

Agricultural University of Athens
Department of Food Science and Human Nutrition
Laboratory of Food Process Engineering

Development of microbial and enzymatic processes for
valorisation of industrial side streams for the production of
microbial lipids and esters

A thesis submitted for the degree of Doctor of Philosophy

by

Erminta L. Tsouko



ATHENS 2019

SUPERVISOR

Doctor Apostolos Koutinas

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DOCTORAL DISSERTATION

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Thesis committee

Thesis supervisor

Dr. Apostolos Koutinas, Assistant Professor, Agricultural University of Athens

Thesis co-supervisors

Serafeim Papanikolaou, Associate Professor, Agricultural University of Athens
Ioannis Kookos, Professor, University of Patra

Other members

Ioanna Mantala, Associate Professor, Agricultural University of Athens
Vasiliki Evaggeliou, Associate Professor, Agricultural University of Athens
Nikolaos Stoforos, Professor, Agricultural University of Athens
Denise M.G Freire, Professor, Federal University of Rio de Janeiro, Brazil

Γεωπονικό Πανεπιστήμιο Αθηνών
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Ανάπτυξη μικροβιακών και ενζυμικών διεργασιών για την
αξιοποίηση παράπλευρων βιομηχανικών ρευμάτων προς
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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΕΡΜΙΝΤΑ Α. ΤΣΟΥΚΟ



ΑΘΗΝΑ 2019

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ

Απόστολος Κουτίνας, Αναπληρωτής Καθηγητής Γ.Π.Α

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Απόστολος Κουτίνας (Αναπληρωτής Καθηγητής Γ.Π.Α)

Σεραφείμ Παπανικολάου (Αν. Καθηγητής Γ.Π.Α)

Ιωάννης Κούκος (Καθηγητής Πανεπιστημίου Πατρών)

Ιωάννα Μαντάλα (Αν. Καθηγήτρια Γ.Π.Α)

Νικόλαος Στοφόρος (Καθηγητής, Γ.Π.Α)

Βασιλική Ευαγγελίου (Αν. Καθηγήτρια, Γ.Π.Α)

Denise M.G Freire (Professor, Federal University of Rio de Janeiro, Brazil)

ABSTRACT

This doctoral dissertation focused on the valorisation of renewable resources including liquid and solid industrial waste and by-products streams for the biotechnological production of value-added products. Their potential utilisation as raw materials for the chemical industry was evaluated. Extraction of phenolic compounds from by-product streams of palm oil production was assessed in terms of total phenolic content and antioxidant activity index. Phenolics incorporation into various edible oils aimed to prolong their shelf life.

The first part of this thesis demonstrated the production of a generic nutrient-rich feedstock using by-product streams from palm oil production as a substitute for commercial fermentation supplements. Solid-state fermentations of palm kernel cake (PKC) and palm-pressed fiber (PPF) were conducted in tray bioreactors and a rotating drum bioreactor by the fungal strain *Aspergillus oryzae* for the production of crude enzymes. The production of protease was optimized (319.3 U/g) at an initial moisture content of 55%, when PKC was used as the sole substrate. The highest free amino nitrogen (FAN) production (5.6 mg/g) obtained via PKC hydrolysis using the crude enzymes produced via solid-state fermentation was achieved at 50 °C. Three initial PKC concentrations were tested in hydrolysis experiments, leading to total Kjeldahl nitrogen to FAN conversion yields up to 27.9%. Sequential solid-state fermentation followed by hydrolysis, was carried out in the same rotating drum bioreactor, leading to the production of 128.8 U/g of protease activity during fermentation and 201.5 mg/L of FAN during hydrolysis. Microbial oil production was successfully achieved with the oleaginous yeast strain *Lipomyces starkeyi* DSM 70296 cultivated on the produced PKC hydrolysate mixed with commercial carbon sources, including glucose, xylose, mannose, galactose, and arabinose.

A sequential part of the thesis, dealt with the extraction of phenolic compounds from various side streams derived from palm oil production including PKC, PPF, palm kernel shells (PKS) and empty fruit bunches (EFB). Among these streams, PKC showed the highest total phenolic content with a value of 5.2 mg of gallic acid equivalents (GAE)/g of dry sample, whereas the EFB presented the lowest value (1.8 mg GAE/g dry sample). The extraction time as well as the liquid-to-solid ratio were investigated to optimize the phenolic extraction. PKC exhibited the highest total phenolic content (5.4 mg GAE/g dry sample) with a liquid-to-solid ratio of 40:1 during 20 minutes of extraction. The main phenolic compounds of the extracts deriving from all by-product streams were also identified and quantified employing HPLC-DAD. Catechin and 4-hydroxybenzoic acid were detected in all samples. The

addition of 0.8% of PKC extract increased more than 50 % the induction time of sunflower oil.

A subsequent objective of this study was the valorisation of various protein-rich agricultural residues for the production of microbial oil-based polyol esters with lubrication properties. Palm kernel cake was initially used as substrate to evaluate lipid production via solid state fermentations using the fungal strains *Cunninghamella echinulata*, *Mortierella ramanniana* sp., *Mortierella isabellina* and *Thamnidium elegans*. Based on fermentation efficiency and fatty acid composition, *C. echinulata* was selected for further optimisation of fermentative conditions (i.e. temperature, initial moisture content, ratio of palm kernel cake and palm pressed fiber). Under the optimum conditions, microbial oil production in tray fermentations was 129.1 mg/g of fermented solids (gfs), with a γ -linolenic content of 5.3% (w/w). Cotton seed cake and castor seed cake were also evaluated for microbial lipid production in tray fermentations. The highest biomass production (304.1 mg/gfs) with a lipid content of 40.1% (w/w) was achieved when cotton seed cake was used. Subsequently, microbial lipid production using cotton seed cake was also evaluated in packed bed bioreactors, leading to a fungal biomass production of 320.5 mg/gfs with an intracellular lipid content of 37.7% (w/w). The microbial oil was extracted from the fermented solids and used for polyol ester production catalyzed by Lipomod 34MDP in a solvent free system. The highest conversion yield of 80% was achieved after 2 h reaction. The produced esters were characterized via Nuclear Magnetic Resonance analysis. The present study showed the perspective of valorising agricultural residues into value added oleochemicals with potential use as biolubricants.

Next, spent sulphite liquor (SSL), the major waste stream of pulp and paper industry, was valorized for the production of microbial oil rich in γ -linolenic acid (GLA) by five oleaginous fungi of the genus *Mortierella* sp., *Cunninghamella* sp. and *Thamnidium* sp. The aim was the utilization of fungal oil as substrate for the enzymatic synthesis of isopropyl and 2-ethylhexanol esters employing a solvent-free system catalyzed by commercial lipases. The reaction conditions evaluated, were temperature (30 - 70 °C), microbial oil to alcohol molar ratio (1:2.84 - 1:4) and biocatalyst amount (5 - 20%, w/w). Under the optimized conditions, subsequent transesterifications were conducted using lipid classes obtained via fractionation of microbial oil, including neutral lipids (NL), glycolipids and sphingolipids (GL+SL) and phospholipids (PL). Results showed that the presence of GL+SL and PL fractions negatively affected the enzymatic synthesis of the esters. Transesterification of the NL fraction with isopropanol and 2-ethylhexanol led to the maximum conversion yield of

80.2% and 73.8%, respectively. Subsequently, the conversion yield was maximized (90% and 80% for isopropyl and 2-ethylhexyl esters respectively) by the utilization of hydrolyzed microbial oil. The reuse and stability of biocatalyst in 6 repeated reactions as well as the physicochemical properties of the produced esters were also evaluated using the hydrolyzed microbial oil. The present study demonstrates the perspective of utilizing renewable resources for the production of specialty fatty acid esters rich in GLA with potent application in food, cosmetics and oleochemicals industries.

The last part of the thesis was related to the exploitation of SSL as the sole carbon source for the production of microbial oil. Initially, the effect of different C/FAN ratios and lignosulphonates (LS) concentrations on microbial oil production and fatty acid composition by *Lipomyces starkeyi*, *Cunninghamella echinulata* and *Mortierella isabellina* was evaluated in shake flasks fermentations. The highest lipid and total dry weight (TDW) production of *L. starkeyi* was 11.2 g/L and 2.8 g/L, respectively, using at C/FAN ratio of 173. In fed-batch fermentation, *L. starkeyi* showed the highest lipid production (40.9 g/L) at a C/FAN ratio of 33. In shake flask fermentations of *M. isabellina*, biomass production and microbial oil content were highly inhibited when 10 g/L and 50 g/L of LS were added in the fermentation media. In the case of *C. echinulata*, lipid and GLA contents were positively affected with increasing C/FAN ratios (until a threshold), leading to the highest values of 60% and 18.8%, respectively. A simultaneous phenolic removal (61%) of the substrate was also observed by the fungal strain in this case. TDW seemed to be induced while GLA formation was significantly decreased (8.9%) with increasing LS concentration. Fed-batch cultures of *C. echinulata* in bioreactor resulted in TDW production of 12.2 g/L with a lipid content of 56% in a media containing 5 g/L LS. Fermentation efficiency was significantly affected in the case of 90 g/L LS leading to reduced TDW production (9.9 g/L) and lipid content (27%). Fed-batch experiments demonstrated that GLA formation was strongly affected probably due to the applied aeration and agitation.

Scientific area of the thesis: Design of Food and Biorefinery Processes

Keywords: oleaginous fungi and yeasts; fermentation; microbial oil; γ -linolenic acid; renewable agro-industrial waste streams; enzymatic biocatalysis; Novozyme 435; fractionation of microbial oil, isopropyl and 2-ethylhexyl esters; lignosulphonates; oil

biotransformation; enzymes; packed-bed bioreactor; phenolic compounds, antioxidant activity, induction time, sunflower oil

ΠΕΡΙΛΗΨΗ

Η παρούσα διδακτορική διατριβή επικεντρώθηκε στην αξιοποίηση των ανανεώσιμων πρώτων υλών, συμπεριλαμβανομένων υγρών και στερεών βιομηχανικών αποβλήτων και υποπροϊόντων για τη βιοτεχνολογική παραγωγή προϊόντων υψηλής προστιθέμενης αξίας. Αξιολογήθηκε η πιθανή αξιοποίησή τους ως πρώτη ύλη για τη χημική βιομηχανία όπως εστέρες με εφαρμογές στον τομέα των καλλυντικών και εστέρες με βιολιπαντικές ιδιότητες. Η εκχύλιση των φαινολικών ενώσεων από τα ρεύματα υποπροϊόντων από την παραγωγή φοινικέλαιου αξιολογήθηκε ως προς τη συγκέντρωση ολικών φαινολικών και την αντιοξειδωτική τους ικανότητα. Η ενσωμάτωση φαινολικών σε διάφορα βρώσιμα έλαια είχε ως στόχο την παράταση της διάρκειας ζωής τους.

