2000). The evaluation of palm ester synthesis using cetyl, oleyl and behenyl alcohols, catalysed by Novozyme 435 or Lipozyme, was carried out regarding temperature and initial lipase concentration.

Effect of temperature: Transesterification reactions were initially carried out at four temperatures (40, 50, 60, 70 °C) using 1:3 molar ratio of palm oil:alcohol and initial lipase activity of 3 U/g (Figure 8.6). The melting points of behenyl and cetyl alcohols prevented the evaluation of these alcohols at reaction temperatures lower than 70 °C and 60 °C, respectively. The highest wax ester conversion yield was 81.6% (Figure 8.6) when Novozyme 435 was used in the transesterification reaction carried out at 70 °C between palm oil and behenyl alcohol. The transesterification with cetyl alcohol catalysed by Novozyme 435 led to final conversion yields of 75.2% at 70 °C and 65.1% at 60 °C. The temperature affected significantly ($P < 0.05$) the conversion yield of cetyl derived wax esters. The conversion yields of palm wax esters derived from oleyl alcohol were lower than the other alcohols, varying from 53% at 40 °C to 59% at 60 °C. Statistical analysis showed that no significant differences ($P < 0.05$) were found for oleyl wax esters among the different temperatures.

The different results obtained from the three alcohols can be related to the structure of the enzyme's binding site, which determines the specificity of the enzyme. The substrate binding pocket of *Candida antarctica* lipase B is an elliptical and steep funnel (Pleiss et al., 1998). Oleyl alcohol is an unsaturated substrate and the fold in the alcohol chain may produce steric conflicts reducing thus the product formation (Pedersen et al., 2002). The results obtained from the reactions using Lipozyme demonstrated that the highest yields of 51.5%, 49.1% and 63.7% were achieved using oleyl, cetyl and behenyl alcohols, respectively, at 70 °C. ANOVA demonstrated that the conversion yields were significantly influenced ($P < 0.05$) by the temperature in the case of oleyl alcohol. Specifically, the Tukey's test was shown that the conversion yield at 60 °C was significantly different ($p < 0.05$) from the yields performed at the other temperatures. The binding pocket of *Rhizomucor miehei* lipase is a shallow bowl (Pleiss et al., 1998) which can reduce the steric hindrance. In this way, the results obtained with oleyl and cetyl alcohol were similar. The better results attained with behenyl alcohol can be related with the high activity of this enzyme for longer fatty acids (Pleiss et al., 1998).

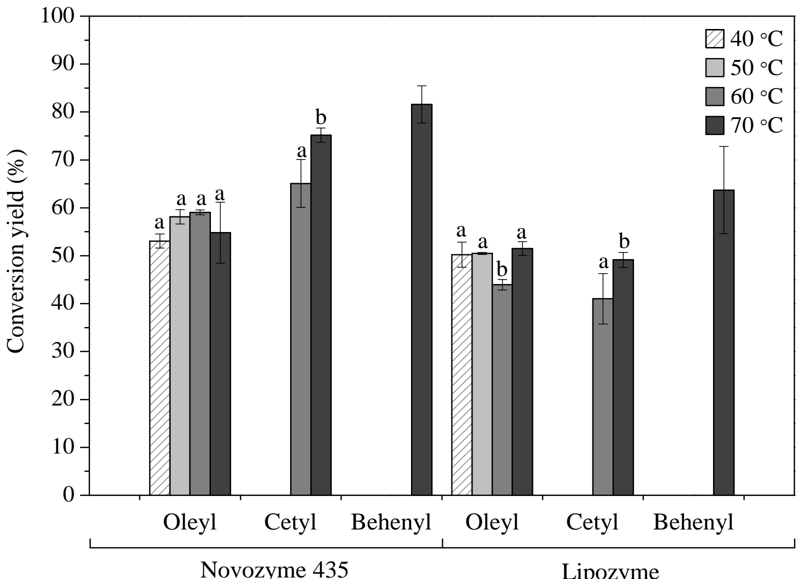

Figure 8.6 Effect of temperature (40, 50, 60, 70 °C) on the conversion yield of transesterification of palm oil with oleyl, cetyl and behenyl alcohols catalysed by Novozyme 435 and Lipozyme lipases with an initial enzyme activity of 3 U/g. Different letters on the bars indicate significant differences between temperature treatments for each alcohol and each lipase ($P < 0.05$)

The conversion yields obtained with Novozyme 435 (a random lipase, with no 1,3-positional selectivity) were higher than those using Lipozyme (a 1,3-positionally specific enzyme). Similar results were reported also in synthesis of other molecules such as biolubricants (Aguieiras et al., 2011). The higher conversion yields obtained at 70 °C for cetyl and behenyl alcohols in reactions catalyzed by Novozyme 435 can be also explained due to high activity of this enzyme in high temperatures as previously observed (Aguieiras et al., 2011). The high conversion yield observed at 70 °C for cetyl alcohol can be related to the reduction on the viscosity of the medium at higher temperatures. The increase in temperature can improve substrate diffusion, reducing mass transfer limitations and favoring interactions between enzyme and substrates (Duan et al., 2010). This phenomenon was not observed in reactions with oleyl alcohol due to low melting point of this alcohol (19 °C).

Most of the literature-cited studies focusing on wax ester production from palm oil, palm kernel oil or specific fatty acids and oleyl alcohol, utilised Lipozyme RM IM as biocatalyst in the presence of co-solvent (hexane). Gunawan et al. (2004) reported that the optimum temperature for oleyl palm esters production was around 50 °C that led to conversion yields in the range of 78 - 85% at 5 - 7 h in the presence of hexane as solvent.

Keng et al. (2009) reported conversion yields higher than 92% at 50 °C and 5 h reaction duration using hexane as solvent for the production of oleyl palm esters by Lipozyme RM IM. Novozyme 435 has been also studied as biocatalyst for wax ester synthesis mainly in esterification reactions using free fatty acids. The literature-cited optimum temperature of Novozyme 435 in solvent-free esterifications of myristic, lauric, palmitic or stearic acids with cetyl alcohol was in the range of 65 – 80 °C (Petersson et al., 2005; Serrano-Arnaldos et al., 2016).

Effect of enzyme concentration: The effect of initial enzyme activity on wax ester synthesis was evaluated at 50 °C for reactions performed with oleyl alcohol and 70 °C for reactions carried out with cetyl and behenyl alcohols (Figure 8.7). In the case of cetyl and behenyl palm esters produced by Novozyme 435, the initial lipase activity did not influence significantly ($p< 0.05$) the conversion yields that varied within the range of 75.1 - 79.6% for cetyl esters and 78.6 - 81.6% for behenyl esters. In this case, it is supposed that the reaction equilibrium was attained. The conversion yield of oleyl wax esters synthesis by Novozyme 435 increased with increasing initial enzyme concentration up to 65.6% conversion yield at an initial lipase activity of 4 U/g, indicating that the enzyme concentration affected significantly ($p< 0.05$) the reactions performed with oleyl alcohol. These results could be explained by taking into consideration the steric hindrance as was previously pointed out. In this case, the reaction may have not reached equilibrium, and the utilisation of higher enzyme concentration could increase the conversion yield. Significant effect ($p< 0.05$) of the enzyme concentration was also found on the production of oleyl and cetyl wax esters by Lipozyme, as the conversion yields showed an increasing trend with increasing enzyme activity. The conversion yields were up to 57.9% and 52.9%, in the case of oleyl and cetyl wax esters, respectively, at initial enzyme activity of 4 U/g. This behaviour can be related to the presence of inhibition effects at long reaction times due to competitive inhibition by the formation of inactive binary or ternary between the enzyme and the alcohol or the ester (Garcia et al., 2000). Increasing the enzyme concentration could improve the conversions. In the case of behenyl palm ester synthesis by Lipozyme, the conversion yield achieved at 2 U/g was significantly different ($p< 0.05$) than those at 3 U/g and 4 U/g. The higher yields were 64% at 3 U/g and 63.5% at 4 U/g. Figure 2 shows that the use of Lipozyme led to lower wax ester conversion yields than Novozyme 435, especially in the case of cetyl and behenyl palm esters. Thus, Novozyme 435 was selected for wax ester synthesis using the three microbial oils.

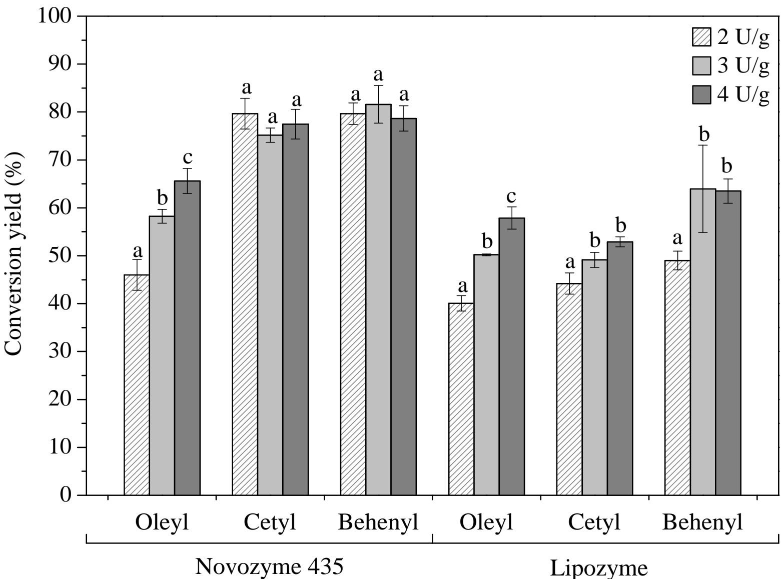

Figure 8.7 Effect of initial enzyme activity (2, 3 and 4 U/g) on the conversion yield of transesterification reactions carried out with palm oil and oleyl, cetyl and behenyl alcohols catalysed by Novozyme 435 and Lipozyme lipases. The temperatures used were 50 °C for reactions with oleyl alcohol and 70 °C for reactions with cetyl and behenyl alcohols. Different letters on the bars indicate significant differences between enzyme activity treatments for each alcohol and each lipase ($p < 0.05$)

8.3.3 Wax esters production using microbial oils as raw material

Based on the best transesterification results achieved with palm oil and taking into consideration that higher wax ester productivity that was achieved at 4 U/g enzyme concentration, transesterification experiments with microbial oils commenced with cetyl and behenyl alcohols for the production of wax esters, catalysed by 4 U/g of Novozyme 435 at 70 °C. The transesterifications carried out with behenyl alcohol and microbial oils resulted in higher conversion yields than cetyl alcohol (Figure 8.8). At the same reaction conditions, the conversion yields of behenyl derived wax esters achieved in the case of *L. starkeyi* oil (77.6%), *R. toruloides* oil (70.6%) and palm oil (78.6%) were significantly lower ($p < 0.05$) than the one achieved with *C. curvatus* oil (87.3%) (Figure 8.8).

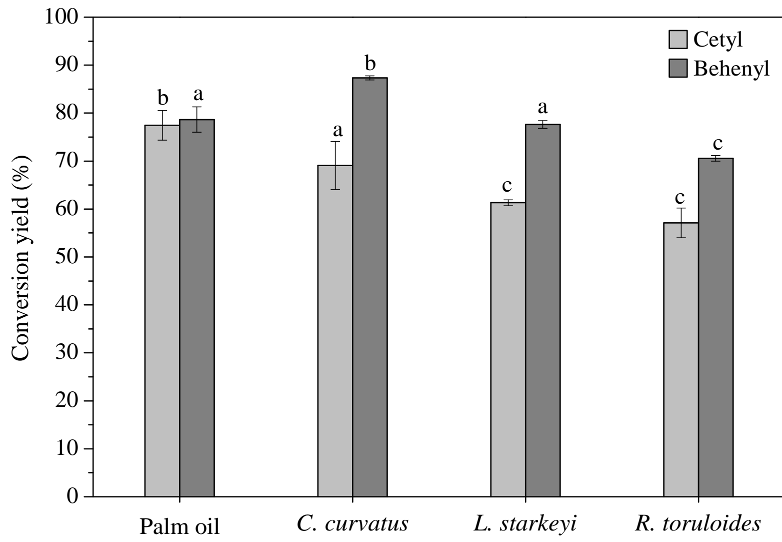

Figure 8.8 Conversion yields of wax esters produced from palm oil and the microbial oils of *C. curvatus*, *R. toruloides* and *L. starkeyi*, using cetyl and behenyl alcohols. The reaction conditions were 70 °C and 4 U/g of Novozyme 435 lipase. Different letters on the bars indicate significant differences between oils for each alcohol ($p < 0.05$)

The different conversion yields obtained using palm oil and microbial oils can be related to their different composition. Specifically, the microbial oils do not contain only NL (Table 8.3). The presence of S & G and P as additional lipid classes results in lower ester conversion yield than palm oil during transesterification with cetyl alcohol. Nagle and Lemke (1990) reported that the conversion of triglycerides to biodiesel through transesterification is higher than 99%, while the conversion yield was reduced to less than 70% in the case of phospholipids. Talukder et al. (2009) showed that the activity of Novozyme 435 is decreased in the presence of phospholipids. Wax esters from palm oil presented higher conversion yield, since it contains lower amounts of phospholipids than microbial oils (Talukder et al., 2009). Moreover, the lower conversion yield obtained using the microbial oil of the red yeast *R. toruloides*, may also be related to the fact that lipid-borne compounds, such as carotenoids, affect negatively the lipase stability (Xu et al., 1998). Other components that influence lipases are free fatty acids, lipid polymers, lipid hydroperoxides, tocopherols, chlorophyll and citric acid (Xu et al., 1998). Behenyl alcohol was chosen for subsequent wax ester production studies from microbial oil, since the conversion yields achieved with this alcohol are higher than cetyl alcohol for all microbial oils used.