Το πρώτο μέρος της διατριβής επικεντρώθηκε στην αξιοποίηση παραπροϊόντων από την παραγωγή φοινικέλαιου προς παραγωγή υποστρώματος ζύμωσης πλούσιου σε θρεπτικά συστατικά με σκοπό να αντικαταστήσει εμπορικά θρεπτικά μέσα που χρησιμοποιούνται στις συμβατικές βιομηχανικές ζυμώσεις. Οι ζυμώσεις στερεάς κατάστασης χρησιμοποιώντας φυτικές ίνες μεσοκαρπίου μετά την εξαγωγή του φοινικέλαιου (palm pressed fibers-PPF) και άλευρα ενδοσπερμίου μετά την εξαγωγή του πυρηνέλαιου (palm kernel cake-PKC) με το στέλεχος του μύκητα *Aspergillus oryzae* διεξήχθησαν σε κωνικές φιάλες των 250 mL. Στόχος ήταν η ανάπτυξη της ενζυμικής διεργασίας για την παραγωγή πρωτεολυτικών ενζύμων. Η παραγωγή πρωτεολυτικών ενζύμων βελτιστοποιήθηκε (319,3 U/g) σε αρχική υγρασία στερεού 55%, όταν το PKC χρησιμοποιήθηκε ως μοναδικό στερεό υπόστρωμα ζύμωσης. Διαδοχικές ζυμώσεις στερεής κατάστασης σε αναλογία PKC:PPF ίσης προς 70:30 πραγματοποιήθηκαν σε 15 L βιοαντιδραστήρα περιστρεφόμενου τυμπάνου οδηγώντας σε πρωτεολυτική ενεργότητα 128,8 U/g. Σε επόμενο στάδιο, τα παραχθέντα ακατέργαστα πρωτεολυτικά ένζυμα χρησιμοποιήθηκαν για την ενζυμική υδρόλυση των πρωτεϊνών που περιέχονται στο PKC με σκοπό την δημιουργία υδρολυμάτων πλούσιων σε άζωτο και θρεπτικά συστατικά. Οι ενζυμικές υδρολύσεις έδειξαν ότι η υψηλότερη παραγωγή αζώτου που περιέχεται στις ελεύθερες αμινομάδες αμινοξέων και πεπτιδίων (free amino nitrogen-FAN) (5,6 mg/g) επιτεύχθηκε με το PKC στους 50 °C. Τρεις αρχικές συγκεντρώσεις PKC εξετάστηκαν, οδηγώντας σε απόδοση μετατροπής ολικού αζώτου (Kjeldahl) προς FAN έως και 27,9%. Ακολούθως, αξιολογήθηκε η ικανότητα της ζύμης *Lipomyces starkeyi* DSM 70296 να αναπτύσσεται και να παράγει μικροβιακά λιπίδια στα παραχθέντα υδρολύματα του PKC μέσω ασυνεχών υγρών ζυμώσεων και την προσθήκη συνθετικών πηγών άνθρακα όπως αραβινόζη, ξυλόζη, γαλακτόζη, μαννόζη και γλυκόζη. Η

παραγωγή μικροβιακής βιομάζας κυμάνθηκε από 20,6 g/L έως 22,4 g/L και η συγκέντρωση λιπιδίων από 5,7 g/L έως 7,3 g/L.

Το επόμενο μέρος της διδακτορικής διατριβής αφορούσε στην εκχύλιση φαινολικών ενώσεων από διάφορα παράπλευρα ρεύματα προέρχομενα από τη διεργασία παραγωγής φοινικέλαιου, συμπεριλαμβανομένων των PKC, PPF, άλευρα ενδοκαρπίου (palm kernel shells-PKS) και άδεια τσαμπιά μετά την παραλαβή του φοινικόκαρπου (empty fruit bunches-EFB) και την αξιολόγηση των αντίστοιχων φαινολικών εκχυλισμάτων ως προς την συγκέντρωση ολικών φαινολικών και την αντιοξειδωτική τους ικανότητα. Μεταξύ των ρευμάτων αυτών, το PKC έδειξε την υψηλότερη ολική συγκέντρωση φαινολικών με τιμή 5,2 mg ισοδύναμα γαλλικού οξέος (gallic acid equivalents-GAE)/g ξηρού δείγματος, ενώ τα EFB παρουσίασαν τη χαμηλότερη τιμή (1,8 mg GAE/g). Ο χρόνος εκχύλισης καθώς και ο λόγος διαλύτη εκχύλισης προς στερεό ερευνήθηκαν για τη βελτιστοποίηση της εκχύλισης φαινολικών από το PKC. Οι κύριες φαινολικές ενώσεις των εκχυλισμάτων από όλες τις ροές υποπροϊόντων, ταυτοποιήθηκαν και ποσοτικοποιήθηκαν χρησιμοποιώντας HPLC-DAD. Το 4-υδροξυβενζοϊκό οξύ και η κατεχίνη ανιχνεύθηκαν σε όλα τα δείγματα. Η μελέτη έδειξε ότι η προσθήκη 0.8% εκχυλίσματος PKC σε ηλιέλαιο οδήγησε σε αύξηση του χρόνου επαγωγής του πάνω από 50%.

Στόχος της μελέτης αυτής ήταν επίσης η αξιοποίηση διαφόρων πλούσιων σε πρωτεΐνες γεωργικών υπολειμμάτων για την παραγωγή εστέρων πολυολών με λιπαντικές ιδιότητες από μικροβιακό λίπος. Το PKC χρησιμοποιήθηκε ως υπόστρωμα για να αξιολογηθεί η παραγωγή μικροβιακού λίπους μέσω ζυμώσεων στερεάς κατάστασης από διάφορα στελέχη μυκήτων όπως *Cunninghamella echinulata*, *Mortierella ramanniana* sp., *Mortierella isabellina* και *Thamnidium elegans*. Με βάση την αποτελεσματικότητα της ζύμωσης και τη σύνθεση των λιπαρών οξέων, το στέλεχος *C. echinulata* επιλέχθηκε για περαιτέρω βελτιστοποίηση των συνθηκών ζύμωσης (θερμοκρασία, αρχική περιεκτικότητα σε υγρασία, αναλογία PKC και PPF). Υπό τις βέλτιστες συνθήκες, η παραγωγή μικροβιακού λίπους ανήλθε σε 129,1 mg/g ζυμωθέντων στερεών (fermented solids-fs), με περιεκτικότητα σε GLA 5,3%. Στην συνέχεια, στερεά υπολείμματα βαμβακόσπορου (cotton seed cake-CoSC) και ρίκινου (castor seed cake-CaSC) αξιολογήθηκαν επίσης για παραγωγή μικροβιακών λιπιδίων σε ζυμώσεις στερεάς κατάστασης. Η μέγιστη παραγωγή βιομάζας (304,1 mg/gfs) με περιεκτικότητα λιπιδίων 40,1% επιτεύχθηκε με την χρήση CoSC. Η παραγωγή μικροβιακών λιπιδίων διεξήχθη επίσης σε βιοαντιδραστήρες σταθερής κλίνης όπου χρησιμοποιήθηκε ως στερεό υπόστρωμα ζύμωσης το CoSC. Σε αυτή την περίπτωση, η παραγόμενη μυκητιακή βιομάζα ανήλθε σε 320,5 mg/gfs με περιεχόμενο ενδοκυτταρικών

λιπιδίων 37,7%. Το μικροβιακό λίπος εκχυλίστηκε από τα ζυμωμένα στερεά και στη συνέχεια χρησιμοποιήθηκε για την ενζυμική παραγωγή εστέρων πολυολών σε ένα σύστημα απουσία διαλυτών καταλυόμενο από το ένζυμο Lipomod 34-MDP. Η υψηλότερη απόδοση μετατροπής (80%) επιτεύχθηκε μετά από αντίδραση 2 ωρών. Η παρούσα μελέτη έδειξε την προοπτική αξιοποίησης γεωργικών υπολειμμάτων προς παραγωγή ελαιοχημικών υψηλής προστιθέμενης αξίας με δυναμική χρήση ως εστέρες με βιολιπαντικές ιδιότητες.

Ακολούθως, το υγρό απόβλητο από την βιομηχανία χαρτοπολτού και χάρτου (spent sulphite liquor-SSL) αξιοποιήθηκε προς παραγωγή μικροβιακών λιπιδίων πλούσιων σε GLA, μέσω πέντε μυκήτων του γένους *Mortierella* sp., *Cunninghamella* sp. και *Thamnidium* sp. Ο στόχος ήταν η χρησιμοποίηση των λιπιδίων ως υποστρώμα για την σύνθεση εστέρων ισοπροπανόλης και 2-αιθυλο-1-εξανόλης σε σύστημα απουσία διαλύτη, καταλυόμενο από εμπορικές λιπάσες (Novozyme 435). Οι συνθήκες αντίδρασης που αξιολογήθηκαν ήταν η αναλογία μικροβιακού λίπους προς αλκοόλη (1 : 2,84 – 1 : 4), η ποσότητα βιοκαταλύτη (5 - 20%, κ.β.) και η θερμοκρασία (30 - 70 °C). Υπό τις βέλτιστες συνθήκες, διεξήχθησαν μετεστεροποιήσεις χρησιμοποιώντας κλάσεις λιπιδίων που λαμβάνονται μέσω κλασμάτωσης, συμπεριλαμβανομένων των ουδέτερων λιπιδίων (neutral lipids-NL), των γλυκολιπιδίων και των σφιγγολιπιδίων (glycolipids and sphingolipids-GL + SL) και των φωσφολιπιδίων (phospholipids-PL). Τα αποτελέσματα έδειξαν ότι η παρουσία των GL + SL και PL επηρέασε αρνητικά την ενζυμική σύνθεση των εστέρων. Η μετεστεροποίηση του κλάσματος NL με ισοπροπανόλη και 2-αιθυλεξανόλη οδήγησε στη μέγιστη απόδοση βιομετατροπής 80,2% και 73,8% αντίστοιχα. Στη συνέχεια, η απόδοση βιομετατροπής μεγιστοποιήθηκε (90% και 80% για τους εστέρες ισοπροπανόλης και 2-αιθυλεξανόλης, αντίστοιχα) με τη χρήση υδρολυμένου μικροβιακού ελαίου. Η επαναχρησιμοποίηση και η σταθερότητα του βιοκαταλύτη σε 6 επαναλαμβανόμενες αντιδράσεις καθώς και οι φυσικοχημικές ιδιότητες των παραγόμενων εστέρων αξιολογήθηκαν χρησιμοποιώντας το υδρολυμένο μικροβιακό λίπος. Η παρούσα μελέτη έδειξε την προοπτική χρήσης ανανεώσιμων πρώτων υλών για την παραγωγή εστέρων λιπαρών οξέων πλούσιων σε GLA με πιθανές εφαρμογές σε βιομηχανίες τροφίμων, καλλυντικών και ελαιοχημικών.

Το τελευταίο μέρος της διατριβής σχετίζεται με την αξιοποίηση του SSL ως μοναδικής πηγής άνθρακα για την παραγωγή μικροβιακών λιπιδίων. Αρχικά, μελετήθηκε η επίδραση διαφόρων αναλογιών άνθρακα:αζώτου και διαφορετικών συγκεντρώσεων λιγνοσουλφονικών ουσιών στην παραγωγή μικροβιακών λιπιδίων και στην σύσταση των λιπαρών οξέων των μικροβιακών στελεχών *L. starkeyi*, *C. echinulata* and *M. isabellina* σε ζυμώσεις ασυνεχούς καλλιέργειας. Η υψηλότερη παραγωγή λιπιδίων και βιομάζας με το

στέλεχος *L. starkeyi* ήταν 11,2 g/L και 2,8 g/L, αντίστοιχα, χρησιμοποιώντας λόγο άνθρακα:αζώτου 173. Σε μικροβιακές ζυμώσεις ημισυνεχούς λειτουργίας, ο *L. starkeyi* έδειξε την υψηλότερη συγκέντρωση λιπιδίων (40,9 g/L) σε αναλογία άνθρακα:αζώτου 33. Σε ζυμώσεις ασυνεχούς καλλιέργειας, με τον μύκητα *M. isabellina*, η παραγωγή βιομάζας και η λιποπεριεκτικότητα παρεμποδίστηκαν όταν 10 g/L και 50 g/L λιγνοσουλφονικών ουσιών προστέθηκαν στο θρεπτικό μέσο της ζύμωσης. Στην περίπτωση του μύκητα *C. echinulata*, η λιποπεριεκτικότητα και το ποσοστό σε GLA ευνοήθηκαν με την αύξηση των αναλογιών άνθρακα:αζώτου (μέχρι ένα όριο), οδηγώντας σε μέγιστες τιμές 60% και 18,8% αντίστοιχα. Ταυτόχρονη απομάκρυνση φαινολικών ουσιών (61%) παρατηρήθηκε από το μυκητιακό στέλεχος στην περίπτωση αυτή. Η παραγόμενη βιομάζα ευνοήθηκε ενώ το ποσοστό των λιπιδίων σε GLA μειώθηκε σημαντικά (8,9%) με την αύξηση της συγκέντρωσης των λιγνοσουλφονικών ουσιών. Οι μικροβιακές ζυμώσεις ημι-συνεχούς λειτουργίας με τον μύκητα *C. echinulata* είχαν ως αποτέλεσμα την παραγωγή βιομάζας 12,2 g/L με περιεκτικότητα λιπιδίων 56% όταν προστέθηκαν 5 g/L λιγνοσουλφονικά. Η αποτελεσματικότητα της ζύμωσης επηρεάστηκε σημαντικά στην περίπτωση που προστέθηκαν 90 g/L λιγνοσουλφονικών, οδηγώντας σε παραγωγή βιομάζας 9,9% g/L και λιποπεριεκτικότητα 27%. Η παραγωγή σε GLA επηρεάστηκε σημαντικά στα πειράματα που έλαβαν μέρος στον βιοαντιδραστήρα.

Επιστημονική περιοχή της διατριβής: Σχεδιασμός Διεργασιών Τροφίμων και Βιοδιωλιστηρίων

Λέξεις κλειδιά: ελαιογόνοι μύκητες και ζύμες; ζύμωση; μικροβιακό λίπος; γ-λινολενικό οξύ; ανανεώσιμα αγροβιομηχανικά απόβλητα; ενζυμική βιοκατάλυση; Novozyme 435; κλασμάτωση μικροβιακού λίπους, εστέρες ισοπροπανόλης και 2-αιθυλο-1-εξανόλης; λιγνοσουλφονικά; βιομετατροπή του μικροβιακού λίπους; ένζυμα; βιοαντιδραστήρας σταθερής κλίνης; φαινολικά συστατικά, αντιοξειδωτική δράση, χρόνος επαγωγής, ηλιέλαιο

Ανάπτυξη μικροβιακών και ενζυμικών διεργασιών για την αξιοποίηση παράπλευρων βιομηχανικών ρευμάτων προς παραγωγή μικροβιακών ελαίων και βιογενών εστέρων.

Η έγκριση της διδακτορικής διατριβής από το Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου του Γεωπονικού Πανεπιστημίου Αθηνών δεν υποδηλώνει αποδοχή των απόψεων του συγγραφέα (ν.5343/1932, αρ.202, παρ. 2).

Η πνευματική ιδιοκτησία αποκτάται χωρίς καμία διατύπωση και χωρίς την ανάγκη ρήτρας απαγορευτικής των προσβολών της. Πάντως κατά το ν.2121/1993, όπως μεταγενέστερα τροποποιήθηκε ιδίως με το αρ.81, ν.3057/2002 καθώς και με τα αρ. 1, 2 και 4, ω.3524/2007 και την διεθνή σύμβαση της Βέρνης (που έχει κυρωθεί με το ν.100/1975), απαγορεύεται η αναδημοσίευση και γενικά η αναπαραγωγή του παρόντος έργου, με οποιονδήποτε τρόπο (ηλεκτρονικό, μηχανικό, φωτοτυπικό, ηχογράφηση ή άλλο) τμηματικά ή περιληπτικά, στο πρωτότυπο ή σε μετάφραση ή άλλη διασκευή, χωρίς γραπτή άδεια του συγγραφέα.

Το μη αποκλειστικό δικαίωμα αναπαραγωγής αντιγραφής (για λόγους ασφάλειας και συντήρησης) και διάθεση της παρούσας διδακτορικής διατριβής υπό ηλεκτρονική μορφή, για εκπαιδευτική, ερευνητική και ιδιωτική χρήση και όχι για χρήση που αποσκοπεί σε εμπορική εκμετάλλευση, παραχωρείται στην Βιβλιοθήκη και Κέντρο Πληροφόρησης του Γεωπονικού Πανεπιστημίου Αθηνών.

Dissemination activities and research visits

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Research visits

In 2015 I spent two months at the Institute of Bioactive Polymer Systems Biopos e.V., Center Teltow-Seehof (Teltow, Germany) within the framework of E-COST (European Cooperation in Science and Technology), Cost Action TD1203 'Food waste valorisation for sustainable chemicals, materials and fuels' founded by European Union.

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CHAPTER 1

Introduction

Renewable energy and nuclear power are the world's fastest growing sources of energy thus fossil fuels are expected to dominate until 2040 with an energy market share of 78%. The industrial sector including mining, manufacturing, agriculture, and construction, holds the largest share of energy consumption (50%) and it is projected to increase by 18% within the period 2015-2040. Food sector and pulp and paper industry are characterized as energy-intensive manufactures with a gross output increment of 3.5% annually (U.S. Energy Information Administration, IEO, 2017).

World energy-related CO₂ emissions are predicted to grow at an average of 0.6% annually within 2015-2040. Consequently, apart from fossil resources depletion as a result of the growing demands for fuels and energy, other aspects with respect to severe environmental issues including global warming and toxic waste generation, have emerged seeking for renewable resources which are considered as a very fast-growing source of energy for the period 2012-2040 (U.S. Energy Information Administration, IEO, 2017).

Increased public awareness and the establishment of strict policy regulations for fossil-based production have paved the way towards alternative resources that provide clean energy, green chemicals and high biodegradability. Research has mainly focused on renewable materials that can be found in abundance and at low cost and do not compete with the food chain. Recent developments on bio-based production have mainly targeted to the production of biofuels, i.e biodiesel and bioethanol. Thus, economic efficiency could be attained with the co-production of both biofuels and high added-value chemicals, simulating the petrochemical refinery. Renewable biomass and its fractionation and bioconversion to chemical and biochemical intermediates that are further supplied into a variety of other end-products, is of utmost importance to meet sustainability. Industrial biotechnology including the development and establishment of innovative technologies and biotechnological pathways, is the key for sustainable production of fuels, chemicals and polymers, establishing the bio-economy era.

The bioeconomy concept is reflected in three visions (Bugge et al., 2016). The first vision relates to the importance of bio-technology research and application and commercialisation of bio-technology in various sectors. The bio-resource vision focuses on the role of research, development, and demonstration (RD & D) related to biological raw materials in agriculture, forestry, and bioenergy, as well as on the establishment of new value chains. The last vision, bio-ecology, highlights the importance of ecological processes that optimise the use of energy and nutrients, promote biodiversity, and avoid monocultures and soil degradation.

One-third of plastic waste globally is not collected or managed. The alternative of a circular economy would turn goods that are at the end of their service life into resources for others, minimizing waste and replacing production with sufficiency. A study of seven European nations found that a shift to a circular economy would reduce each nation's greenhouse-gas emissions by up to 70% and grow its workforce by about 4% - the ultimate low-carbon economy (Stahel, 2016).

CHAPTER 2

State of the art

2.1. Renewable resources

2.1.1. Biorefinery concept

Biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy. The development of lignocellulosic based biorefineries for the production of chemicals, biopolymers and high-added value products via fractionation of the renewable biomass (Kachrimanidou et al., 2015; Alexandri et al., 2016), could pave the way towards innovative technologies as well as sustainable and economically viable fermentative processes. The establishment of consolidated bioprocesses in conventional industrial plants is dependent on the exploitation of all residual streams generated as co-products, by-products or waste. Selection of an efficient microbial strain, cost-effective, nutrient rich and abundant feedstock as well as optimisation of process parameters including bioreactors design, are considered critical factors for the successive and cost-feasible implementation of fermentative procedures.

Several upstream processing schemes exploiting the full potential of complex biological entities have been developed i.e cereal conversion into bioethanol, biodegradable plastics and platform chemicals (Arifeen et al., 2007; Koutinas et al., 2007; Du et al., 2008; Lin et al., 2012). In addition, pretreatment technologies that have been developed for the generation of fermentation feedstocks for bioethanol production could be adapted in the case of microbial oil-derived biodiesel production (Lloyd and Wyman, 2005; Zhu et al., 2009). Protein-rich industrial waste streams should be used for the production of fermentation media enriched in organic sources of nitrogen (e.g. amino acids, peptides), phosphorus, minerals, vitamins and trace elements. Such nutrient supplements for fermentation processes could be produced from oilseed residues generated after oil extraction in 1st generation biodiesel production plants (i.e protein-rich rapeseed or sunflower cakes), meat-and-bone meal, sewage sludge, protamylase (residual stream enriched in amino acids and peptides that is generated during the industrial production of starch from potatoes), corn steep liquor and residual yeast from potable or fuel ethanol production plants. Protein and other nutrients are also contained together with carbon sources in various food waste streams (i.e waste bread, whey). Therefore, in many cases, a single waste stream from the food industry could be sufficient for the production of nutrient-complete fermentation media for metabolites production i.e microbial oil (Tsakona et al., 2014). It should be stressed that organic N-sources may enhance lipid accumulation (even two or three times higher than the amount of lipids accumulated with inorganic N-sources) in certain oleaginous microorganisms (i.e *Rhodospiridium toruloides*, *Trichosporon cutaneum* and *Trichosporon fermentans*) (Evans and Ratledge, 1984a, 1984b; Zhu et al., 2008).

Products from biorefineries can be placed in a value pyramid of bioproducts depending on their value and production volume. The top of the pyramid includes high-value products i.e. pharmaceuticals and fine chemicals which are needed in small amounts while the bottom includes fuels that have a low value but are needed in large volumes. Products with intermediate volume and value i.e. chemicals, performance materials and food, are placed in between. Due to the small profit margin between feedstock and the lowest valued products, the economics of a biorefinery is highly dependent on production of co-products higher up in the pyramid, enabling efficient utilisation of the entire feedstock (Almqvist, 2017).

Based on the maximum theoretical conversion yields of glucose to microbial oil (0.33 g/g) and bioethanol (0.51 g/g) and the lower heating values (LHV) for microbial oil-derived biodiesel (37.5 MJ/kg) and bioethanol (26.7 MJ/kg), then the lower heating value per kg glucose that could be generated via fermentative production of microbial oil and bioethanol is 9% higher in the case of ethanol. However, the overall energy balance could be favourable in the case of microbial oil-derived biodiesel because it is expected that the energy required to produce biodiesel after microbial oil fermentation would be lower than the energy required to purify bioethanol from fermentation broths. This will also result in surplus lignin that will be used for chemical production when lignocellulosic biomass is used as raw material. In the case of bioethanol production, all lignin is required for energy generation for the plant. In addition, biodiesel production from microbial oil would create a sustainable supply of glycerol that is regarded as an important building block for the chemical industry. For instance, biodiesel production from microbial oil could be combined with biodegradable polymer (i.e. polyhydroxyalkanoates) and platform chemical (i.e. 1,3-propanediol, succinic acid, itaconic acid) production from crude glycerol generated during biodiesel production (Papanikolaou et al., 2000; Chatzifragkou and Papanikolaou, 2012).

In the 1980s, Davies and Holdsworth (1992) reported an economic analysis for microbial oil production (\$0.8 – 1/kg) from waste lactose (200,000 m³ whey per year) utilising the yeast strain *Candida curvata*. Based on this cost and using an order-of-magnitude approximation, microbial oil production cost in 2008 would have been \$1.4 - 1.8/kg (this value does not include the biodiesel production cost from SCO) in the case that whey is used as carbon source. Ratledge and Cohen (2008) reported that the minimum price of microbial oil produced from yeast or fungi could be \$3/kg. Koutinas et al. (2014) presented a thorough evaluation of microbial oil production and its potential utilisation as feedstock for biodiesel generation. Based on published experimental data concerning glucose-based renewable resources in combination with the yeast

strain *Rhodospiridium toruloides*, it was reported that industrial implementation of microbial oil production is dependent on the fermentation media used and on the productivities and final microbial oil concentrations achieved. It was estimated that for a glucose price of \$400/t, the oil production cost and biodiesel production cost were in the range of \$5.5/kg oil and \$5.9/kg biodiesel, respectively. If it is considered that biodiesel production from microbial oil is still at an early research stage, then the above economic considerations demonstrate that microbial oil production deserves more thorough research and development.

2.1.2. Lignocellulosic resources

Lignocellulosic biomass is the most abundant renewable resource in nature. Pretreatment constitutes an essential step prior to cellulose conversion procedures, causing a reduction in the crystallinity, enabling likewise the action of specific enzymes to hydrolyze cellulose. Considering that pretreatment is one of the most expensive steps for the conversion of lignocellulosic biomass to fermentable sugars, several attempts have been made to render the process more efficient and cost-effective. By rough classification, several physical, physicochemical, chemical and biological methods have been investigated concerning pretreatment of the lignocellulosic biomass, aiming at the optimisation of sugar hydrolysis and recovery or improvement of enzymatic saccharification, minimization of the inhibitory by-products and cost viability. A detailed review regarding the potential of the aforementioned methods is provided by Sun and Cheng (2002).

During hydrolysis of lignocellulosic biomass, formation of inhibitory compounds, such as phenolic compounds from lignin degradation, furfural from pentoses dehydration, hydroxyl-methyl-furfural (HMF) from hexoses degradation, acetic acid and heavy metals, hamper cell growth and product formation (Palmqvist and Hahn-Hagerdal, 2000 II). Therefore, it is important to identify suitable detoxification methods (Palmqvist and Hahn-Hagerdal, 2000 I; Mussatto and Roberto, 2004) in order to enhance fermentation yields and productivities.