The profile of wax ester composition reflects the fatty acid profile of the initial oil used (Table 8.3). Particularly, the wax esters produced from palm oil were mainly consisted of behenyl palmitate (49.5%) and behenyl oleate (34%). Lower quantities of behenyl stearate (5.7%), behenyl linoleate (6.7%) were also detected and traces of behenyl laurate and behenyl myristate (up to 2.1%). The microbial oil derived wax esters contained mainly behenyl oleate (52.2 - 53.5%) and behenyl palmitate (27.1 - 37.1%), followed by behenyl stearate (4 - 12.6%), behenyl linoleate (2 - 6.9%) and minor amounts of behenyl myristate (0.5 - 2.8%) and behenyl laurate (0.1 - 0.2%).

8.3.4 Kinetic profile of palm and microbial esters synthesis

Since the effect of enzyme concentration was not found significant ($p < 0.05$) in transesterifications carried out with behenyl alcohol by Novozyme 435, the kinetic profile of palm esters synthesis using the lower (2 U/g) and the higher (4 U/g) enzyme concentration was studied and it is presented in Figure 8.9. The production of behenyl palm esters reached the highest conversion yields at 4 h (77%) and 12 h (80%) of reaction when 4 U/g and 2 U/g of Novozyme 435 were used, respectively. Although, a similar conversion yield was achieved at different initial enzyme activities, in the case of palm oil ester production, the duration of the transesterification reaction was longer when the lower initial enzyme activity was used. Specifically, the productivity of behenyl palm esters was 19.2% per h when 4 U/g Novozyme 435 were utilised and 6.7% per h in the case of 2 U/g Novozyme 435.

The reaction profiles of wax ester production during transesterification of behenyl alcohol with the microbial oils of *R. toruloides* and *C. curvatus* are presented in Figure 8.9. The production of wax esters with behenyl alcohol and *C. curvatus* oil using 4 U/g of Novozyme 435 reached a conversion yield of 86% at 3 h of reaction, while the highest conversion yield of wax esters obtained from *R. toruloides* oil was about 67% after 4 h of reaction.

The progress of the transesterification reaction between behenyl alcohol and *C. curvatus* oil was also observed on a TLC plate. It is noticeable that after 4 h the band of triglycerides is no longer present in the TLC plate, which confirms the conversion of triglycerides of microbial oil into wax esters (Figure 8.10).

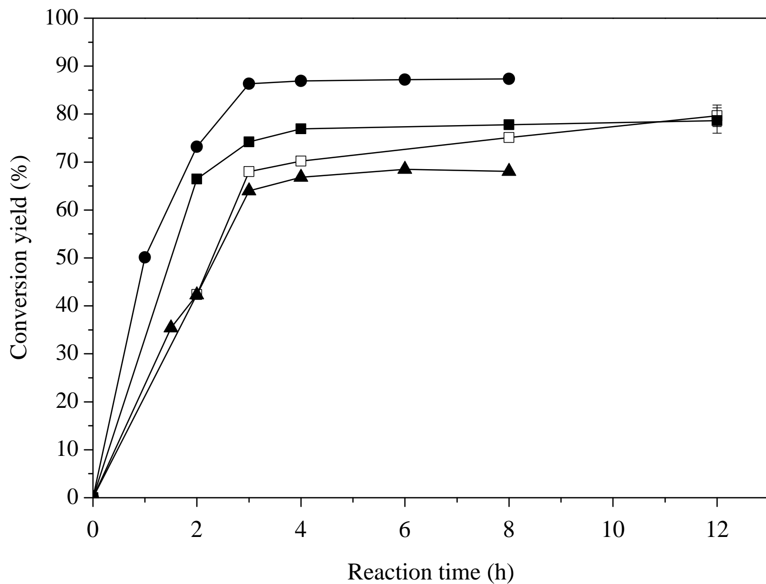

Figure 8.9 Reaction profiles of behenyl esters conversion yields produced from (□) palm oil using 2 U/g of Novozyme 435 and (■) palm oil, (●) *C. curvatus* oil and (▲) *R. toruloides oil* using 4 U/g of Novozyme 435. All experiments were carried out at 70 °C

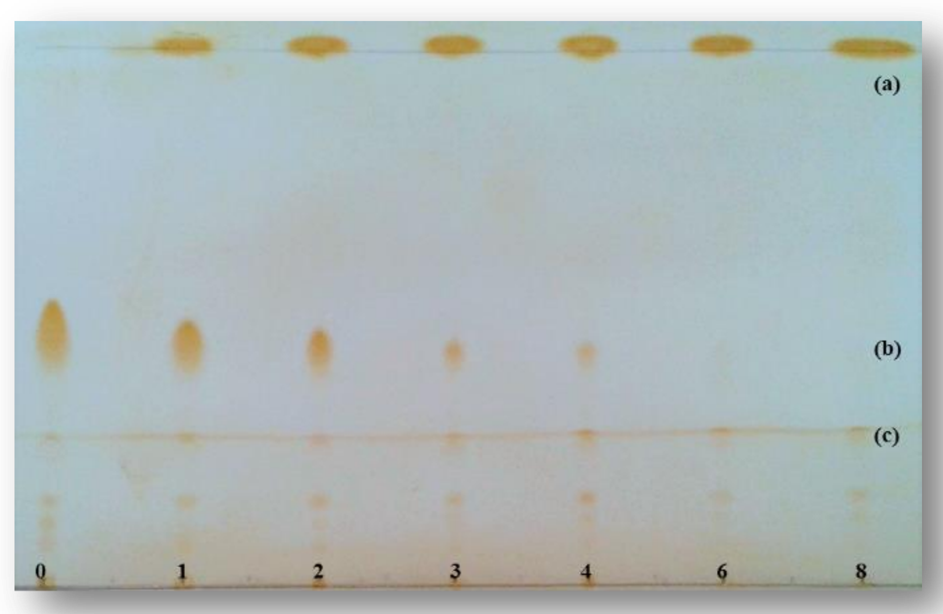

Figure 8.10 TLC silica gel plate presenting the reaction progress during transesterification of *C. curvatus* oil by Novozyme 435 using behenyl alcohol at 0 - 8 h of reaction. Lanes: (a) esters, (b) triglycerides, (c) alcohol. Reaction conditions: 70 °C, Novozyme 435 at 4 U/g

8.3.5 Evaluation of enzyme stability

The main advantages of immobilised biocatalysts are the easy recovery from the reaction mixture and their sequential utilisation in multiple batch reactions. The transesterification efficiency of Novozyme 435 after eight repeated transesterifications of palm oil and *C. curvatus* oil with behenyl alcohol are presented in Figure 8.11. Petroleum ether was selected to wash out the lipase after each batch reaction stopped (Deng et al., 2011). The enzyme stability was investigated using 2 U/g and 4 U/g of Novozyme 435 in the case of wax esters produced from palm oil and behenyl alcohol. The palm esters conversion yield was more than 70% for up to six cycles using either 2 U/g or 4 U/g of Novozyme 435 (Figure 8.11). The conversion yield was reduced significantly in the seventh and eighth cycle.

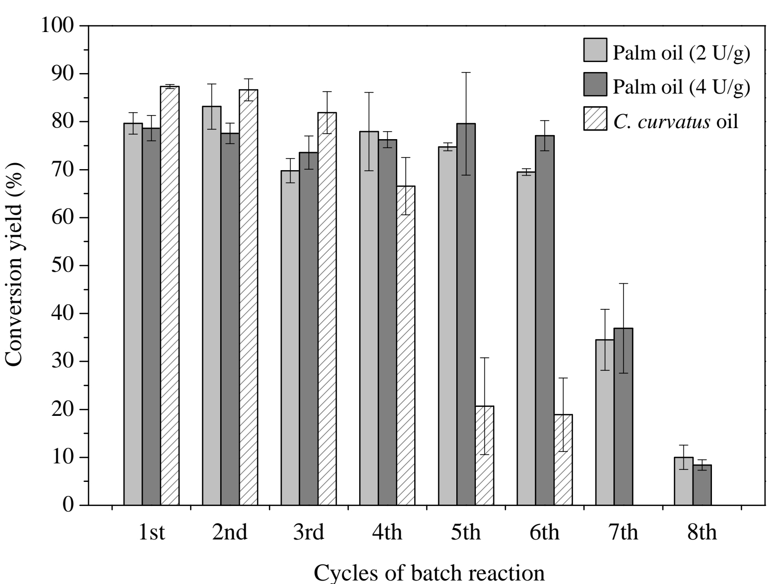

Figure 8.11 Stability of Novozyme 435 lipase during transesterification reactions of behenyl alcohol with palm oil or *C. curvatus* oil. The reaction conditions were 70 °C and Novozyme 435 at 2 U/g and 4 U/g for palm oil and 4 U/g for *C. curvatus* oil

It has been reported that the glycerol by-product produced in transesterification can be adsorbed onto the enzyme support leading to modification of the microenvironment of the enzyme and consequently to a decrease in its activity (Dossat et al., 2002). However, since a significant reduction in conversion yield was only observed after the seventh cycle, the washing of the biocatalyst with petroleum ether appeared to be efficient for glycerol removal. In this case, the reduction of the conversion can be attributed to the biocatalyst deactivation after several cycles of reaction at high temperatures as previously reported (Aguieiras et al.,

2011; Duan et al., 2010). Moreover, it should be considered the dissociation and dissolution of the biocatalyst support due to solvent washing after each cycle and to the utilisation of a solvent-free system (Verdasco-Martín et al., 2016).

In the case of wax esters produced from *C. curvatus* oil, the conversion yield was higher than 80% for three cycles (Figure 8.11). The conversion yield of *C. curvatus* oil to wax esters was reduced to 66.6% in the fourth cycle and then it was further decreased to less than 20.7% in the fifth cycle. The conversion yields achieved with *C. curvatus* oil was higher than the respective conversion yields achieved with palm oil in the first three repeated batches, but a lower conversion yield was observed in the fourth cycle. The results suggested that some components present in microbial oil (e.g. phospholipids) may adsorb on the support material of the enzyme and exert a deleterious effect on the immobilized biocatalyst. This indicates that the washing step with petroleum ether was inefficient and led to enzyme activity reduction.

8.3.6 Physichochemical characterisation of wax esters

The wax esters produced via transesterification of behenyl alcohol with palm oil and microbial oils were further characterised regarding their melting point, iodine value, acid value and saponification value. The melting point is one of the most widely used tests to determine the quality and type of wax (Speight, 2002). The melting point, as determined by DSC analysis (Figure 8.12), was similar for all behenyl wax esters tested and varied from 52.9 °C to 59.7 °C. The melting points of the three different microbial oil derived behenyl esters were closer to the melting points of spermaceti (41 - 49 °C) and beeswax (61 - 64 °C) (Anonymous, 2016c; Sharma, 1991), which implies that the microbial oil derived behenyl esters could be evaluated further in specific industrial applications of spermaceti and beeswax. The DSC analysis revealed two peaks for each wax ester. Ruguo et al. (2011) reported that the appearance of different peaks reflects different chemical composition.

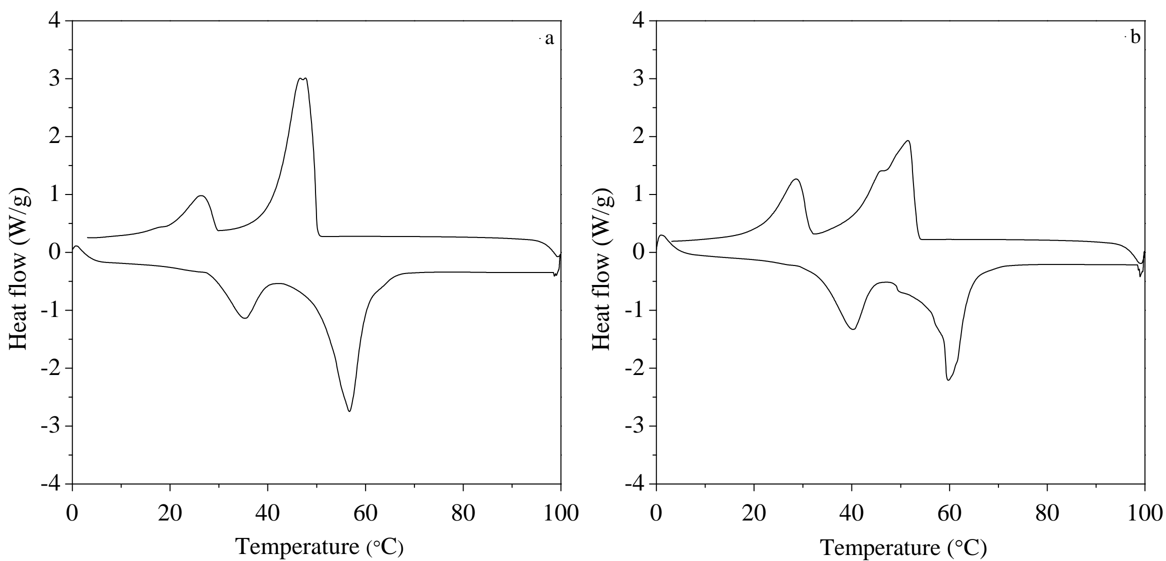

Figure 8.12 Differential scanning calorimetry (DSC) curves of behenyl esters of (a) palm oil and (b) *C. curvatus* oil

The results concerning the iodine value, acid value and saponification value are presented in Table 8.4. The iodine value represents the degree of unsaturation and thus the occurrence of double bonds in the esters. Thus, the higher the iodine value the higher the unsaturation. The iodine values of behenyl esters produced from palm oil (20.6 g iodine per 100 g) and microbial oils (23.4 - 27.5 g iodine per 100 g) were similar to the respective values of lanolin (15 - 36 g iodine per 100 g), higher than beeswax (9 - 11 g iodine per 100 g), carnauba wax (5 - 14 g iodine per 100 g) and spermaceti oil (3 - 9 g iodine per 100 g), and lower than jojoba oil (80 - 85 g iodine per 100 g) (Anonymous, 2016; Mahalingam et al., 2008; Sharma, 1991; Spencer and List, 1988). Keng et al. (2009) reported that high iodine values are preferable for wax esters used in cosmetics application as they have improved moisturizing properties.

Behenyl esters produced from either palm oil or microbial oils presented lower acid values (1.4 - 2 mg KOH per g) than beeswax (17 - 21 mg KOH per g) and carnauba wax (2 - 8 mg KOH per g), higher acid values than lanolin (≤ 1 mg KOH per g) and jojoba oil (0.7 mg KOH per g), and similar acid values to spermaceti oil (0.5 - 2.8 mg KOH per g) (Anonymous, 2016c; Mahalingam et al., 2008; Sharma, 1991; Spencer and List, 1988). The acid value indicates the amount of free fatty acids present in the wax. High content of free fatty acids is considered a disadvantage as it impairs oxidative stability of the compounds and promotes rancidity (Keng et al., 2009).

The saponification values of microbial oil derived wax esters using behenyl alcohol (141.8 - 145.2 mg KOH per g) were higher than beeswax (90 - 102 mg KOH per g), carnauba wax (78 - 95 mg KOH per g), lanolin (82 - 120 mg KOH per g) and jojoba oil (90 - 95 mg KOH per g) (Anonymous, 2016c; Mahalingam et al., 2008; Sharma, 1991; Spencer and List, 1988). The wax ester that has saponification value closer to those of microbial oil derived behenyl esters obtained in this study is spermaceti oil (121 - 135 mg KOH per g) (Sharma, 1991). The saponification value indicates whether the esters contain shorter or longer chain fatty acids than other wax esters. The esters that are composed of long chain fatty acids exhibit lower saponification values than the esters that contain short chain fatty acids (Sonntag, 1982). Based on the saponification values presented in Table 2, the behenyl wax esters produced from microbial oils contain lower carbon chain fatty acids (lower molecular weight) than natural waxes. Ungcharoenwiwat et al. (2016) reported that the saponification value of the wax esters produced by transesterification of jatropha oil and oleyl alcohol was 110.22 mg KOH per g.

Table 8.4 Physicochemical properties of natural and bio-based wax esters

Wax Esters	Melting point (°C)	Iodine Value [b]	Acid value [c]	Saponification value [d]	Reference
Beeswax (or yellow wax)	61-65	3-11	17-22	87-102	Anonymous 2016c; Mahalingam et al. 2008; Sharma 1991
Carnauba wax	80-88	5-14	2-8	78-95	
Lanolin	38-44	18-36	≤ 1	82-120	
Spermaceti	41-49	3-9	0.5-2.8	121-135	
Jojoba oil	7-9	80-85	0.7	90-95	Spencer and List 1988
Palm oil - oleyl	23.9 [a]	69.7	0.6	93.1	Keng et al. 2009
Palm oil - behenyl	56.8 [a]	20.6	2.0	143.4	This study
C. curvatus oil - behenyl	59.7 [a]	27.5	1.7	144.0	
R. toruloides oil - behenyl	52.9 [a]	25.3	1.4	141.8	
L. starkeyi oil - behenyl	56.0 [a]	23.4	1.8	145.2	

[a] melting point corresponds to T_{max} as determined by DSC; [b] expressed as g iodine per 100 g; [c] expressed as mg KOH per g; [d] expressed as mg KOH per g

The viscosity of waxes is an important parameter especially in applications involving coating or dipping processes as it influences the quality of the obtained coating (Speight, 2002). Wax esters from palm oil presented pseudoplastic flow properties as viscosity decreases with increasing shear rate (Figure 8.13a). The dynamic viscosity of behenyl esters from palm oil and microbial oils of *C. curvatus* and *R. toruloides* presented similar behaviour when it was studied in relation with temperature (Figure 8.13b). It is noticeable that the viscosity of all esters was similar up to 55 °C.

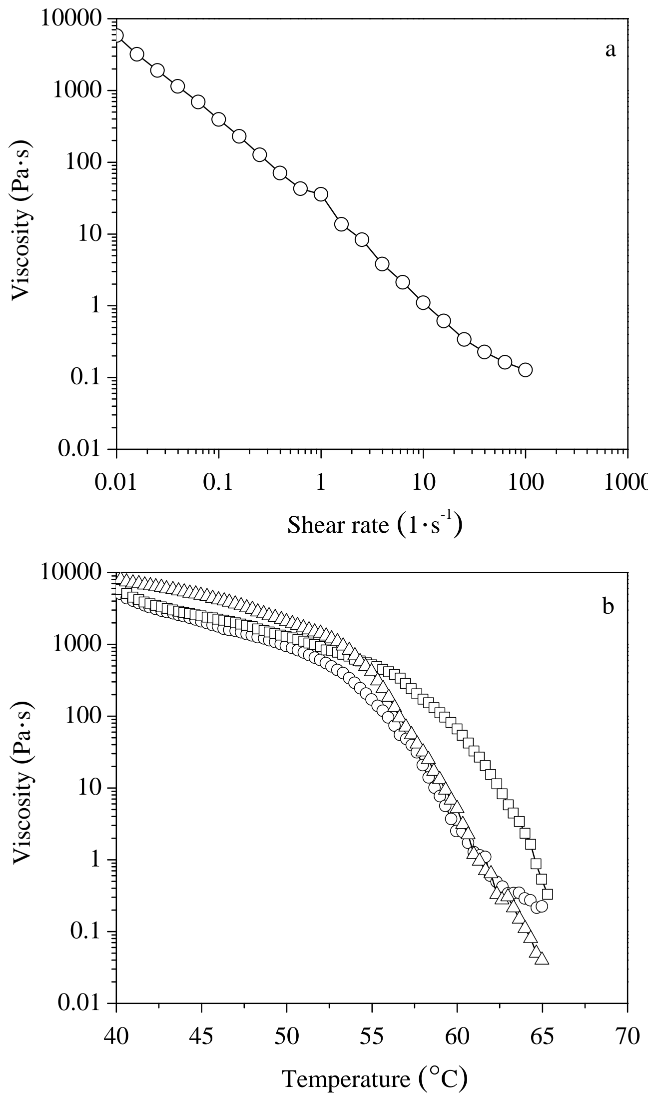

Figure 8.13 (a) Viscosity of behenyl palm esters (○) in relation with shear rate and (b) viscosity of wax esters derived from behenyl alcohol and palm oil (∆), behenyl alcohol and *R. toruloides* oil (□) and behenyl alcohol and *C. curvatus* oil (○) in relation with temperature

The results showed that the wax esters produced by enzymatic route could be evaluated in cosmetic, food (e.g. production of oleogels, structuring components and healthy substitutes for fats) and coating applications. Behenyl esters, such as behenyl olivate and behenyl beeswax, are currently used in cosmetic applications. For instance, behenyl beeswax is ideal for use in emulsions and stick-type cosmetics (Fiume et al., 2015). Doan et al. (2017) evaluated the application of natural waxes for the production of oleogels for certain fat-based food products. An enzymatically produced wax ester blend, consisting of behenyl oleate and behenyl linoleate, has been utilised in confectionery products (chocolate coating), in order to replace fats and reduce the calorie intake (Hepburn et al., 2000). This blend can be also applied to various food products, such as ice creams, dressings, sauces, bakery fats and cheese (Hepburn et al., 2000).

The fatty acids composition of microbial oils produced by oleaginous yeast and fungi could be manipulated through selection of the appropriate microbial strain or through optimisation of fermentation conditions. This could lead to the production of wax esters with different properties and diversified applications.

8.4 Wax esters production from microbial oil using non-commercial enzymes

The use of non-commercial enzymes was realised in esterification reactions of the hydrolysed microbial oil (HMO) derived from *R. toruloides*. The microbial oil was produced via the fermentations described in Chapter 7 based on VHP sugar, molasses and soybean cake valorisation. The lipases VEG and LipB were employed for hydrolysis and esterification of microbial oil, respectively, using oleyl and cetyl alcohols.

The fatty acid composition of the microbial oil has been presented in Chapter 7 and the results showed that it mainly contained oleic acid (51%), palmitic acid (29%) and stearic acid (13%). The hydrolysis of microbial oil was successfully performed by VEG leading to a free fatty acid fraction with acidity of 98%. The HMO was then recovered and used as raw material for the production of wax esters.

The effect of temperature (35 °C, 40 °C and 50 °C) was evaluated in esterification reactions (Figure 8.14a). A conversion yield of 94% for oleyl esters was achieved at 40 °C, which was found significantly higher than the other temperatures tested ($p < 0.05$). In the case of cetyl wax esters production,esterification was evaluated at 40 °C and 50 °C, since the dissolution of the high melting temperature cetyl alcohol at 35 °C in a solvent-free system

was impossible. The conversion yield of microbial oil derived cetyl esters was 91.3% at 50 °C and 81.6% at 40 °C. The statistical analysis showed that temperature affected significantly the production of cetyl wax esters ($p < 0.05$). This may be attributed to the reduction of the viscosity of the reaction mixture at 50 °C.