Considering the biochemistry of degradation of xylose in order to serve as substrate for the *de novo* lipid accumulation, this compound can be either metabolized through the phosphoketolase reaction, yielding approximately 1.2 moles of acetyl-CoA per 100 g of xylose (~ 0.66 moles) utilized, or the pentose phosphate pathway, forming 1.0 mole of acetyl-CoA per 100 g of xylose utilized. Therefore, the maximum theoretical yield of microbial oil produced per glucose consumed is around 0.32 g/g. This value is higher in the case of xylose (~ 0.34 g/g), due to the

fact that oleaginous microorganisms utilize exclusively the phosphoketolase pathway for xylose assimilation (Papanikolaou and Aggelis, 2011).

Several agro-industrial residues have been utilized as feedstock for the production of microbial oil. A thorough review has already been provided by Tsouko et al. (2016). More specifically, corn cob hydrolysates have been used for the cultivation of *Trichosporon* sp. Sugar cane bagasse hydrolysates were assessed for their suitability as fermentation media in the case of *Geotrichum fermentans*, *Yarrowia lipolytica* and *Lipomyces starkeyi*. Wheat straw hydrolysates were evaluated as potential fermentation media for the cultivation of *Cryptococcus curvatus*, *Rhodotorula glutinis*, *Rhodospiridium toruloides*, *Lipomyces starkeyi*, and *Yarrowia lipolytica*. Rice straw and rice hull hydrolysates were used for the production of microbial oil by *Geotrichum fermentans* and *Mortierella isabellina*. Finally, corn stover hydrolysates were utilized by *Cryptococcus humicola*, *C. laurentii*, *Rhodospiridium toruloides* and *Rhodotorula graminis* for microbial oil production.

The industrialization of SCO production from lignocellulosic resources is dependent on the development of fed-batch or continuous fermentation strategies in order to achieve high cell density cultures or high productivities (Huang et al., 2013). The fermentation procedure can be further improved via mild pretreatment method that generates low concentrations of inhibitors. In a next step, prediction of fermentability based on the analysis of the hydrolysates would be helpful and finally an efficient detoxification method can be employed for the purification of highly inhibiting compounds prior to fermentation (Palmqvist and Hahn-Hagerdal, 2000 II). Additional factors that should be considered for overcoming the inhibitory effect of the lignocellulosic by-products are strain mutation, genetic modification and identification of yeast strains that are resistant to inhibitors (Sitepu et al., 2014).

2.1.3. By-products from palm oil processing

Palm tree (*Elaeis guineensis*) is cultivated in 45 countries all over the world and it covers a land area of 19.8 million hectares (Anonymous a 2016). The top producers are Indonesia and Malaysia (Figure 2.1). Seeing the mass balance per 1 hectare (ha) of oil palm plantation (Figure 2.1) it should be taken into much consideration the fact that the solid waste to product ratio is quite high equal to 6.6.

Palm biomass including empty fruit bunches (EFB), palm pressed fiber (PPF), palm kernel cake (PKC), palm kernel shells (PKS,) oil palm fronds, trunks, leaves and roots as well as palm oil

mill effluent (POME) correspond to 90% of the whole tree while the rest 10% comprises the oil. Empty palm fruit bunches constitute a by-product from the initial processing to obtain palm oil fruit. Two main products are manufactured from the oil palm fruit: palm oil (PO) extracted from the mesocarp of the fruit generating palm pressed fiber residues (PPF) and palm kernel oil (PKO) extracted from the endosperm, resulting in palm kernel cake (PKC) as by-product stream. The worldwide production of palm oil and palm kernel oil for 2015/2016 amounted to 62 million t and 7 million t, respectively (Anonymous a 2016). Based on mass balances (Yusoff 2006; Basiron and Weng 2004), the approximate quantities of PPF and PKC correspond to 53 million t and 9.9 million t, respectively.

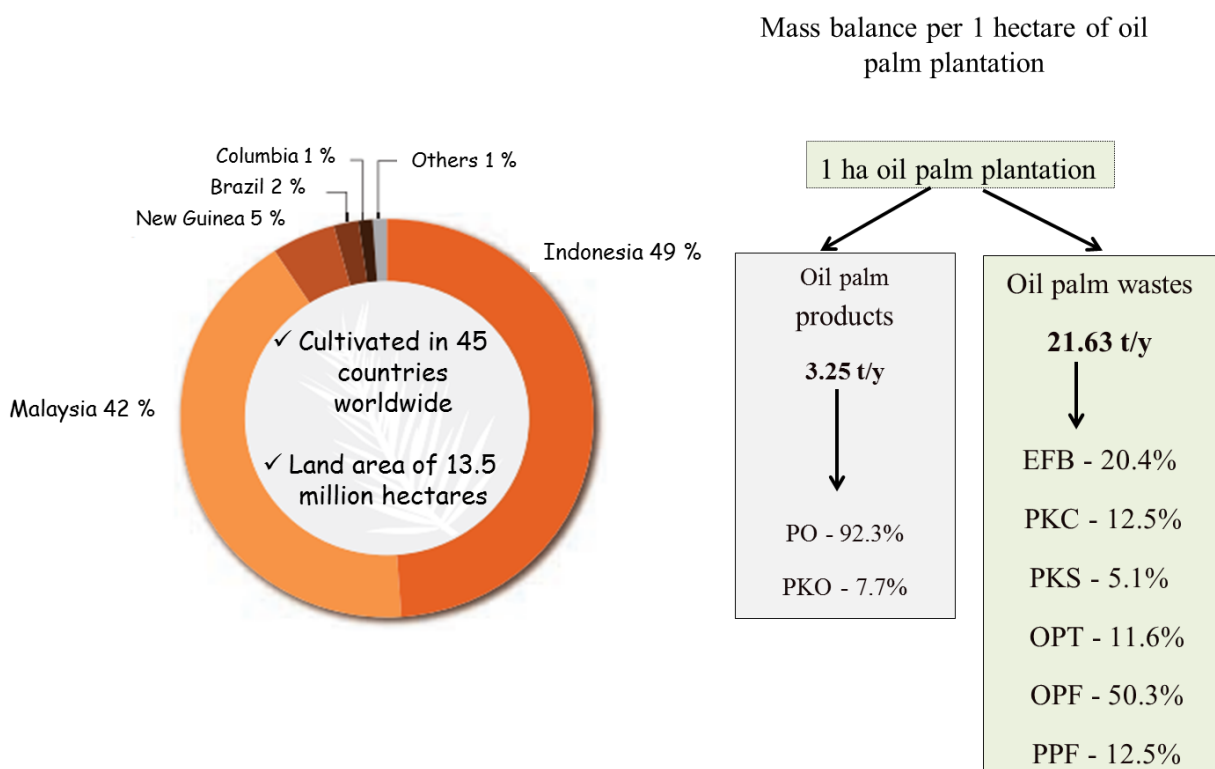


Figure 2.1 World distribution of palm tree and the mass balance per 1 hectare of oil palm plantation annually (Ofori-Boateng and Lee 2013).

The solid residues from palm oil production processes that amount to 428 million tons per year globally (Anonymous a 216) are conventionally used for incineration, disposal in landfills, steam and electricity generation (Prasertsan et al., 1996), applications as animal feed (Agunbiade et al., 1999) or implementation as substrate for fungal cultivation (Tabi et al., 2008). Table 2.1 depicts the composition of palm solid residues. As it can be seen, they are rich in lignin, cellulose and hemicellulose but they have relatively low protein content.

Table 2.1 Characterization of oil palm solid residues.

Composition (% dry basis)	Substrate					
	Palm kernel cake	Empty fruit bunches	Palm kernel shells	Oil palm fonds	Oil palm trunks	Palm pressed fiber
Ash	3.0-6.0 ^{a-e}	7.3 ^a	3.2-6.7 ^{a,j}	-	-	8.4 ^g
Moisture	4.0-14.0 ^{b-e}	40.0 ^g	9.4 ^l	50-60 ^g	24.5 ^g	13.8-29.4 ^{g,h}
TPC (mg GAE/g db) ¹	> 0.2 ^g	0.7-1.5 ^{g,m}	-	-	-	0.05-4.0 ^g
Crude Protein	12.0-21.0 ^{b-g,h}	< 4 ^g	-	4.0-10 ^g	< 3 ^g	6.2-9.0 ^{g,h}
Lipids	5.0-17.0 ^{b,c}	-	-	-	-	-
ADF ²	45.7-52.4 ^{a,d,i}	-	-	-	-	47.2 ^a
ADL ³	14.0-23.8 ^{c,d,f}	21.2 ^g	45.6 ^l	20.5 ^g	18.3 ^g	21.3-21.8 ^{g,h}
Neutral detergent fiber ⁴	43.0-85.0 ^{d,f,i}	65.5 ^g	-	83.5 ^g	76.3 ^g	77.6 ^h
Cellulose	16.6-30.0 ^{d,f}	41.0 ^g	33.0 ^j	49.8 ^g	45.0 ^g	40.0 ^g
Hemicellulose	24.0 ^f	24.0 ^g	23.8 ^j	25.3 ^g	25.0 ^g	22.1 ^g
Free Fatty acids ^g	8.3	< 2	-	< 2	< 2	39.0

^aTabi et al., 2008; ^bSundu et al., 2006 ^cJørgensen et al., 2009; ^dRibeiro et al., 2011; ^eSabu et al., 2005; ^fLawal et al., 2010; ^gOfori-Boateng and Lee 2013; ^hHo et al., 1991; ⁱAgunbiade et al., 1999; ^jKim et al., 2010

¹total phenolic content (TPC); gallic acid equivalents (GAE)

²Acid detergent fiber (ADF); ³Acid detergent lignin (ADL); ⁴Neutral detergent fiber (NDF)

Potent utilisation (Figure 2.2) as fermentative feedstock for the production of ethanol, butanol, organic acids, microbial oil and polymers (Jorgensen et al., 2010; Shukor et al., 2016), enzymes production (proteases, tannases, cellulases) (Zhu et al., 2014), high fiber bread and recovery/extraction of phytochemicals for pharmaceutical and nutritional purposes could lead to effective waste management, sustainable development and economic enhancement of the palm oil industry (Ofori-Boateng and Lee 2013).

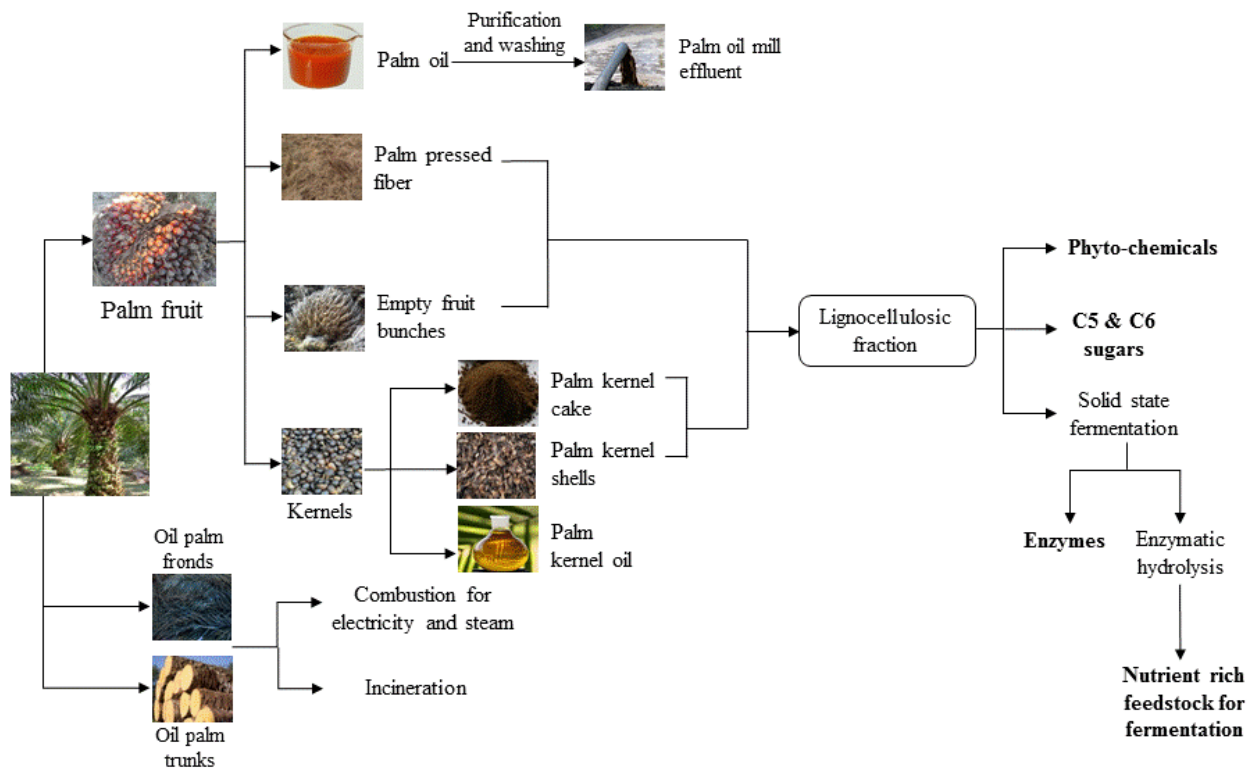


Figure 2.2 Generation of palm oil residues and their potent utilisation.

2.1.4. Waste streams from the acidic sulphite wood pulping process

Wood constitutes the most common feedstock in pulp production, with shift gradually driven towards hardwood i.e eucalyptus, birch, beech and aspen (Jönsson, 2016). Chemical pulping holds the 77% of global pulp production with Kraft and sulphite pulping accounting for 95% and 5% (7×10^6 t in 2012) respectively (Ragnar et al., 2000).

Sulphite pulping (Figure 2.3) occurs under strong acidic conditions and elevated temperatures (135 - 145 °C), including the delignification of wood biomass most commonly with magnesium bisulphite liquor. During the process, several reactions take place such as sulfonation of lignin leading to lignosulphonates (LS) formation, lignin depolymerization and hemicellulose hydrolysis. Along with the cellulose rich pulp, a spent cooking liquor, called weak spent sulphite liquor (SSL), is generated mostly containing LS and monosaccharides from hemicellulose. The weak SSL is condensed in multiple-effect evaporators and the resulting liquor is called thick SSL (Almqvist, 2017).

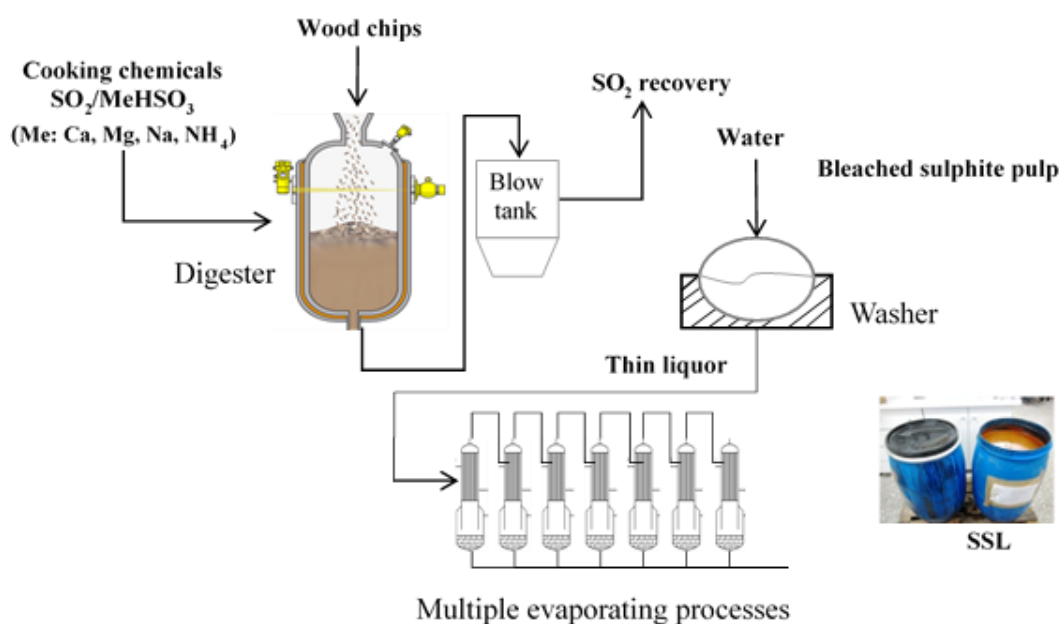


Figure 2.3 Spent sulphite liquor (SSL) generation through wood pulp manufacture.

The sugar fraction of weak SSL varies between 3% and 4% depending on the wood species used for the pulping process. In the case of softwood (wood from gymnosperms), the sugars are mainly hexoses, while when hardwood i.e. *Eucalyptus globulus* (wood from angiosperms) is pulped, more than 50% of sugars are pentoses and mostly xylose (Helle et al., 2004). Galactose, glucose, mannose and arabinose are also present but in lower concentrations. When *Eucalyptus globulus* is used for the pulping process, the main components of SSL are LS (400 g/L), C5 and C6 sugars (150 g/L), phenolic compounds, dissolved solids and other organic compounds (Palmqvist and Hahn-Hagerdal., 2000 I, II). LS are salts of liginosulphonic acid resulting from lignin sulphonation, during the acidic sulphite pulping process. This process involves the protonation of the benzyl ether bond of lignin followed by substitution in the same position with nucleophile HSO_3^- , leading to the formation of sulphonated lignin (Bjørsvik and Liquori, 2002). Acetic acid, formic acid and levulinic acid are also found in low quantities or traces (Palmqvist and Hahn-Hagerdal, 2000 I, II). Among the volatile compounds, furfural and hydroxymethylfurfural are present in minor quantities (Pereira et al., 2013).

Europe is one of the biggest wood pulp producers (26% of total pulp production) producing in 2016 about 26.1 million t via chemical pulping processes (Anonymous 2016 b) with liquid wastes corresponding to 185 million t (Koutinas et al., 2014). In 2015, the world production of bleached sulphite pulp amounted to 2 million t (Xavier et al., 2010). The sulphite pulping

process generates approximately 8 - 9 m³ of weak SSL per t of pulp produced (Chipeta et al., 2005).

Exploitation of SSL is restricted and thus pretreatment via nanofiltration or ultrafiltration, is a vital step for the reduction of its high organic load (Jönsson, 2016) prior to utilisation as carbon sources in bioprocesses. Production of high value added metabolites i.e succinic acid (Alexandri et al., 2016; Ladakis et al., 2018), poly-hydroxybutyrate (Weissgram et al., 2015) and ethanol (Helle et al., 2004), could enhance economics of pulp and paper industry in addition to revenues coming from LS market as dispersants, precipitates, binders, and adhesives (Jönsson, 2016).

2.1.5. Other agro-industrial by-product streams

Cotton (*Gossypium hirsutum* L.) cultivation is one of the major fiber sources for the textile industry with cotton lint as the main product and cottonseed as a by-product of ginning. Cottonseed accounts for the 60% of the plant mass. Cottonseed oil processing generates hulls (26%), cakes (46%) and linters (9%). Global cottonseed output in 2017/18 reached 41.8 million t while cottonseed cake amounted to 14.3 million t (Anonymous, 2017/2018).

Cottonseeds contain approximately 17 - 21% of oil but only 15% can be recovered while residual oil remains in the cake (Pütün et al., 2006). Cottonseed cake is rich in protein (30 - 44%) (Aguieiras et al., 2019) with a moisture content of 6%, ash of 5.2% and cellulose of 27.6% (Pütün et al., 2006).

The common practice for waste management of the solid residues is their application as fertilizers or partial substitutes for soybean meal in animal feed while the oil is considered as a source of secondary revenues corresponding to 15 - 25% of the crop value (He et al., 2015). The formulation of value-added products i.e wood adhesives (Cheng et al., 2013), bioplastics and films (Yue et al., 2012), superabsorbent hydrogel (Zhang et al., 2010) and antioxidant meal hydrolysates (Gao et al., 2010) has mainly focused on the exploitation of the protein or peptides fraction contained in CoSC. Fermentation approaches are rather restricted due to the presence of toxic gossypols, which are phenolic aldehydes that permeates cells and acts as inhibitors for several dehydrogenase enzymes (Sun et al., 2015). Grewal and Khare (2017) demonstrated the effective production of 2-pyrrolidone synthesis from γ -aminobutyric acid produced by *Lactobacillus brevis* under SSF utilising cottonseed cake. Agueiras et al. (2019) reported the SSF production of lipases cultivating *Rhizomucor miehei* on cottonseed cake. Other studies

aimed to the reduction of gossypol levels in cottonseed cake employing various microorganisms under SSF (Zhang et al., 2007; Sun et al., 2008).

Castor (*Ricinus communis*) is mainly cultivated for the production of non-edible oil corresponding to 0.15% of vegetable oils globally (Patel et al. 2016). Castor oil is of great economic importance mainly due to its high ricinoleic acid (90%) which has a hydroxyl group (C12) providing polarity and high stability and also making attainable its chemical derivatization, unlike other vegetable oils (Ogunniyi, 2006).

Castor oil, comprising 46 - 55% of the castor seed, finds a wide range of applications in the manufacture of paints, varnishes, cosmetics, germicides, lubricants, adhesives, fungicides, insecticides, printing inks and plastics (Silva et al., 2014). Castor seed cake deriving after oil extraction from castor seeds, is mainly used as a fertilizer. It is not recommended as animal feed due to its content of toxic protein (ricin) and toxic allergen (castor bean allergen). These toxic components are not carried into the oil.

The composition of castor seed cake has been reported by de Castro et al. (2016) as follows: 8.8% moisture, 6.2% ash, 43.5% protein 8.0% lipids, 6.8% cellulose, 4.4% hemicellulose and 9.9% lignin. So far, this toxic waste stream has been evaluated for the production of lipases with *Penicillium simplicissimum* under SSF, after its efficient detoxification (Godoy et al., 2009) resulting in lipases activity of 44.8 U/g. Also the potent application of castor seed cake for xylanase production by *Aspergillus japonicas* has been reported (Herculano et al., 2016).

2.2. Microbial oil and microorganisms

Microbial oil refers to intracellular storage lipids mainly comprising of triacylglycerols (TAGs). Lipids are produced by all living macro- and microorganisms for essential structural and functional roles such as the formation of permeable membranes of cells and organelles in the form of a lipid bilayer (Bogdanov et al., 2014).

There are many microalgae, yeasts (i.e. *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodotorula*, *Rhodospiridium*, *Trichosporon*), fungi (i.e. *Mortierella*, *Cunninghamella*) and bacteria (i.e. *Rhodococcus*, *Mycobacterium*) that can accumulate high amounts of microbial oil that has similar fatty acid composition to vegetable oils (Papanikolaou and Aggelis, 2011). Microorganisms can be characterized as oleaginous in the case that they are able to accumulate microbial oil to more than 20% of their total cellular dry weight (Ratledge, 1991).

The first attempts to use microbial oil in industrial-scale operations mostly referred to the utilisation of fatty materials as substitutes of rarely found lipids of the Plant or Animal Kingdom (i.e. microbial replacements of lipids containing γ -linolenic acid like borage oil or substitutes of exotic fats like cocoa-butter) (Papanikolaou and Aggelis, 2010).

Microalgae that are cultivated both autotrophically via photosynthesis and CO₂ fixation as well as heterotrophically utilising various organic carbon sources to accumulate intracellular lipids, cannot compete with oleaginous yeasts and fungi because their cultivation requires a large cultivation area and long fermentation duration (Koutinas et al., 2014). Although bacteria may achieve high growth rates and are genetically tractable due to their less complex genome, the majority of bacterial strains accumulate relatively low amount of microbial oil (up to 40%) (Meng et al., 2009). Particular yeast strains (e.g. *Rhodospiridium* sp., *Rhodotorula* sp., *Lipomyces* sp.) may biosynthesize around 70% of microbial oil (Leiva-Candia et al., 2015). Mainly yeasts and some fungi may offer appropriate cell factories for the production of microbial oil although the former are superior in terms of growth rates, yields and productivities.

Microbial oils provide various applications for a successful establishment of more sustainable or bio-based economy including biodiesel production (Tsouko et al., 2016) and oleochemicals i.e. biolubricants (Papadaki et al., 2018) and wax esters (Papadaki et al., 2017).

There is a remarkable plethora of pure or crude agro-industrial substrates that can be used by oleaginous microorganisms for microbial growth and accumulation of lipids. A thorough insight has been given by Tsouko et al. (2016). In many cases, microbial oils have similar fatty acid profile with vegetable oils. SCO is mainly composed of TAGs with a fatty acid composition rich in C16 and C18 fatty acids and more specifically, palmitic (16:0), palmitoleic (C16:1), stearic (18:0), oleic (18:1) and linoleic (18:2) acids (Meng et al., 2009). The microbial oil produced by *C. curvatus* has similar composition to palm oil (Davies, 1988). The microbial oil produced by *Yarrowia lipolytica* contains stearic, oleic, linoleic and palmitic acid (Papanikolaou et al., 2002). The fatty acid profile of lipids produced by *Lipomyces starkeyi* contains 33% palmitic and 55% oleic acid (Li et al. 2008).