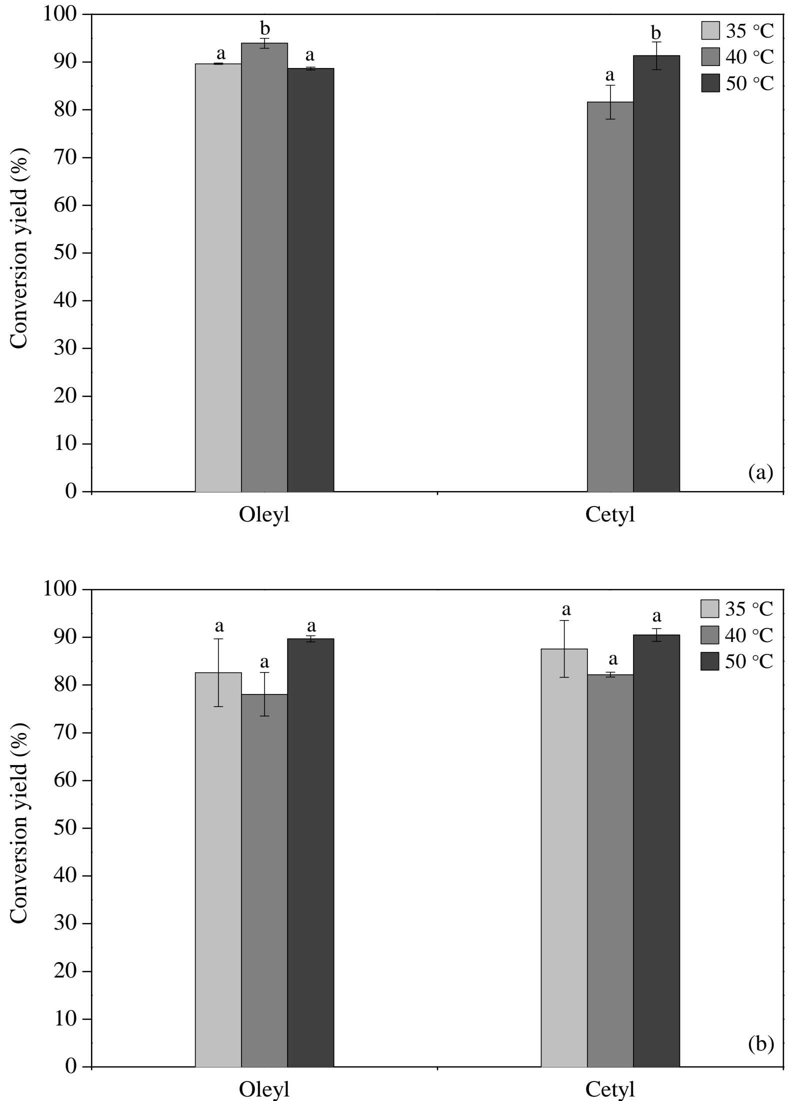

Figure 8.14 Conversion yields of microbial oil derived wax esters catalyzed by the non-commercial LiB lipase using cetyl and oleyl alcohols at different temperatures (35, 40 and 50 °C), in the presence of (a) 0% and (b) 30% limonene. Different letters on the bars indicate significant differences between the treatments for each alcohol ($p < 0.05$)

Figure 8.14b presents the conversion yields achieved at 35 °C, 40 °C and 50 °C after the addition of 30% of limonene as green solvent during esterification for the production of oleyl

and cetyl wax esters. The highest conversion yields for oleyl esters (89.7%)and cetyl esters (90.5%) were achieved at 50 °C. The addition of limonene adversely affected the esterification of oleyl esters, since the conversion yield was significantly lower ($p< 0.05$) than the highest yield (93.9% at 40 °C) achieved in a solvent free reaction system. The conversion yield of the cetyl wax ester was not influenced significantly ($p< 0.05$) by the addition of limonene as green solvent. It should be stressed that the utilisation of limonene facilitated the synthesis of cetyl wax esters at35 °C (87.5%), which is desirable inbioprocess development with lower energy consumption and reduced operating costs. The cetyl wax esters contained mainly cetyl oleate and linoleate (58.3%), cetyl palmitate (29.5%) and cetyl stearate (8.0%).

Wax ester synthesis via transesterification of microbial oils using the commercial lipase Novozyme 435 has been stated in section 8.3. The behenyl esters conversion yield achieved with *R. toruloides* derived lipids (70.6%) and *C. curvatus* derived lipids (87.3%) was lower than the corresponding one reported in this section.In the present experimental set-up, the enzymatic hydro-esterification process was employed to produce wax esters, since it has been successfully applied in the synthesis of biodiesel (Aguieiras et al., 2014). The advantage of the hydro-esterification process is the prevention of glycerol adsorption on the immobilized biocatalyst, which leads to the reduction of lipase activity and operational stability (Hérnandez-Martin and Otero, 2008; Talukder et al., 2009). Limonene has been utilised as bio-based solvent in many applications, including esterification of fatty acids. Paggiola et al. (2014) reported that the utilisation of limonene as green solvent promoted the rate of hexyl laurate synthesis catalyzed by Novozyme 435 as compared to conventional solvents. According to these results, further investigation of limonene addition to enzymatic conversions for wax esters synthesis would be interesting.

8.4.1 Physichochemical characterisation of wax esters

The determination of their physicochemical properties of the produced cetyl wax esters was also carried out. The acid value was found low (3.5 mg KOH per g of sample), which indicates that only a low quantity of free fatty acids was not esterified. Behenyl wax esters from microbial oil of *R. toruloides*presented in section 8.3.6 and natural waxes, such as carnauba wax,presented also low acid values (1.4 and 4 - 8 mg KOH per g, respectively) (Sharma, 1991). The saponification value was 131.5 mg KOH per g demonstrating that the wax esters contained mainly short chain length fatty acids (C16 and C18), similarly to the *R. toruloides* microbial oil derived behenyl wax esters (141.8 mg KOH per g), the natural wax

esters lanolin (82 - 120 mg KOH per g) and spermaceti wax (121 - 135 mg KOH per g) (Anonymous, 2016c; Sharma, 1991). The iodine value was 23.8 g I_2 per 100 g, which was similar to the wax esters produced from microbial oils and behenyl alcohol (section 8.3.6). The melting temperature profile was also analysed by DSC showing a wide range of melting temperatures. The $T_{onset}$and T_{com} temperatures of the wax esters were 6.9 °C and 46.3 °C, respectively, whereas the DSC curve revealed two T_p temperatures at 14.1 °C and at 41.6 °C.

8.5 Biolubricants production from microbial oil

The polyol esters synthesis through enzymatic catalysis is described in this section. The microbial oils of *C. curvatus* and *R. toruloides*, derived via fermentation carried out on hydrolysates of confectionery industry wastes, were used as raw materials together with neopentyl glycol (NPG) and trimethylolpropane (TMP) for polyol esters production.

The production of biolubricants was monitored during enzymatic reaction by recording the reduction of the acidity caused by the esterification of free fatty acids because analytical standards for the esters formed are not commercially available. Therefore, the microbial oil was enzymatically hydrolysed into free fatty acids. The initial acidity of the microbial oil was 11% and after 24 h of enzymatic hydrolysis the acidity was increased to 96%. Figure 8.15 presents the conversion yield of HMO of *C. curvatus* to TMP and NPG esters. The conversion yield of both esters reached 78.5% and 68.5% in the case of NPG and TMP, respectively, after 24 h. At 72 h, the conversion yield of NPG and TMP esters were 82.7% and 75.9%, respectively.

Figure 8.16 demonstrates the conversion yield of HMO of *R. toruloides* to TMP and NPG esters. Furthermore, the highest conversion yield was also achieved in the case of NPG-esters (85.7%) than TMP esters (82.9%) after 24 h. The conversion yield was increased to 88% for NPG esters and 83% for TMP esters at 72 h. The NPG esters were produced at a higher rate than TMP esters in the first 15 min of the enzymatic reaction when the HMO of *R. toruloides* was used. This could be explained by the higher solubility of NPG in the HMO than TMP. However, the esterification of HMO of *R. toruloides* with TMP reached a similar conversion yield with NPG esters after 24 h. The addition of surplus quantity of free fatty acids could increase the modification of the polyols for each reaction, but it would also lead to an acid product that must be treated further in order to produce a commercially acceptable biolubricant, which could lead to an increased cost of the final product (Alkio et al., 2000).

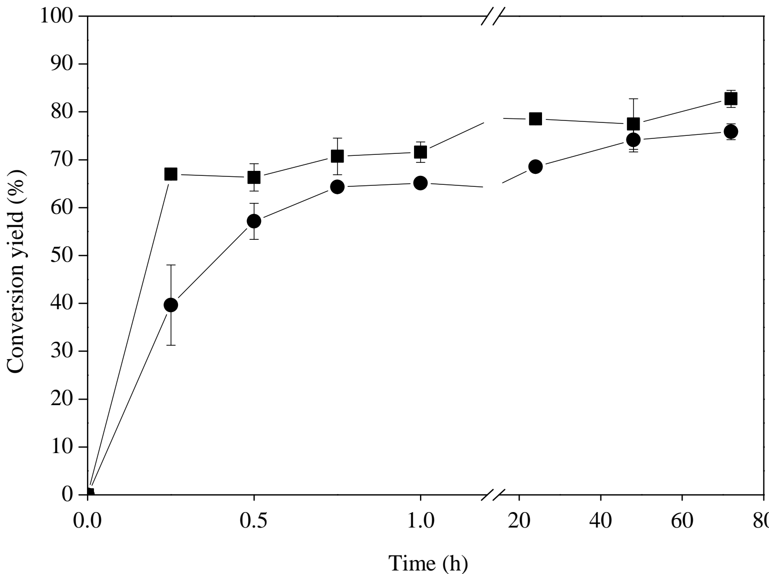

Figure 8.15 Time course of biolubricant production by enzymatic esterification using the hydrolysed microbial oil of *C. curvatus* with neopentyl glycol (NPG) (■) and trimethylolpropane (TMP) (●)

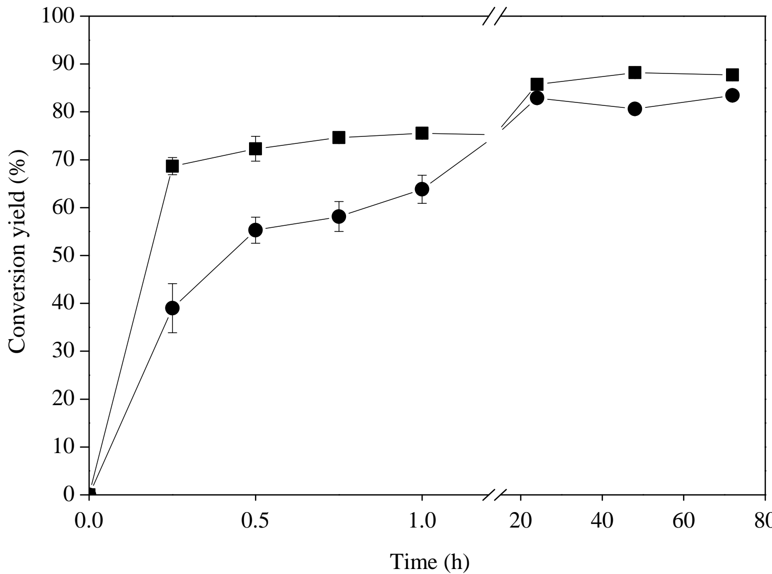

Figure 8.16 Time course of biolubricant production by enzymatic esterification using the hydrolysed microbial oil of *R. toruloides* with neopentyl glycol (NPG) (■) and trimethylolpropane (TMP) (●)

This study reports for the first time the enzymatic production of polyol esters as biolubricants using microbial oil produced by oleaginous yeasts. Although there are no many studies concerning the utilization of NPG polyol, the esterification of fatty acids derived from vegetable oils with TMP has been described in literature-cited publications. Uosukainen et al. (1998) reported a conversion yield of 75% using TMP and rapeseed oil after 24 h reaction that was carried out at similar molar ratio (3:1) and temperature (37 ºC) to those described in this work, but higher concentration of a lipase produced by *Candida rugosa* (40%). A high conversion yield (90%) was achieved after 68 h when the temperature was increased to 47 ºC (Uosukainen et al., 1998). The esterification of oleic acid with TMP by 2% of *Candida antarctica* lipase at 60 ºC led to a conversion yield of 80% after 50 h (Åkerman et al., 2011). Enzymatic esterification is more advantageous than chemical conversion due to the milder operating conditions employed. For instance, the synthesis of TMP triesters using palm oil and calcium methoxide as catalyst (Chang et al., 2012) or oleic acid and methylbenzene as catalyst (Quiao et al., 2017) requires high temperatures (180 ºC) in order to achieve high conversion yields.

8.5.1 Nuclear magnetic resonance (NMR) analysis

The composition of TMP and NPG esters concerning the content of tri-, di- and mono-esters was determined via NMR analysis (Tables 8.5 and 8.6). The esterification (24 h) carried out using the HMO of *C. curvatus* with TMP produced mainly triesters (68.1%, w/w) and diesters (22.1%, w/w), while the non-esterified TMP was only 0.5% (w/w) corresponding to 13.25% of free hydroxyl groups (Table 8.5). The monesters fraction was not detected when TMP was esterified with HMO of *C. curvatus*. The product formed after 24 h between HMO of *R. toruloides* and TMP consisted of triesters (58.2%, w/w), diesters (20.7%, w/w) and monoesters (10.8%, w/w), while the non-esterified TMP was 2% (w/w) corresponding to 25.77% of free hydroxyl groups (Table 8.5).