2.3. Essential fatty acids

Essential fatty acids (EFAs) also known as polyunsaturated fatty acids (PUFAs), belong to the omega-6 and omega-3 families. They cannot be synthesized in humans and must be obtained

from dietary sources. The main dietary sources of omega-6 fatty acids include vegetable oils e.g sunflower, sesame, and corn, integral part of cereals, legumes and vegetables. Omega-3 fatty acids can be obtained from vegetable oils e.g linseed, rapeseed, canola, peanut or olive, dry fruits, oily cold-water fish and their corresponding oils. There is a critical role of EFAs and their metabolic products for maintenance of structural and functional integrity of central nervous system and retina (Singh 2005). They are closely involved with modulation of membrane structure, formation of short-lived regulating molecules e.g prostaglandins and leukotrienes, control of the water impermeability of the skin and other membranes e.g blood-brain barrier, and regulation of cholesterol transport and synthesis (Horrobin 1992).

γ -Linolenic acid (GLA) is an omega-6, all-cis-6,9,12-octadecatrienoic acid, also designated as 18:3 (n-6). GLA is a colorless, essential fatty acid, with chemical formula C₁₈H₃₀O₂ and molecular weight 278.44 g/mol. Primary sources of GLA comprise human milk, borage oil (~21%), black currant seed oil (~18%), evening primrose oil (~9%), hemp seed oil, spirulina and *Durio graveolens* fruits (12%) (Sergeant et al., 2016). GLA production from plant seeds is influenced by region, climate and seasons, resulting in variable quantities and qualities of the oils. Increasing market demand for GLA-lipids, inadequate supply of GLA from agricultural and animal sources and lack of GLA in humans, has led to the biotechnological production of GLA employing appropriate microorganisms capable of producing it in high concentrations up to 24%. Lower filamentous fungi of the division *Zygomycota* can accumulate significant quantities of intracellular lipids rich in GLA (Čertík et al., 2012).

The inflammatory and immunomodulating effects of gamma-linolenic acid (GLA) have been widely reported in in vitro and in vivo animal models while clinical literature has been less conclusive (Sergeant et al., 2016; Fan and Chapkin, 1998). GLA could modulate systemic inflammatory response and improve oxygenation, in acute lung injury cases (Rice et al., 2011), contribute positively in atopic eczema and dermatitis, platelet aggregation and alterate the fatty acid composition of membrane phospholipids modulating the function of the immune cells by changing the membrane fluidity, changing the activity of the membrane-associated enzymes, and producing immunoregulating eicosanoids (Hernandez 2016). GLA provides numerous applications in food industry as dietary supplement, nutraceuticals as encapsulation preparations, cosmetics for the formulation of natural ingredient-based cosmetics and in the medical sector. GLA-based lipids are of paramount interest for the food industry. Food supplementation with GLA can be attained directly via the production of PUFA-producing edible microorganisms in foods e.g whole cells, they can be extracted from their substrate-

matrix e.g agricultural feedstock, after solid state fermentation and used in foods as oil or as emulsions, or they can pass to animal products through the utilisation of feed rich in PUFAs. Thus, GLA-producing fungi are rich in proteins, dietary fibers, trace elements, vitamins, antioxidants, and cholesterol-free oil, enhancing their role as additives to functional food and feed.

2.4. Biochemistry of lipid accumulation in oleaginous microorganisms

2.4.1. General remarks

When various carbon sources are utilized for the production of SCO, accumulation of lipid in the microbial cells or mycelia (the so-called *de novo* lipid accumulation) is triggered by exhaustion of nitrogen in the growth medium, allowing the conversion of sugar to storage lipid (Ratledge, 1994; Papanikolaou and Aggelis 2011). In contrast, when growth is conducted on hydrophobic carbon sources (e.g. fats, oils), accumulation of storage lipids (the so-called *ex novo* lipid accumulation) is a primary anabolic process occurring simultaneously with the production of lipid free material, being independent from the nitrogen exhaustion in the medium (Fickers et al., 2005).

In *de novo* lipid accumulation, there is an increasing interest upon the potential of transforming abundant renewable materials (e.g waste glycerol, flour-rich waste streams, cellulose and hemicellulose hydrolysates) into SCO. The process of *ex novo* lipid accumulation aims at adding value to low cost fatty materials so that speciality high-value lipids (e.g. cocoa-butter or other exotic fats substitutes) will be produced (Papanikolaou and Aggelis 2010).

The lipids produced by oleaginous microorganisms are mainly composed of neutral fractions, principally triacylglycerols (TAGs) and to a lesser extent, steryl esters (SEs) (Ratledge, 1994; Ratledge and Wynn, 2002). As a general remark, when growth is carried out on various hydrophobic substances, the microbial lipid produced contains lower quantities of accumulated TAGs compared with growth elaborated on sugar-based substrates (Fakas et al. 2008). In any case, accumulation of storage lipids is accompanied by morphological changes in the oleaginous microorganisms, since large lipid globules can generally appear during the lipid accumulating phase. Storage lipids, unable to integrate into phospholipid bilayers, cluster to form the hydrophobic core of the so-called “lipid bodies” (Mlickova et al., 2004). Storage lipids serve as energy donators for the cells, covering their energy requirements for maintenance and proliferation, given that all essential nutrients are available in the fermentative environment.

2.2.1. Lipid synthesis pathway

De novo accumulation of cellular lipids is an anabolic biochemical process in which, by virtue of quasi-inverted β -oxidation reaction series, acetyl coenzyme A (CoA) issued by the intermediate cellular metabolism, generates the synthesis of intracellular fatty acids (Figure 2.4). Fatty acids will be then esterified to synthesize structural (phospholipids, sphingolipids etc) and reserve lipids (TAGs and SEs) (Ratledge, 1994; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2009).

Acetyl-CoA is the precursor for biogenesis of intracellular fatty acids. In microalgae and non oleaginous microorganisms acetyl-CoA is synthesized from acetate by the acetyl-CoA synthetase. In oleaginous microorganisms, the donor of acetyl-CoA is citrate, which becomes available in the cytosol after a series of biochemical events induced by nutrient (especially nitrogen) limiting conditions (Ratledge, 1988, 1994; Ratledge and Wynn, 2002). This limitation provokes a rapid decrease of the intracellular adenosine monophosphate (AMP), since, by virtue of AMP-desaminase, the microorganism cleaves AMP into inosine monophosphate (IMP) and NH_4^+ ions for nitrogen utilisation, in the form of NH_4^+ ions, as a complementary nitrogen source, necessary for the synthesis of cell material (Evans and Ratledge, 1985).

The sharp decrease of intracellular AMP alters the Krebs cycle function. The activity of both isocitrate dehydrogenases of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP^+) (enzymes responsible for the transformation of isocitric to α -ketoglutaric acid) is lost, since they are allosterically activated by intracellular AMP, and this event results in the accumulation of citrate inside the mitochondrion (Botham and Ratledge, 1979; Evans and Ratledge, 1985; Wynn et al., 2001).

Citrate is cleaved by ATP-citrate lyase (ACL - key enzyme of lipid accumulation process) in acetyl-CoA and oxaloacetate, with acetyl-CoA being converted, by an inversion of β -oxydation process, to cellular fatty acids. Absence or malfunction of ACL can lead to an increase in citrate concentration, which subsequently is either exerted in the growth medium or accumulated in the cytosol provoking inhibition of the glycolysis catabolic pathway (e.g. at the level of phosphofructokinase) that favors the biosynthesis of intracellular polysaccharides.

In general, production of citric acid by citrate producing strains is a process carried out when extra- and hence intracellular nitrogen is depleted (overflow metabolism phenomenon), while studies of the intracellular enzyme activities and coenzyme concentrations have somehow

identified and clarified the biochemical events leading to citric acid biosynthesis (Finogenova et al., 2002; Morgunov et al., 2004) and indeed it has been demonstrated that citric acid secretion and SCO accumulation are processes indeed identical into their first steps.

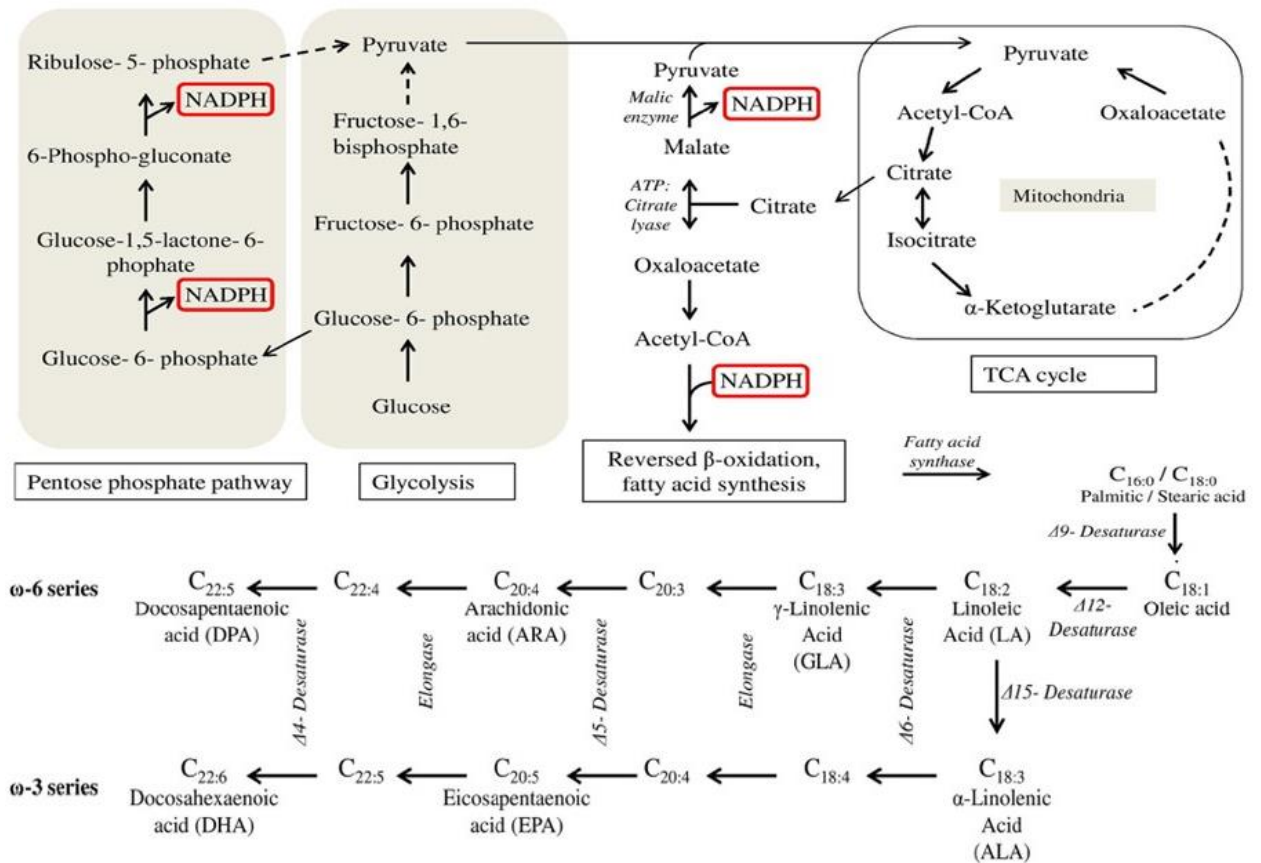


Figure 2.4 Biosynthesis of fatty acids in oleaginous eukaryotic microorganisms (Ochsenreither et al. 2016) and biosynthesis of polyunsaturated fatty acid via the metabolism of α -linolenic acid (ALA) and linoleic acid (LA).