Table 8.5 NMR analysis of biolubricants produced at 24 h via esterification of trimethylolpropane (TMP) and hydrolysed microbial oils of *R. toruloides* and *C. curvatus*

Molecules	***R. toruloides***		***C. curvatus***	
	Molar (%)	**Weight (%)**	**Molar (%)**	**Weight (%)**
Fatty acids	15.1	8.3	23.1	9.3
Trimethylolpropane	7.4	2.0	2.5	0.5
Trimethylolpropane trimester	47.8	58.2	51.2	68.1
Trimethylolpropane diesters	15.9	20.7	23.2	22.1
Trimethylolpropane monoesters	13.8	10.8	0.0	0.0

Table 8.6 NMR analysis of biolubricants produced at 24 h via esterification of neopentyl glycol (NPG) and hydrolysed microbial oils of *R. toruloides* and *C. curvatus*

Molecules	***R. toruloides***		***C. curvatus***	
	Molar (%)	**Weight (%)**	**Molar (%)**	**Weight (%)**
Fatty acids	10.4	7.8	15.3	8.3
Neopentyl glycol	11.7	3.3	0.3	0.1
Neopentyl glycol diesters	44.2	53.6	64.8	78.0
Neopentyl glycol monoesters	33.7	35.3	19.6	13.6

8.5.2 Physicochemical characterization of biolubricants

The highest conversion yields were obtained using NPG when HMO from either *C. curvatus* or *R. toruloides* was employed. For this reason, the NPG esters were selected for the evaluation of their lubricant properties via physical and chemical characterization.

DSC analysis was employed in order to determine the melting points of the products. The thermogram of NPG - *R. toruloides* esters showed the main peak at -10.47 ºC (Figure 8.17a), but other smaller peaks appear around -23 ºC and -2 ºC. These peaks may represent a mixture of esters including mono- and di-esters (Zheng et al., 2011). Considering the lower melting point of unsaturated esters (Knothe and Dunn, 2009), it could be indicated that most of the peaks towards to the left of Figure 8.17a correspond to these esters. Other peaks representing melting temperatures over 0 ºC also appeared and these may be attributed mostly to saturated esters. The thermogram of NPG - *C. curvatus* esters also presented several peaks with melting temperature lower than zero with the main peak appearing at -8 ºC (Figure 8.17b). The melting temperatures observed for this product are slightly higher than those observed in the case of NPG - *R. toruloides* esters. Both microbial oils mainly contain

palmitic acid and oleic acid. Thus, the melting temperatures of the polyol esters are similar to those reported for esters derived from these fatty acids (Knothe and Dunn, 2009).

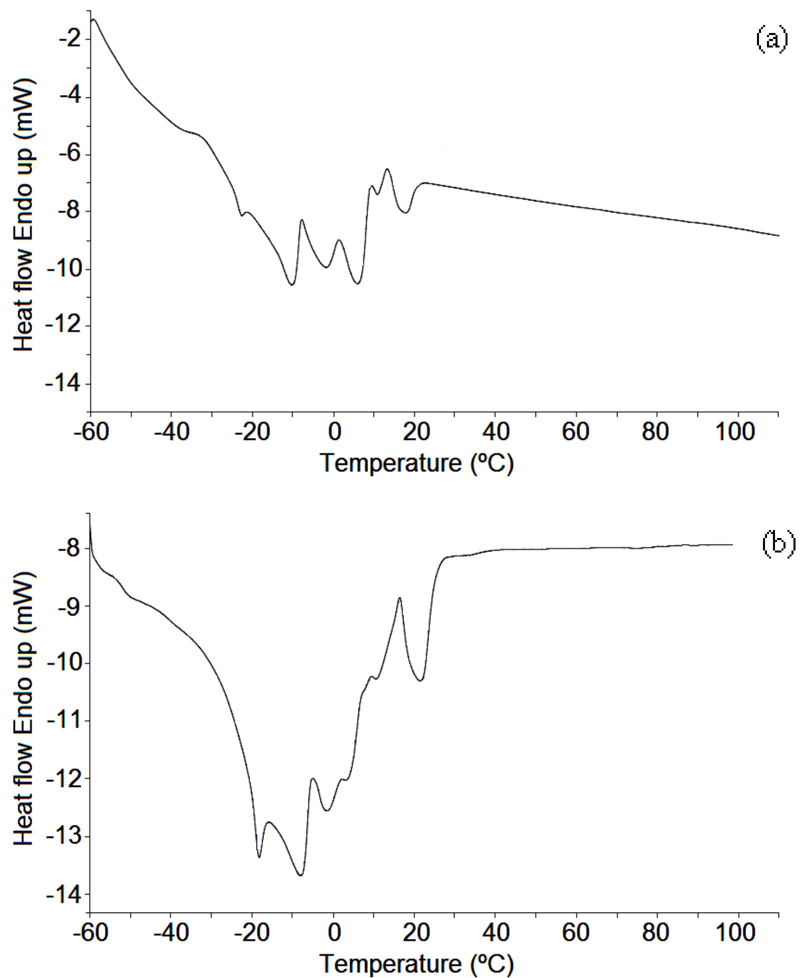

Figure 8.17 Differential scanning calorimetry (DSC) analysis for neopentyl glycol (NPG) esters produced from the hydrolysed microbial oil of (a) *R. toruloides* and (b) *C. curvatus*

The viscosity, pour point and oxidative stability were also determined (Table 8.7). The viscosity or viscosity index plays important role in lubrication, optimizing the working efficiency of the machinery and also preventing rubbing and collision between the components (Ting and Chen, 2011). Biolubricants do not have a specific pre-defined minimum value of viscosity, and specific applications can be chosen based on the lubricants classification according to their ISO grades (Bock, 2007). Lubricants in current commercial use reach a range of ISO from lower than 10 to higher than 1000 based on viscosity values (mm/s^2) at 40 ºC. Both NPG esters of *R. toruloides* (22.33 mm/s^2) and *C. curvatus* (23.84 mm/s^2) showed similar viscosity values at 40 ºC, which classifies them to the ISO VG 22

(19.8 – 24.2 mm/s^2). This ISO grade allows the utilization of these esters as chainsaw lubricant, total loss lubricant, mold release fluid for concrete and hydraulic fluid (VanVoorst and Alam, 2000). Cavalcanti et al. (2018) reported that the NPG esters derived from soybean oil was also classified to VG 22. The NPG esters of *R. toruloides* and *C. curvatus* oils had similar viscosity index (181-183). The viscosity index of the NPG esters produced via enzymatic esterification of NPG with free fatty acids derived from soybean oil was 214 (Cavalcanti et al., 2018).

Table 8.7 Physical and chemical characterization of neopentyl glycol (NPG) esters produced using the hydrolyzed microbial oils of *R. toruloides* and *C. curvatus*

Properties	***R. toruloides* esters**	***C. curvatus* esters**
Viscosity 40 ºC (mm^2/s)	22.33	23.84
Viscosity 100 ºC (mm^2/s)	5.26	5.54
Viscosity index	181.0	183
Acidity index (mg KOH/g)	10.36	12.13
Pour Point (ºC)	3.0	2
Cloud Point (ºC)	4.0	6
Oxidative stability (h)	3.29	4.18

The cold flow temperature properties of the NPG-based esters were determined by the cloud point and pour point analysis. The pour point indicates the lowest temperature at which the lubricant flows and in practice this is the lowest temperature at which the biolubricant is pumpable (Mobarak et al. 2014). The cloud point is the temperature at which dissolved solids, like wax crystals, start to solidify giving a cloudy appearance. The determination of cloud point is essential for cold temperature applications, since the presence of solidified waxes cause thickening of the biolubricants. Furthermore, the operation temperature should be kept above the cloud point in order to prevent clogging of filters (Mobarak et al. 2014).

The pour point of the NPG esters (2 – 3 °C) derived from both HMO used in this study (Table 8.7) is considered high for lubrication at low operating temperatures. However, at this pour point, the biolubricants could be used for lubrication of chainsaw and also as dust-suppressant fluids in countries or seasons where the temperature does not drop below 5 °C.

Another possibility is the utilization of biodegradable additives, which can decrease the pour point to more desirable temperatures (Asadauskas and Erhan 1999; Macpherson 1997). Pour point depressants can improve the biolubricant performance at low temperatures even when they are used in very low concentrations. Soybean oil-based lubricants produced using different alcohols presented variable pour points according to their structure, but all of them had their pour point temperatures decreased after the addition of 1% pour point depressants (v/v), achieving reduction up to 39 ºC (-6 ºC before and -45 ºC after pour point depressant addition) (Hwang and Erhan, 2001).

The cloud point of NPG esters produced from HMO of *R. toruloides* was 4 ºC, while the cloud point of NPG esters from HMO of *C. curvatus* was 6 ºC (Table 8.7). The utilization of depressants can also decrease the cloud point of the products and extend their range of applications (Ming et al., 2005). The biolubricants produced from canola oil presented lower cloud point (-3 ºC) and pour point (-9 ºC) than the biolubricants produced in this study (Sharma et al., 2015). Generally, the poor cold flow properties of the biolubricants produced in this study should be improved in order to facilitate their applications in cold weather conditions. Besides addition of depressants, the NPG-HMO based esters could also be used in different blends with other esters in order to optimize their lubrication properties and applications.

The determination of oxidation stability is critical for the combustion process of engines (Sharma et al., 2015). Oxidation leads to an increase in the viscosity of lubricant and deposits varnish and sludge decreasing the lubricant lifetime. Thus, the oxidative stability of the product is one of the properties that decides the quality of the product and potential applications. The evaluation of oxidative stability showed that the induction times for NPG esters derived from HMO of *R. toruloides* was 3.29 h, while the oxidative stability of the NPG esters derived from the HMO of *C. curvatus* was 4.18 h. Sripada et al. (2013) reported oxidative induction times at 110^0 C for TMP esters produced with either methyl oleate (2.08 h) or canola biodiesel (0.74 h) using sodium methoxide as catalyst (Sripada et al., 2013). TMP esters derived from cooking oil had an induction time of 3.5 h at 120 ºC, which was decreased to 1.7 h when the temperature was increased to 130 ºC (Wang et al., 2014). The oxidative stability is related to the fatty acid composition of the oil used (Singh et al., 2014). For instance, the oxidative induction time of NPG esters derived from palm fatty acid distillate was found >69 h (Fernandes et al., 2018), due to the high saturation degree (48.7%) of the raw material as compared to the microbial oils used in this study the saturation degree of

which is up to 37.6%. The oxidative stability of the products can be improved by using additives, such as the commercially available antioxidant MP90, which significantly improved the induction time of TMP esters from cooking oil to 15.8 h at 130 °C (Wang et al., 2014).

Chapter 9

Development of bio-based oleogels as novel food formulations

9 Development of bio-based oleogels as novel food formulation

9.1 Introduction

The most common modifications of vegetable oils to achieve the required properties for food applications are hydrogenation or partial hydrogenation. However, these processes lead to the production of modified oils rich in saturated and *trans* fatty acids, which have deleterious effect on human health.The increased consumption ofsaturated fatty acidsislinked tocoronary heart diseases and cancer. Although *trans* and *cis* fatty acids have the same molecular composition and their absorption is similar, the *trans* isomers are metabolized differently and contribute to higher cholesterol (Wang, 2011; Hartel et al., 2018). In 2015, the FDA stripped of the GRAS (Generally Recognized as Safe) character of *trans* fatty acids, which led R&D activities towards the development of alternative ways of structuring edible oils (Food and Drug Administration, 2015; O'Sullivan et al., 2016). Oleogelation is a promising technology for edible formulations (O'Sullivan et al., 2016).

Oleogel technology has great potential for application in food products, such as margarines, dough products, chocolate spreads, sausages, cheese products, cookies, cakes, fillings for bisquits, muffins and ice creams (Fayaz et al., 2017; Mert and Demirkesen, 2016; Moriano and Alamprese, 2017; Oh et al., 2018; Panagiotopoulou et al., 2016; Zulim Botega et al., 2013). Oil-based food products require the crystalline triacylglycerol network resulting from oleogelation as an ingredient to achieve the desired texture (Hwang et al., 2015). Among all these application, the production of margarines has great technological and economic importance and it is believed that would be the major food product, along with spradable fats, where oleogels will be applied (da Silva et al., 2018; Hwang et al., 2015). In the case of margarines and spreads, the oil phase from the oleogel will consist of a small amount of an oleogelator and a healthy vegetable oil (Hwang et al., 2015). The extra virgin olive oil was selected for oleogel preparation, as it is distinguished for its health benefits and sensory properties. Moreover, soybean oil has attracted the interest for the production of oleogels due to its economic importance, availability, low cost and the rich composition in polyunsaturated fatty acids (Chaves et al., 2018). The soybean-based oleogels have been applied as deep-fat frying medium for fried noodles resulting in lower oxidation and oil uptake by the fried noodles (Lim et al., 2017).

The objective of the present study was the development of a novel biorefinery concept based on the valorisation of side-streams, derived from the processing of sugarcane and oilseeds, for the production of oleogels with desirable properties for food applications.