2.4.2. Biogenesis of essential fatty acids

Essential fatty acids biogenesis (Figure 2.4) starts with linoleic acid (LA) in the case of omega-6 series and α -linolenic acid (ALA) in the case of omega-3 series, followed by desaturation and elongation reactions. More specifically, the LA pathway starts with the addition of a double bond by Δ^6 desaturase resulting in GLA formation. An elongase then facilitates the addition of two-carbons to GLA forming dihomo- γ -linolenic acid (DGLA) which is further converted to arachidonic acid (AA), via the addition of a double bond by Δ^5 desaturase (Bellou et al., 2016). The activity of Δ^5 desaturase in human platelets, monocytes and neutrophils is rather limited and can be influenced by certain diseases, so supplementation with GLA or DGLA, does not

increase AA levels in these cells (Surette et al., 1996). ALA is converted into the oxylipin precursors, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) through an identical to LA enzyme system (Figure 2.4). PUFAs are mainly esterified in triacylglycerols (sn-1, sn-3 position) and secondarily in glycerophospholipids (sn-2 position) and secreted into the blood. After their hydrolysis by the cytoplasmic phospholipase A2 (cPLA2), the released fatty acids enter cells underlying the capillary endothelium or bind to plasma albumin (Lagarde et al., 2013).

Free PUFAs are oxygenated by three enzymes groups carboxygenase, lipoxygenase, and cytochrome P450 into distinct classes of oxylipins. Oxylipins are synthesized *de novo* from PUFAs in an activation dependent manner (Massey and Nicolaou, 2013). Oxylipins constitute signaling molecules in animals and plants. Animal oxylipins include prostaglandins, leucotriens, and thromboxans, and have specific roles in the regulation of physiological processes. Plant oxylipins are involved in defense reactions as well as they act as fungicides, bactericides, and insecticides, or as volatile signals to attract predators (Heldt 2011).

2.4.3. Triacylglycerol synthesis

The fatty acids may be released from phospholipids and are used as building blocks for various lipids, including storage TAGs, via the Kennedy pathway (α -glycerol phosphate acylation) (Ratledge, 1988; Davies and Holdsworth, 1992; Müllner and Daum, 2004). After biosynthesis of fatty-CoA esters, free fatty acids are activated by CoA and are subsequently used for the acylation of the glycerol backbone to synthesize TAGs. In the first step of TAGs assembly, glycerol-3-phosphate (G-3-P) is acylated by G-3-P acyltransferase (GAT) at the sn-1 position to yield 1-acyl-G-3-P (lysophosphatidic acid-LPA), which is then further acylated by lysophosphatidic acid acyltransferase (also named 1-acyl-G-3-P acyltransferase-AGAT) in the sn-2 position to yield phosphatidic acid (PA). This is followed by dephosphorylation of PA by phosphatidic acid phosphohydrolase (PAP) to release diacylglycerol (DAG). In the final step DAG is acylated either by diacylglycerol acyltransferase or phospholipid diacylglycerol acyltransferase to produce TAGs (Davies and Holdsworth, 1992; Müllner and Daum, 2004; Fakas et al., 2009b).

2.4.4. Lipids turnover

Degradation of storage lipids occurs in cases that carbon sources are depleted from the fermentation media or their uptake rate is rather low (Papanikolaou and Aggelis 2011). The β -

oxidation pathway (FA degradation) requires lipase and sterol ester hydrolases for the cleavage of esters, as well as acyl CoA oxidases. Lipid turnover in microalgae may occur under light starvation conditions (Bellou et al., 2016).

2.5. Microbial oil production in fed-batch cultures using various yeast strains

A successful fed-batch strategy presupposes choosing an efficient fermentation medium and an appropriate feeding mode. Fed-batch cultures could lead to higher cell densities than traditional batch operations and simultaneously alleviate the inhibitory effect caused by high nutrient concentration, regulating the flow rate of the feeding medium. Efficiently metabolizable carbon source e.g glucose, could lead to enzyme repression via the increase of the intracellular concentration of ATP, called catabolite repression. This phenomenon can be avoided by keeping the carbon source concentration low via fed-batch processes.

Fed-batch operations, depending on the feeding mode that is implemented, can be categorized into processes without feedback control and processes with feedback control (Yamanè and Shimizu, 1984). The production of high lipid titers depends on the C/N ratio employed at the beginning of the fermentation and during the feeding stage. Maintaining a desirable C/N ratio and a constant carbon source concentration in the bioreactor during the feeding stage, could enhance lipid biosynthesis and productivity. The nutrient that is most frequently limited is nitrogen and in a few cases phosphorus, zinc or ferrous ion.

Literature-cited publications have focused on the development of fed-batch processes for microbial oil production using various yeast strains with industrial potential, feeding strategies and renewable resources (Table 2.2). Anschau et al., 2014 reported a TDW of 82.4 g/L and a lipid content of 46.9% with a productivity of 0.28 g/L/h, when cultivating *L. starkeyi* on commercial xylose and glucose (70:30), in an attempt to simulate the composition of the hemicellulosic fraction from sugarcane bagasse. The first three feeding pulses contained sugar and nutrient solutions and the last pulse involved only sugars while fermentation was initiated with an inoculum concentration of 3 g/L. Wiebe et al. 2012 reported constant and intermittent fed batch cultivation by *Rhodosporidium toruloides* using glucose, xylose and arabinose as carbon sources. Lipid production was 15.7 g/L with a biomass of 27.0 g/L while lipids yield and productivity were relatively low. In another study, *Cryptococcus* sp. was grown in corncob hydrolysate containing 4% glucose followed by feeding with corncob hydrolysate containing 2% glucose (Chang et al., 2013). TDW reached 10.8 g/L after 144 h of incubation with a lipid

content of 61.3%. Brandenburg et al. (2016) established an efficient pH-stat fed-batch process promoting *L. starkeyi* growth (22.0 g/L) and microbial oil accumulation (8 g/L) under an initial batch phase using xylose as the carbon sources with sequential feeding with birch wood hydrolysate. The same strain was evaluated for lipids production on unsaccharified sweet potato starch (Wild et al., 2010). Reported values of biomass (12 g/L) and lipids concentration (4.8 g/L) were accompanied by rather low lipids yield (0.16 g/g) and productivity (0.096 g/L/h). A biphasic fed-batch fermentation supplying glucose for growth and xylose for lipids accumulation was suggested by Probst and Vadlani (2017). Maximum growth of *L. starkeyi* (60.9 g/L) with a lipids content of 60.1% demonstrated the efficiency of this strategy. *R. toruloides* was cultivated on a 15 L fermenter applying a fed-batch model with an initial nutrient-rich media of pure glucose followed by discontinuous feeding. After 134 h of fermentation, biomass production and lipid content were respectively 106.5 g/L and 67.5% while productivity reached 0.54 g/L/h (Li et al., 2007). Zhang et al. (2011) carried out fed-batch fermentations for microbial oil production in a 30 L bioreactor with *Cryptococcus curvatus* using glucose as the sole carbon source obtaining a biomass, lipid content and productivity of 104.0 g/L, 82.7 % and 0.47 g/L/h, respectively. Intermittent feeding of a highly concentrated glucose solution was employed in this case. Meesters et al. (1996) employed a two stage fed-batch process using glycerol as carbon source to produce high cell densities of *C. curvatus* (118 g/L) with a productivity of 0.59 g/L/h but a low lipid content of 25%. Pan et al. (1986) employed an oxygen-enriched air technique without feedback control. Feeding solution contained high concentration of nutrients and was intermittently supplied. Biomass and lipids achieved were respectively 185.0 g/L and 74 g/L.

Table 2.2 Literature-cited publications focusing on the development of fed-batch processes for microbial oil production using various yeast strains with industrial potential, feeding strategies and renewable resources.

Strain	Substrate	Biomass (g/L)	Lipids (g/L)	Lipid content (%)	Lipids yield (g/g)	Biomass yield (g/g)	Productivity (g/L/h)	References
<i>L. starkeyi</i> NRRL Y-11557	Potato starch	12.0	4.8	40.0	0.16	0.41	0.096	Wild et al., 2010
<i>L. starkeyi</i> DSM 70296	G ³⁰ X ⁷⁰	82.4	38.6	46.9	0.16	0.43	0.279	Anschau et al., 2014
<i>L. starkeyi</i> DSM 70297	Xylose	94.6	37.4	39.5	0.17	0.77	0.227	Anschau et al., 2015
<i>L. starkeyi</i> CBS 1807	X:BP F:BWH	22.0	8.0	38.2	0.10	0.29		Brandenburg et al., 2016

<i>L. starkeyi</i> ATCC 56304	G _{batch} X _{lipid}	60.9	36.6	60.1	0.17	0.28	0.14	Probst and Vadlani 2017
<i>Cryptococcus</i> sp. SM5S05	Corn cob hydrolysate	10.8	6.6	61.3		-	0.045	Chang et al., 2013
<i>R. toruloides</i> CBS14	Glucose	35.0	26.3	75.0	0.22	-	0.21	Wiebe et al., 2012
	G:X:A (6:55:8)	27.0	15.7	58.0	0.07	-	0.15	
<i>R. toruloides</i> Y4	Glucose	106.5	71.9	67.5	0.23	0.35	0.54	Li et al., 2007
<i>C. curvatus</i> O3	Glucose	104.0	86.0	82.7	-	-	0.47	Zhang et al., 2011
<i>C. curvatus</i> ATCC 20509	Glycerol	118.0	29.5	25	-	-	0.59	Meesters et al. 1996
<i>Rhodotorula</i> <i>glutinis</i> NRRL Y-I091	Glucose	185.0	74	40	-	-	-	Pan et al. 1986

BWH: birch wood hydrolysate; BP: batch phase; F: feeding; A: arabinose; G: glucose; X: xylose

A successful feeding strategy could enhance fermentation yields and productivities and it is highly dependent on the microorganism, fermentation broth and bioreactor configuration applied in the bioprocess. For instance, predicted optimal productivities were demonstrated as independent of the feeding strategy in fed-batch processes for penicillin production. Selection of the feeding mode should be purpose-orientated and balanced with the economics of the bioprocess (Yamanè and Shimizu, 1984).

2.6. Submerged fermentations for enriched in GLA microbial oil, employing various fungal strains

Production of microbial oil rich in EFAs i.e GLA comprise a major target of lipid biotechnology. In contrary to conventional PUFAs sources i.e fish and plants, microbial PUFAs production is sustainable and independent of climatic or seasonal changes. Several fungi, microalgae, and mosses have been reported to produce lipids containing GLA and could comprise potent candidates for scaling up processes as they utilize a wide variety of fermentative substrates with adequate growth rates (Sayegh et al., 2016). Several bacteria are able to produce PUFAs but they are not utilizable because PUFAs comprise structural components of their membranes (Abedi et al., 2014).

Lipogenesis is a secondary metabolic process closely related and influenced by the conditions of the macro and micro phenomena occurring in the environment of the fermentation (Papanikolaou and Aggelis 2011). Factors affecting GLA formation include fermentation time

(Gema et al., 2002; Fakas et al., 2006), mineral supplementation of the fermentative substrate (Muniraj et al., 2015), nitrogen sources (Fakas et al., 2008), temperature (Bellou et al., 2012), C/N ratio, carbon source concentration, physical mutagenesis via UV, X-ray or magnetic field (Al-Hawash et al., 2017), aeration and agitation (Saad et al., 2014).

Zygomycetes have been extensively studied on glucose-based fermentative substrates as well as on renewable resources, for enriched in GLA lipid production. Table 2.3 depict studies that have been so far conducted, considering fungal production of GLA under submerged fermentation mode.