Molasses and soybean cake, which is generated after oil extraction from soybeans, were utilised as carbon and nitrogen sources, respectively, for the production of microbial oil rich in carotenoids by *R. toruloides*. The soybean fatty acid distillate (SFAD), which is a by-product derived from the soybean oil refining process, and the produced microbial oil were enzymatically converted to cetyl wax esters (section 8.4). According to the U.S.A. Food and Drug Administration, cetyl alcohol has been included in the list of food additives and can be safely used for the synthesis of food components when the purity is higher than 98% (CRF, 21CFR172.864). The aim was to develop five different types of oleogels using: a) olive oil and SFAD-wax esters, b) soybean oil and SFAD-wax esters, c) olive oil and microbial oil-wax esters, d) soybean oil and microbial oil-wax esters and e) microbial oil and microbial oil-wax esters. Their properties concerning thermal behaviour, crystal morphology, color, rheology and texture were also evaluated.

9.2 Fatty acid composition of the base oils used for oleogels production

The fatty acid composition of the olive oil, soybean oil and microbial oil used for the preparation of oleogels was analysed by GC and the results are presented in Table 9.1. The microbial oil used in this chapter was recovered from the fed-batch fermentations presented in Chapter 7 using molasses and soybean cake hydrolysate as fermentation feedstocks. Olive oil and microbial oil contained mainly oleic acid in different proportions and around 13.0% palmitic acid, whereas soybean oil contained mainly 54.8% of linoleic acid and 32.5% of palmitic acid. The saturation degree was found higher in olive oil and microbial oil and it was ranged from 29.6% to 36.8%. The soybean oil presented the lower saturation degree of fatty acids (14.7%) due to its abundance in polyunsaturated fatty acids.

Table 9.1 Fatty acid composition of olive oil, soybean oil and microbial oil used for oleogels production

Oils	Fatty acids (%)					Saturation degree
	C16:0	C18:0	C18:1	C18:2	Others	
Extra virgin olive oil	13.0	-	79.7	1.7	5.6	13.0
Soybean oil	11.3	3.4	23.1	55.8	6.4	14.7
Microbial oil	32.5	4.3	54.8	5.6	2.8	36.8

9.3 Crystal morphology

The phenomenon of oleogelation was confirmed by the stability of the mixtures with a turnover at room temperature after 24 h of sample preparation. Representative visual appearance of the olive oil and soybean oil with MO-wax is presented in Figure 9.1. The concentrations of7% and 10% of microbial oil and SFAD derived wax esters were insufficient to entrap the oil phase, whereas 20% was found effective in building a network as the oleogel exhibited non-flowing gel state. The oleogels with 20% wax ester concentration were selected for further evaluation of the properties.

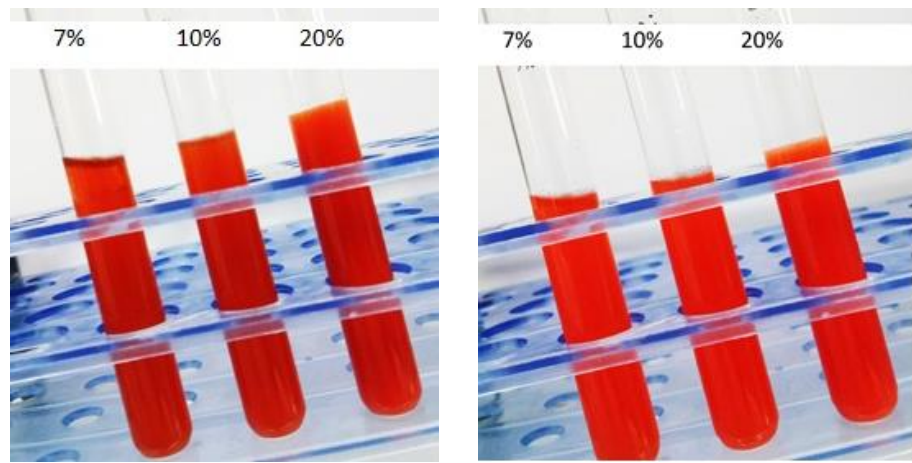

Figure 9.1 Visual appearance of the oleogelation phenomenon prepared from olive oil (left) and soybean oil (right) with microbial oil – wax esters at various concentrations (7%, 10% and 20%, w/w)

Polarized light microscopy was employed for the observation of crystal morphology of the produced cetyl wax esters and the oleogels. As demonstrated in Figures 9.2 and 9.3, the morphology was resembled to flaked-like or plateled crystals. Some crystals appeared like needles in the case of olive oil and soybean oil MO-wax oleogels, but this probably happens when its orientation is vertical to the plane of the microscopic glass slide (Hwang et al., 2015). The crystal size varied between different wax esters and oleogels. In the case of MO-wax esters the majority of the crystals were up to 100 μm (Figure 9.3), while the use of SFAD-wax esters produced crystals with length of more than 100 μm (Figure 9.2). The SFAD-wax esters and the oleogels derived from the use of SFAD-wax esters presented similar morphology, which consisted of large and dense crystals. Different trend was observed in the case of MO-wax esters derived oleogels. Although the olive oil-MO wax

esters and soybean oil-MO wax esters oleogels presented similar microstructure, which is near to the crystal morphology of the MO wax esters (Figures 9.3a,b,c), the microbial oil-MO wax esters oleogel presented different and less dense structure (Figure 9.3d).

Until now only natural wax esters have been evaluated as oleogelators in wax-based oleogels. The most common shape of crystals observed under polarized light microscopy in oleogels prepared with natural wax esters, such as beeswax, is the needle-like crystals (Moghtadaei et al., 2018). Polarized light microscopy has been widely used to study the microstructure of wax-based oleogels, however it has been reported that this method could not provide accurate observations. Indeed, the use of cryogenic scanning electron microscopy and phase contrast microscopy has proved that the needle-like morphology observed in polarized light microscopy was eventually platelet-like (Blake and Marangoni, 2015; Hwang et al., 2015).

The crystal morphology is affected by several factors including the cooling rate and the final cooling temperature of the oleogels (Moghtadaei et al., 2018; Si et al., 2016). Moghtadaei et al. (2018) reported that the oleogel network consisted of clustered mass with large pores when cooling was carried out at room temperature (25 °C). Generally, a faster cooling rate produces denser oleogel networks. However, the most practical cooling rates for the production of margarines and spreadable fats are 3 °C/min, 5 °C/min and 10 °C/min (Hwang et al., 2015).

Moreover, the size and shape of the crystals are influenced by the fatty acid composition, fatty acid distribution and the polymorphic form of the crystals (Deman and Beers, 1987). For instance, the predominance of C16:0 fatty acid at the *sn*-2 position in lard leads to a β crystal form (Podmore, 1987). The β form is considered the most stable crystal type, characterized by platelet-shaped crystals, forming large clumps (> 1 mm diameter) which are responsible for the grainy fat appearance of the products, resulting to the separation of the oil proportion. On the other hand, the β'form is desirable for bakery products, margarines and shortenings because the formation of small needle-shaped (up to 1μm) crystals provides better texture and properties, due to the tendency to entrap large quantities of liquid oil (Lee et al., 2008; O'Brien, 2008).

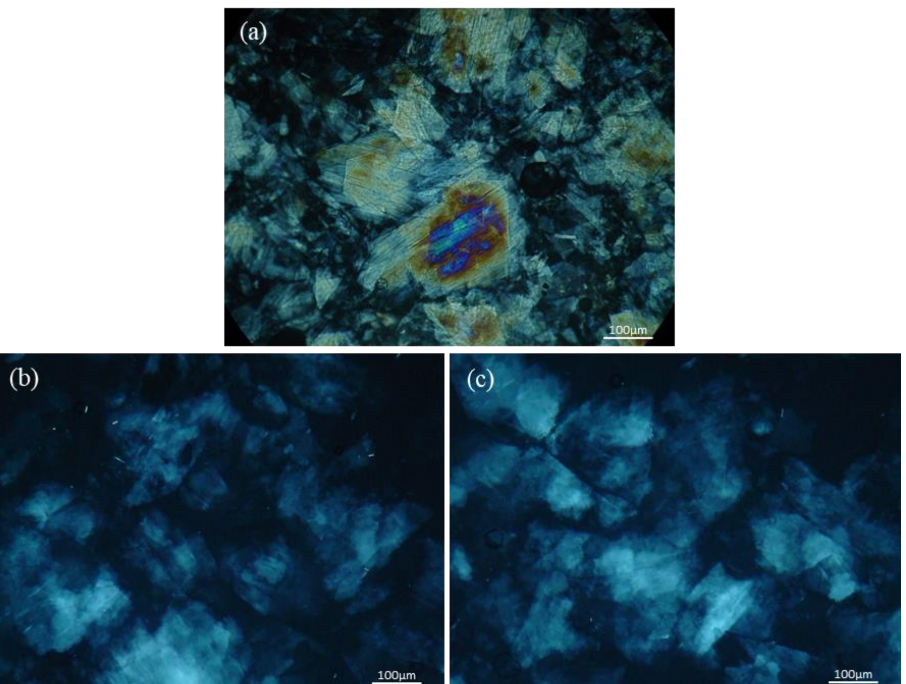

Figure 9.2 Micrographs of crystal formation of (a) SFAD-waxesters, (b) oleogel of olive oil with 20% SFAD-waxesters, and (c) oleogel of soybean oil with 20% SFAD-waxesters, using polarized light microscopy

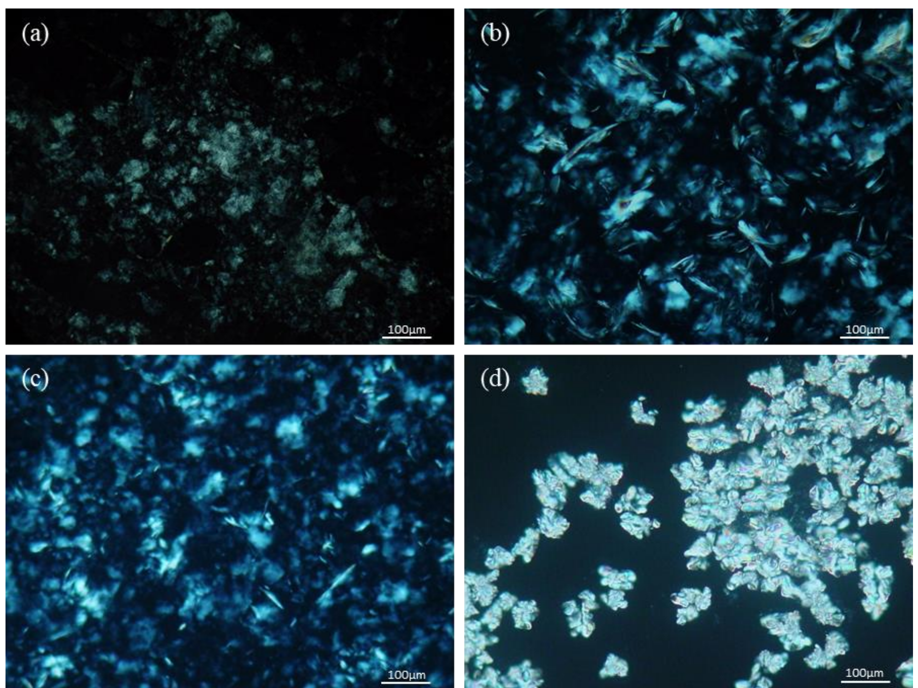

Figure 9.3 Micrographs of crystal formation using polarized light microscopy of the (a) MO-waxesters, (b) oleogel of olive oil with 20% MO-waxesters, (c) oleogel of soybean oil with 20% MO-waxesters and (d) oleogel of microbial oil with 20% MO-waxesters

In this study, olive oil and soybean oil were utilized for the preparation of oleogels, which have been characterized by the *β*crystal form (Boskou, 2011; O'Brien, 2008; Wang, 2011). Furthermore, it would be interesting to study the microbial oil in order to have an overview of the types of crystals forms. The differences between olive oil, soybean oil and microbial oil-based oleogels may be attributed to the diverse fatty acids content, as the crystal behavior is characterized by the degree of saturation and the fatty acid content, the distribution and position of C16:0 on the triglyceride molecule. Soybean oil contains mainly C18:2 fatty acids, whereas olive oil and microbial oil contain C18:1 and C16:0 fatty acids. O'Brien (2008) reported that fats consisting of heterogeneous mixture of triglycerides tend to remain at lower melting crystal form. Another parameter that should be taken into consideration is the phenomenon of polymorphism which could lead to the transformation to the *β* form during processing, especially when the used fat or oil contains homogeneous mixture of triglycerides (O'Brien 2008).

9.4 Color analysis

The color parameters of the oleogel were determined as depicted in Table 9.2. The *L** value express the luminosity of the oleogels, which was lower in oleogels prepared with MO-wax esters as compared to oleogels with SFDA-wax esters. Moreover, *a** measurements were higher in oleogels prepared with MO-wax esters. It is evident in Figure 9.1 that the carotenoids contained in the microbial oil derived wax esters provide a reddish-orange color to oleogels made with MO-wax estersand this results to lower *L** and higher *α** values than the values of SFAD-wax ester based oleogels. The *b**values varied between the different oleogels. The higher values were presented by olive oil and soybean oil derived oleogels due to the higher intensity of yellow color.

Öğütcü and Yılmaz (2014) studied the color of oleogels prepared by extra virgin olive oil and carnauba wax and found that *L** (ranged from 42 to 54), *α** (ranged from -5.6 to -6.4) and *b** values (ranged from 16 to 25) were influenced by the concentrations (3-10%) of the oleogelator used. In the case of SFAD-wax ester oleogels, the results are similar to the study presented by Öğütcü and Yılmaz (2014). However, plant waxes contain natural pigments which contribute to higher *L**and *b**values. The MO-wax ester oleogels presented differences from the literature-cited findings due to the presence of carotenoids in microbial oil produced by the red yeast *Rhodosporidium toruloides* that was used for the production of microbial oil derived wax esters.

Table 9.2 Color analysis of the different oleogels produced from olive oil, soybean oil and microbial oil using SFAD-wax esters and MO-wax esters as oleogelators

Oleogel	*L**	*a**	*b**
Olive oil-SFAD wax	25.7 ± 0.0	2.9 ± 0.1	15.4 ± 1.1
Soybean oil-SFAD wax	27.1 ± 0.1	0.1 ± 0.0	15.5 ± 0.0
Olive oil-MO wax	11.3± 0.5	16.3 ± 0.6	8.5 ± 0.2
Soybean oil-MO wax	11.7 ± 0.9	20.1 ± 1.2	12.0 ± 1.4
Microbial oil-MO wax	11.5 ± 0.1	20.3 ± 0.1	3.3 ± 0.2

Generally, the color of any oleogel can be adjusted by adding oil soluble food pigments (Öğütcü and Yılmaz, 2014). However, in this study the development of carotenoid-rich oleogels may lead to the production of functional food products, due to several health benefits attributed to carotenoids. O′Sullivan et al. (2016) reported that oleogels could be used as novel delivery systems of such bioactive molecules, since they assist in the bioavailability of carotenoids when oleogels contain long chain triglycerides.

9.5 Thermal analysis

DSC analysis was employed for the determination of melting temperatures of the oleogels, since it is an important property for fat products due to the taste effect during consumption (Hartel et al., 2018; Öğütcü and Yılmaz, 2014). The results presented in Table 9.3 show that the melting temperatures T_{on} were 21.1 – 24.3 °C, T_p were 28.5 – 32.3 °C and T_{com} were 29.8 – 35.3 °C for all oleogels.

The melting point of the oleogelator affects significantly the melting temperature of the oleogel. The oleogels prepared by extra virgin olive oil and 10% of carnauba wax had a T_{on} of 63 °C and a T_p of 76 °C (Öğütcü and Yılmaz 2014). The $T_p$melting points of the extra virgin olive oil and carnauba wax were -17.19 °C and around 80 - 87 °C, respectively (Öğütcü and Yılmaz, 2014; Sharma, 1991). In the case of the conventional manufacture of margarines the rapid melting characteristic is attained during hydrogenation (Carr and Vaisey-Genser, 2003). The hydrogenation process of oils targets on higher melting point values, as a result of non-intersoluble triglycerides derived from the formation of *trans* and positional isomers (Nassu and Gonçalves, 1999). The melting properties are essential for the production of fat-based

food products and differ for each application. The main aim is to produce a product with a melting temperature close to that of the human body, which constitutes a critical parameter for the consumer. A melting point up to 35 °C is desirable for fats used in confectionery products, whereas higher temperatures leadto a waxy mouthfeel with reduced flavor notes during consumption (Hartel et al., 2018).

Table 9.3 Melting temperatures of oleogels as determined by differential scanning calorimetry (DSC)

Oleogel	T_{onset}[a] (°C)	T_{peak}[b] (°C)	T_{com}[c] (°C)
Olive oil-SFAD wax	23.2	28.5	29.8
Soybean oil-SFAD wax	24.8	30.1	32.8
Olive oil-MO wax	21.1	29.9	32.3
Soybean oil-MO wax	24.3	32.3	35.3
Microbial oil-MO wax	23.9	28.5	33.5

[a] T_{onset}: onset melting temperature; [b] T_{peak}: maximum peak temperature; [c] T_{com}: completion of melting

9.6 Rheological analysis

The results concerning the viscosity of oleogels are shown in Figures 9.4 and 9.5. The viscosity declined sharply with increasing temperature. The change of the curve slope occurred due to the shift from the solid-state to a viscous liquid-state of the fat crystal network, which occurred under the deforming stress. These points were found around 32 - 35 °C for all oleogels, however the curve slope was different among them. The lower slope was determined in microbial-MO wax ester oleogel (Figure 9.5).

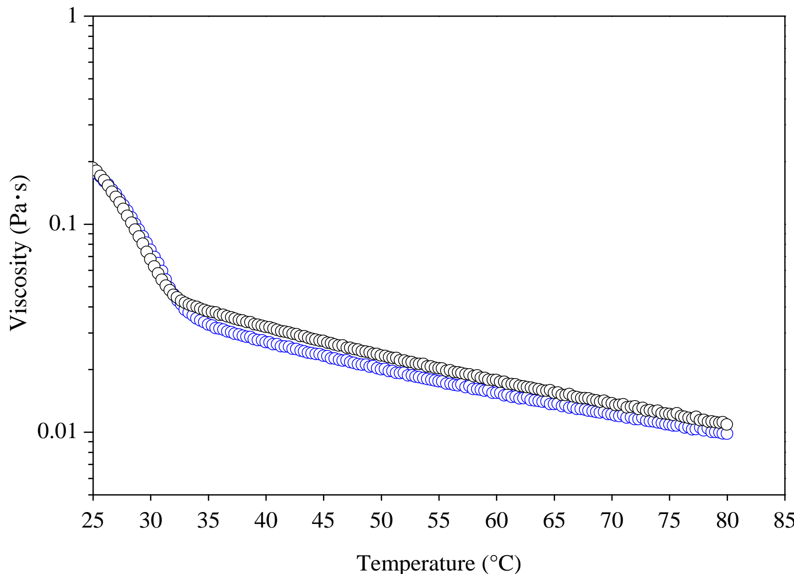

Figure 9.4 Profile change of viscosity versus temperature of the oleogels: olive oil with 20% SFAD-wax esters (○) and soybean oil with 20% SFAD-waxesters (○)

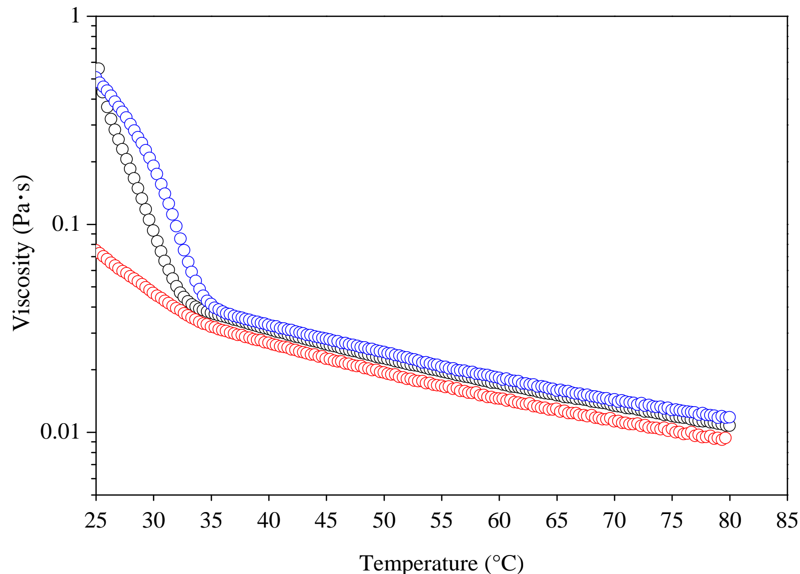

Figure 9.5 Profile change of viscosity versus temperature of the oleogels: olive oil with 20% MO-waxesters (○), soybean oil with 20% MO-waxesters (○) and microbial oil with 20% MO-waxesters (○)

Critical rheological parameters are also G', G'' and tan δ,as they represent the viscoelastic properties of a product (Si et al., 2016). The storage modulus (G') and loss modulus (G'') versus temperature are shown in Figures 9.6 and 9.7. The initial G' values at

room temperature were around 10^3 for SFAD-wax esters derived oleogels, 10^4 for MO-wax esters derived oleogels with olive oil and soybean oil and 10 for microbial oil with MO wax esters oleogel. In all cases, the *G′*was higher than the *G″*at a specific temperature range, which indicates that the produced oleogel presented more solid-likeproperties at this temperature range, since *G′* represents the solid (elastic) behavior of a material (Lee et al., 2008). Subsequently, as the temperature was increased the *G′* value was decreased exhibiting a declined slope, as a result of the crystalline fats melting (Lee et al., 2008). The cross over points at which the *G′* = *G″*, should be close to the T_{com} melting values of each oleogel as demonstrated by DSC. The higher cross over point of *G′* and *G″* was determined for soybean oil-SFAD wax esters oleogel (35 °C) and the lower for the olive oil-SFAD wax esters and microbial oil-MO wax esters oleogels, which was around 28 – 29 °C. The cross over points were found also close to the point of the slope change of the viscosity curve (Figures 9.4 and 9.5) at which a crystal network change occurs. The calculation of tan δ =*G″/G′* determines the predominance of the elastic (solid, tanδ < 1) or viscous (liquid, tanδ > 1) character of a sample (Phuah et al. 2016), whereas the sample becomes a true gel when the tan δ value is lower than 0.1. The tan δ values ranged from 0.38 to 0.73 at 25 °C suggesting that the oleogels behave more like a solid. Si et al. (2016) reported that the low value oftan δ of oleogels indicates astable three-dimensional network structure. The results also showed that the elastic and viscous character of the sample is in balance (tan δ= 1) at a narrow temperature range (32.9 - 34 °C) only in the case of olive oil-MO wax esters oleogel, as the other oleogels presented wider temperature range.

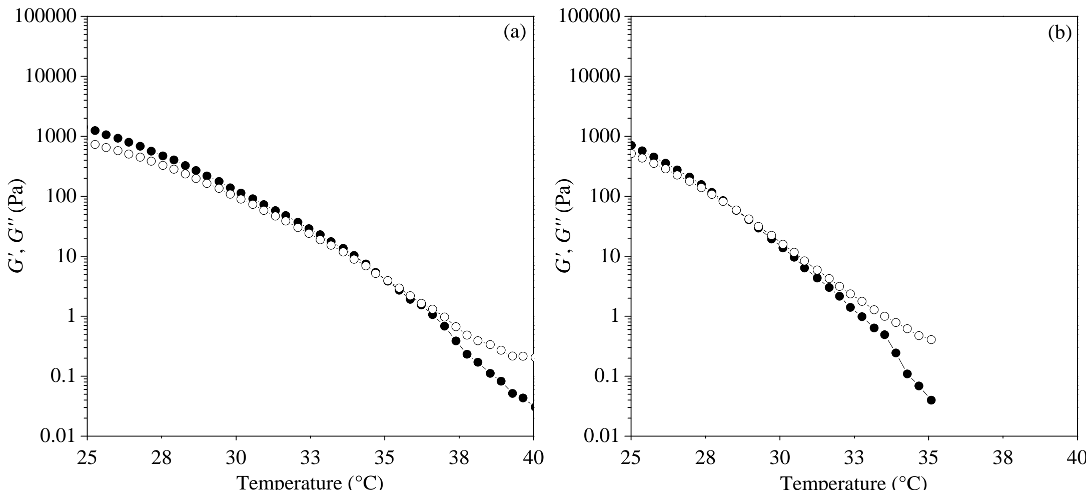

Figure 9.6 Storage modulus (*G′*) (●) and loss modulus (*G″*) (○) versus temperature of the oleogels: (a) soybean oil with 20% SFAD-waxesters and (b) olive oil with 20% SFAD-waxesters

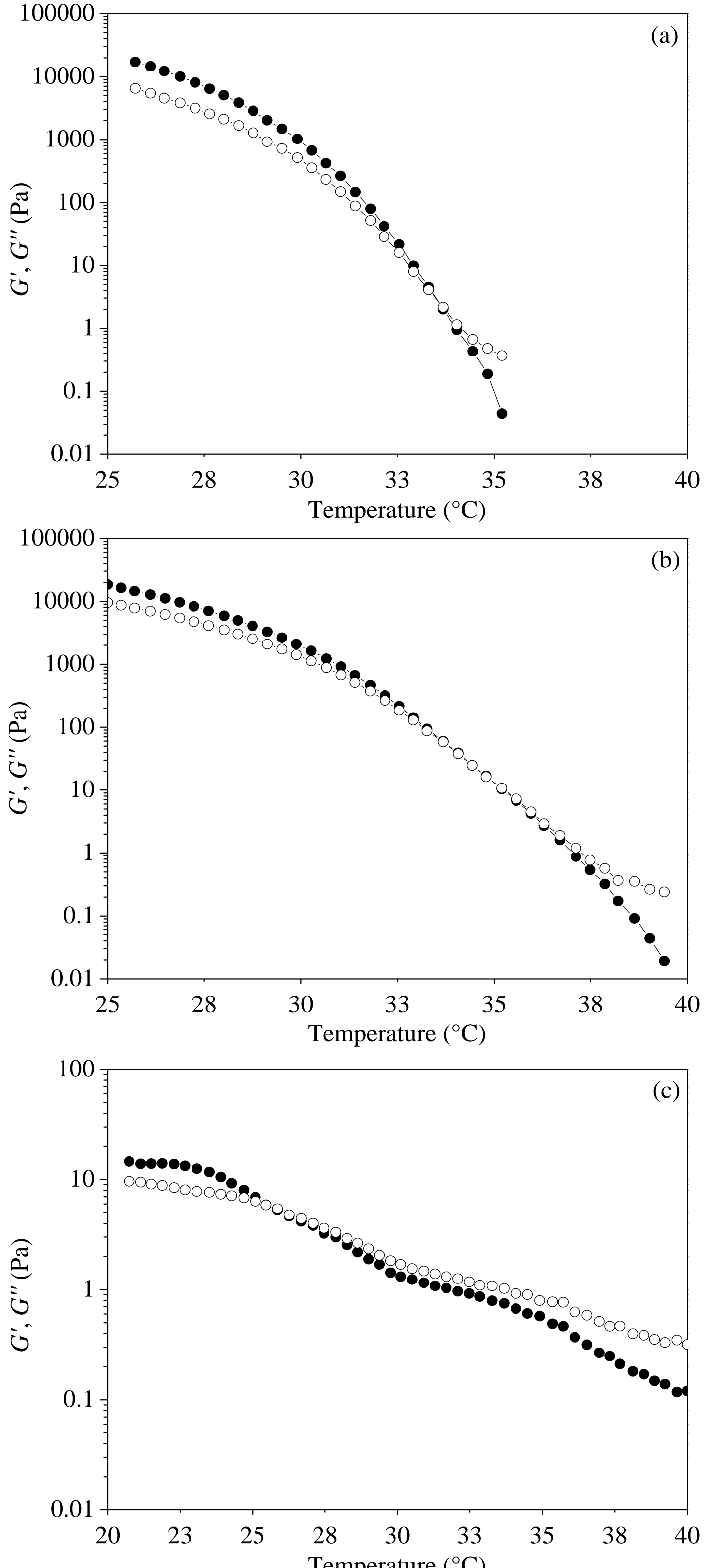

Figure 9.7 Profile change of storage modulus (G') (●) and loss modulus (G'') (○) versus temperature of the oleogels: (a) olive oil with 20% MO-waxesters, (b) soybean oil with 20% MO-waxesters and (c) microbial oil with 20% MO-waxesters

9.7 Texture analysis

The determination of the firmness is essential for the evaluation of the spreadability of the oleogels, thus providing information about their potential utilisation in margarines and spread fat products (Hwang et al., 2018b). The firmness of the oleogel was measured after 24 h at 4 °C and the results showed thatmeasurements were different among the oleogels. At the first day, the higher firmness was determined for microbial oil-MO wax esters (14.5 N) and the lowest one for the soybean oil-MO wax esters (2.9 N) (Figure 9.8). During storage, only the microbial oil-MO and soybean oil-MO wax esters oleogels were stable. However, the later oleogel had very low firmness (1.8 - 2.2 N). In the case of olive oil-MO wax and soybean oil-SFAD wax oleogels, the firmness was decreased by 60% and 82%, respectively, until the 20th day and then was kept constant until the 30th day.

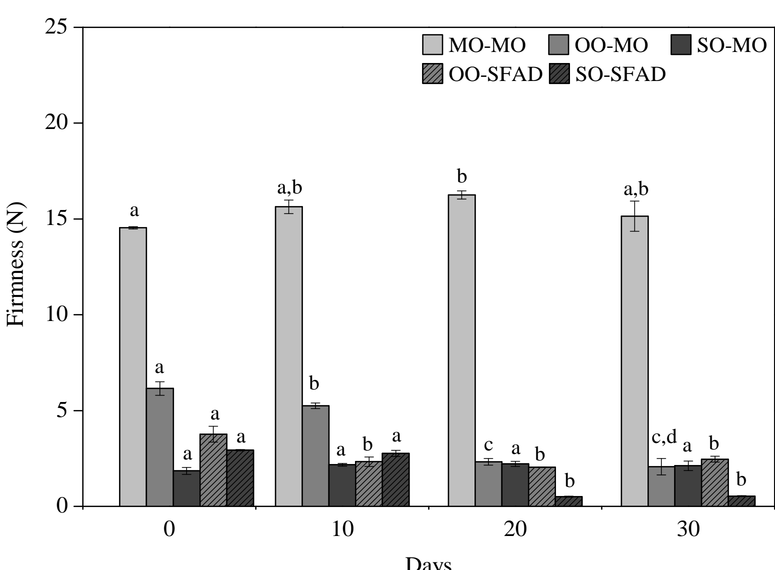

Figure 9.8 Textural properties of microbial oil with 20% MO-wax esters (MO-MO), olive oil with 20% MO-waxesters (OO-MO), soybean oil with 20% MO-wax esters (SO-MO), olive oil with 20% SFAD-wax esters (OO-SFAD) and soybean oil with 20% SFAD-wax esters (SO-SFAD) versus storage time (1 – 30 days) at 4 °C. Different letters on the bars indicate significant differences between the different days for each oleogel ($p < 0.05$)

The firmness of olive oil-based oleogels with 10% carnauba wax ranged from 5.4 N (0 day) to 7 N (30 day) at 4 °C (Öğütcü and Yılmaz, 2014). The authors mentioned that a moderate firmness is desired for spreadable fat products, such as breakfast margarine which had a firmness of 3.9 N (4 °C, 90 days) Öğütcü and Yılmaz (2014). Therefore, the produced

oleogels, except for soybean oil-MO wax esters, had sufficient levels of firmness to be applied in spreadableproducts.

Generally, the stable textural values of oleogels upon storage time is desired. In this study, the decreased firmness may be attributed to the formation of large crystals as indicated by polarized light microscopy, especially for the SFAD-wax derived oleogels. O′Brien (2008) reported that the proper distribution of the solid phase, the size and shape of the crystals are important parameters for the consistency of a plastic fat product. The low firmness demonstrates a weak internal strength between the solid particles of the fats, as a result of the few contact points between the large crystal particles (O′Brien 2008). Moreover, the presence of polar compounds in oils, such as phytosterols, phospholipids, unsaponifiable matters, hydrocarbons, free fatty acids, trace metals and tocopherols, may interfere with the formation of solid fats and thus reduce the firmness of the oleogel (Hwang et al., 2018b). It should be also stressed that the decreased firmness during storage time may not be related to crystal form transitions, considering the fact that any polymorphs transformation could not take place due to the high viscosity of the product at low storage temperatures (McGauley and Marangoni, 2002). However, the firmness of the oleogel could be enhanced by applying faster cooling rates during the preparation of the oleogel, which induces the formation of small crystals and results to a denser fat crystal network (Hwang et al., 2015; Mattice and Marangoni, 2017), increasing the proportion of solid phase of the oleogel(O′Brien, 2008), or even utilizing combined high and low melting wax esters (Tavernier et al. 2017).

Chapter 10

Conclusions and future perspectives

10 Conclusions and future perspectives

The renewable materials generated via the industrial process of cane sugar and vegetable oil production was reviewed in Chapter 2, with particular focus on their current utilisation and the potential development of biorefinery concepts. The biotechnological production of value-added products, such as fumaric acid, microbial oil and carotenoids, was also described highlighting the renewable resources that have been used until now for the production of these bio-based products. The importance of oleochemicals production within the bio-economy framework was also addressed focusing on the current production of wax esters and polyol esters. Finally, published research on the production of oleogels as novel food formulation was described highlighting the necessity of healthier food products manufacture using renewable resources.

Initially, an enzymatic hydrolysis process of soybean cake was developed for the production of a nutrient rich feedstock appropriate for microbial fermentations (Chapter 5). Soybean cake was efficiently hydrolysed by crude enzymes produced by fungal strains producing 0.99 g/L FAN and 200 mg/L IP. The results showed that a temperature of 45 °C and 6.7 U/mL of initial protease activity were the optimum hydrolysis conditions.

In Chapter 6, the production of fumaric acid by *R. arrhizus* was carried out through fermentations using VHP sugar, molasses and soybean cake hydrolysate as feedstock. The nutrient-rich hydrolysate produced via enzymatic hydrolysis of soybean cake using crude enzymes produced by *A. oryzae* led to the highest fumaric acid production (40 g/L), when VHP sugar was utilized as carbon source. The significantly lower fumaric acid concentration and yield observed when untreated or pretreated molasses was used as fermentation feedstock may be attributed to its composition that influences the metabolism of *R. arrhizus*. Molasses-based fermentation media contained higher quantities of various metal ions, compared to VHP sugar media. Moreover, the presence of phenolic compounds in molasses may have led to lower fumaric acid concentrations.

The microbial oil and carotenoids production from the oleaginous yeast *R. toruloides* was studied in Chapter 7. Microbial oil was successfully produced in shake flask fermentations using VHP cane sugar and molasses, as the sole carbon sources. The evaluation of nutrient supplements showed that microbial oil production was favoured when trace elements, phosphate salts and commercial nitrogen sources were added in the fermentation medium. The utilisation of molasses and soybean cake hydrolysate as fermentation feedstock

led to the highest microbial oil (18.4 g/L) concentration, intracellular microbial oil content (49.8%, w/w) and carotenoid content (89.4 μg/g) in bioreactor fed-batch fermentations.

The development of efficient enzymatic synthesis of wax esters and polyol esters using a lipase catalysed solvent-free system was demonstrated in Chapter 8. The production of wax esters was performed using behenyl, cetyl and oleyl fatty alcohols and fatty acid distillates and microbial oils as raw materials. In the case of fatty acid distillates, the highest conversion yield of 80% was attained at 50 °C using 8.8 U/g of a crude biocatalyst immobilised on agro-industrial residues. The utilisation of microbial oils led to a highest conversion yield of 93.9% for oleyl esters and 91.3% for cetyl esters at 50 °C using a non-commercial lipase. The properties of the produced wax esters were found similar to natural waxes indicating their potential use in food applications. The enzymatic synthesis of polyol esters using neopentyl glycol and microbial oil resulted up to 88% conversion yield. Theanalysis of the physicochemical properties showed that the produced esters could be a potential substitute of conventional lubricants.

In Chapter 9, the produced bio-based wax esters, derived from microbial oil and soybean fatty acid distillate, were evaluated as potential oleogelator agents for oleogels production. Different oleogels were produced using extra virgin olive oil, soybean oil and microbial oil as base oils. The analysis of the physical properties demonstrated that all oleogels presented suitable melting temperatures for food applications. The olive oil and soybean oil derived oleogels prepared with microbial wax esters presented good rheological profiles. However, the more stable texture was determined for oleogel produced from microbial oil and microbial oil wax esters. The results indicated that further optimisation of the oleogelation process could lead to the production of bio-based oleogels, which meet the criteria of the circular economy.

This PhD thesis presented a novel integrated biorefinery concept using various renewable resources and novel processes for the production of value-added bio-based products which can be applied in the food industry. Future studies could focus on the following topics:

- The inhibition effects of phenolic compounds and metal ions that are present in molasses on the metabolism of *R. arrhizus* should be studied further in order to optimise fumaric acid production in bioreactor cultures
- Optimisation of fermentation conditions in order to maximize microbial oil and carotenoids production

- Assessment and optimization of microbial oil composition aiming to the production of polyol esters with improved biolubricant properties
- Optimisation of the oleogelation process concerning the cooling ratio temperature and texture stability. It could be also very interesting to study the oxidation stability of the oleogels, the antioxidant activity and the carotenoids bioavailability.

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