C. echinulata was cultivated on various carbon (C) and nitrogen (N) sources and C/N ratios for lipid production rich in GLA. C/N ratio of 33 - 48.5 including soluble starch, yeast extract and NH_4NO_3 resulted in the maximum biomass production of 29.8 g/L with 26.9% lipids and 12% GLA after 120 h (Chen and Chang 1996). Du Preez et al., 1997 evaluated the effect of initial acetic acid concentration on growth, lipid and GLA production by *Mucor circinelloides* via fed batch experiments in a 14 L stirred tank reactor. Acetic acid at 2 g/L, resulted in 15.6% GLA in the neutral lipid fraction. *C. echinulata* was grown on synthetic glucose substrate resulting in 6.7 g/L biomass with a lipid percentage of 44.7% and GLA content of 11.4% (Gema et al., 2002) after 217 h. It was demonstrated that GLA content gradually decreased throughout the lipid accumulation process. Fermentative conditions including carbon sources (glucose, sucrose, starch, lactose), inorganic nitrogen sources, time, aeration (shaking and non-shaking conditions) and addition of sesame oil as GLA promoter, were examined to determine their influence on growth, lipid production and fatty acid composition of *Mucor rouxii* and *Mucor* sp.1b cultures. Glucose in combination with KNO_3 resulted in 6 g/L of biomass with a lipid content of 32.5% and GLA of 13.8% in the case of *M. rouxii*. The supplementation with sesame oil gave 3 folds higher biomass with a lipid concentration of 44.5% but a cessation of GLA synthesis was observed (Somashekar et al., 2003). *M. isabellina* and *C. echinulata* were cultivated on glucose-, pectin-, starch- and lactose-based media. Glucose was the most efficient carbon source for both fungi. *M. isabellina* performed well on all the examined substrates while GLA was produced in low quantities ranging between 2.9 - 6.1%. Glucose, pectin and starch, favored GLA synthesis in *C. echinulata* with values varying between 14.2 - 16.5% at the end of each fermentation (Papanikolaou et al., 2007). Fakas et al., (2008) focused on the utilisation of various nitrogen sources i.e corn gluten, corn steep, whey concentrate, yeast extract and tomato waste hydrolysate to produce fungal oil with *C. echinulata*. GLA content showed great variations at different N-sources and concentrations varying from 2 - 18.3%. Growth on tomato

waste hydrolysate gave the most promising results with respective values of biomass, lipids and GLA, 17.6 g/L, 39.6%, and 11.5% within 240 -280 h of fermentation. *Thamnidium elegans* was successfully grown on crude glycerol. The produced biomass (12.5 g/L) contained 42.0% lipids with a GLA content of 7.3% (Fakas et al., 2009 a) after 270 h. Bellou et al. (2012), studied various *Zygomycetes* on pure glycerol suggesting that PUFAs production in most of them i.e *M. ramanniana* is related to the mycelia growth and is part of the primary metabolism while PUFAs production in *C. echinulata* is not a strictly growth associated activity but it continues occurring even after the biomass stops being produced. Flask fermentations with both fungal strains were more efficient than bioreactor trials resulting in 7.0 g/L of biomass after 216 h. *M. ramanniana* is a strongly competitive oil producer accumulating 53.1% biomass with 4.3% GLA content on NL while *C. echinulata* was very promising for high levels of GLA (15.3 % on NL) but with relatively low oil content (25.1%) (Bellou et al., 2012). The same scientific group continued investigation with *Zygomycetes* cultivating them on olive mill wastewater (OMW) achieving both lipid production and phenolic removal. *T. elegans* and *Zygorhynchus moelleri* performed well when OMW was used as the sole carbon source. Lipid contents of around 50% were achieved while GLA varied between 6.1 - 9.8% (Bellou et al., 2014). *T. elegans* was proved an efficient fungal strain on glucose media, producing 31.9 g/L biomass with a lipid content of 47.1% and GLA percentage of 6.8% after 304 h (Zikou et al., 2013). Saad et al. (2014) optimized lipid and GLA production in a 5 L bioreactor with *C. bainieri* using response surface methodology on a central composite design. Lipid production was significantly influenced by agitation intensity as well as by the interaction between aeration rate and agitation. The best combination of biomass (12.1 g/L), lipid (39.3%) and GLA (29.4%) production was achieved when 1.14 vvm and 400 rpm were applied. Tang et al. (2014) investigated the effect of twenty amino acids on the growth, lipid production and GLA synthesis in *Mucor circinelloides*. Tyrosine led to the highest biomass (17.8 g/L) and lipid production (23.0%) and a high GLA content of 19.8%. Potato processing wastewater was efficiently utilized by *Mucor rouxii* and *Aspergillus flavus* for lipids production rich in GLA. Addition of nutrients improved lipid and GLA yields resulting in respective values of 4.2 g/L and 5.7% for *Mucor rouxii* and 3.5 g/L and 2.9% for *A. flavus* (Muniraj et al., 2015). *M.isabellina* comprises a significant lipid producer accumulating 61.0% intracellular lipids after 545 h, when cultivated on glucose, yet the GLA content of the lipids is poor varying between 1.7 - 5.4% (Gardeli et al., 2017). Zhang et al. (2017) demonstrated the important role of delta-6 desaturase in GLA synthesis by homologous recombination of *Mucor circinelloides*. Mutant Mc-D61 was capable of producing 0.5 g/L lipids containing 43% GLA. GLA was enhanced more than 30% in

comparison to the control strain (Zhang et al., 2017). Fermentations in shake flasks and a 3-L fermenter were conducted for optimisation of GLA production with *C. elegans*. After 96 hours of fermentation using commercial glucose, the maximum GLA production was 882 mg/L in shake flask experiments and 733 mg/L in the fermentor (Varma et al., 2018).

Table 2.3 Submerged fermentations for enriched in GLA microbial oil, employing various fungal strains, carbon sources and cultivation modes.

Strain	Feedstock	Biomass (g/L)	Lipid content (%)	GLA in TFA (%)	References
<i>C. echinulata</i> CCRC 31840	Soluble starch	29.8	26.9	12.0	Chen and Chang 1996
<i>Mucor circinelloides</i> CBS 203.28	Fed batch (14 L) 150-550 rpm	8.5	-	15.6 ²	Du Preez et al., 1997
<i>C. echinulata</i> ATHUM 4411	Glucose	6.7	44.7	11.4	Gema et al., 2002
<i>Mucor rouxii</i> MTCC 386	Glucose&KNO ₃	6.0	32.5	13.8	Somashekar et al., 2003
<i>C. echinulata</i> ATHUM 4411	Starch	13.5	28.0	14.2	Papanikolaou et al., 2007
<i>M. isabellina</i> ATHUM 2935		10.4	36.0	4.0	
<i>C. echinulata</i> ATHUM 4411	Glucose&tomato hydrolysate	17.6	39.4	11.5	Fakas et al., 2008
<i>T. elegans</i> CCF-1465	Crude glycerol	12.5	42.0	7.3	Fakas et al., 2009 a
<i>C. echinulata</i> ATHUM 4411	Pure glycerol	6.9	25.1	15.3 ²	Bellou et al., 2012
<i>M. ramanniana</i> MUCL 9235		7.0	53.1	4.3 ²	
<i>T. elegans</i> CCF-1465	Glucose	31.9	47.1	6.8	Zikou et al., 2013
<i>T. elegans</i> CCF-1465	Olive mill wastewater submerged cultures	5.3	52.7	6.1	Bellou et al., 2014
<i>Zygorhynchus moelleri</i> MUCL 1430		2.9	51.1	9.8	
<i>M. circinelloides</i> CBS108.16	Glucose&tyrosine	17.8	23.0	19.8	Tang et al., 2014

<i>C. bainieri</i> 2A1	Glucose, 1.14 vvm, 400 rpm	12.1	39.3	29.4	Saad et al., 2014
<i>Mucor rouxii</i> DSM1191	Potato processing wastewater	13.5	31.0	5.7	Muniraj et al., 2015
<i>A. flavus</i> I16-3	Glucose, 1.14 vvm, 400 rpm	10.2	34.0	2.9	Saad et al., 2014
<i>M. circinelloides</i> CBS-Mc-D61	Glucose (2 L) fermenter 500 rpm, 0.5 vvm	4.3	12.0	43.0	Zhang et al., 2017
<i>M. isabellina</i> ATHUM 2935	Glucose	28.8	61.0	1.7-5.4	Gardeli et al., 2017
<i>C. elegans</i> CFR C07	Glucose (3 L), 500 rpm, 1 vvm	-	-	733.0 mg/L	Varma et al., 2018
<i>C. elegans</i> CFR C07	Glucose	20.5	-	882.0 mg/L	

¹Total fatty acids (TFA); ²% in the neutral lipids (NL)

2.7. A holistic approach on solid state fermentation practice

Solid state fermentation (SSF) is the oldest known fermentation strategy that has gained substantial attention during the last decades. SSF is a bioprocess occurring under total or almost-total absence of free water, employing renewable resources as carbon and nutrient solid matrices, supplemented with adequate humidity levels to promote microbial growth and metabolic activity of the microorganisms. Selection of an efficient microbial strain, cost-effective, nutrient rich and abundant feedstock as well as optimisation of process parameters including bioreactors design, heat and mass transfer phenomena are considered critical factors for a successive and cost-feasible implementation of SSF.

Several reviews have been published focusing on various aspects of SSF including reviews that overview solid state bioprocesses through a holistic approach (Pandey et al., 2000; Thomas et al., 2013; Soccol et al., 2017) investigating among others, the bioprocesses and products based on SSF and their applications, agro-industrial residues utilisation and enzyme production. Other reviews deal with the design and operation of bioreactors (Pandey 1991; Ali and Zulkaki 2011), physiology of the solid medium and transcription of biosynthetic genes (Barrios-González 2012), microbial strains for the production of secondary metabolites (Barrios-González and Mejía 2008) and microbial oil production (Čertík et al., 2012).

The more ancient application of SSF include the production of traditional fermented foods e.g koji (fermented with *Aspergillus oryzae* rice), soya sauce (fermented soybeans with *Aspergillus oryzae* or *Aspergillus sojae*) or Roquefort (cheese fermented with *Penicillium roqueforti*). Major applications of SSF in the field of bioprocesses include bioremediation and biodegradation of hazardous compounds, delignification and detoxification of agro-industrial residues, biopulping for the improvement of mechanical pulping process and biological upgradation of crop feedstock for improved nutritional value (Nigam and Pandey 2009). SSF offers great potential for production of various value-added products e.g organic acids, enzymes, biopesticides, biofuels, aroma compounds, carotenoid, xanthan, pigments, vitamins, biosurfactants and other bioactive compounds. The most used microbial strains belong to fungal phylums namely Ascomycota (*Aspergillus*, *Penicillium*, *Trichoderma*, *Monascus*), Zygomycota (*Rhizopus*, *Mortierella*, *Cunninghamella*, *Mucor*) and Basidiomycota (white-rot fungi) and bacterial strains including *Bacillus* and *Streptomyces* genus. A great variety of solid by-product or waste streams deriving from the agricultural and industrial sector have been utilized. Cereals (wheat, barley, corn, oat, rice, sorghum), waste streams from vegetable oil

milling industry (sunflower, rapeseed, palm) edible starchy roots (cassava, potato) sugarcane bagasse, cotton, soybean, sugar beet pulp and coffee residues rank among the most important renewable resources that have been applied for biotransformation to high-value added products (Pandey et al., 2000).

SSF processes are influenced by numerous factors including biological, physico-chemical, and environmental ones. The most common inoculating strategy is the spore suspension, although mycelia inoculum has been reported to be more effective in some case e.g phytase production (Krishna and Nokes, 2001) due to the direct disposal of the prerequisite enzymes into the bioprocess. Humidity levels and water activity (a_w) which are positively correlated, are determinants for microbial growth and productivities of the desired products. Generally, low moisture content of the solid substrate hampers diffusion of the nutrients, microbial growth, and enzyme stability while high moisture levels lead to the formation of solid agglomerates, limiting gas transfer into and out of the fermentative substrate (Krishna and Nokes, 2001). Fungi are favored by lower humidity levels (20-70 %) than bacteria (higher than 70%) making them more competitive at the a_w values encountered in SSF procedures. Optimum pH maintenance is scarce in SSF due to the absence of proper equipment for the *in situ* control of pH. Application of urea instead of ammonium salts and an effective buffer solution, could deteriorate the drop of pH (Lonsane et al., 1992). SSF performance is highly dependent on temperature, as high values, could alter protein structure or inhibit enzyme activity. Heat creation by the metabolic activity of the microorganisms, is difficult to be removed due to poor thermal conductivity of solid substrates and also the static nature of SSF. In large-scale SSF heat generation is more intense leading to severe humidity losses, low product yields and limited fungal activity (Auria et al., 1993). Two types of SSF systems can be distinguished depending upon the nature of the solid substrate. Natural ones, with a heterogeneous macromolecular structure, mostly consisted of starch, cellulose, lignocellulose, pectin and other polysaccharides. Cost and availability criteria, render them suitable or not for SSF application. Via mechanical, chemical or enzymatic pretreatment or after their nutrient supplementation, they can be transformed into inert matrices, supplying the microorganisms with carbon, nitrogen and micro-nutritional sources. Agro-industrial renewable resources are generally considered as the most appropriate for SSF processes while inert supports impregnated with a liquid medium, are not widely applicable. The latter, serve only as anchor points for the microbes but they are superior to the formers in terms of easier downstream processes (Krishna 2005). Another factor of vital importance and closely related to heat and mass transfer

phenomena is particle size of the substrate. Particle size defines the accessible to the microorganism surface as well as the porosity of the solid matrix that determines the available space for gas transfer. Although small particle size tends to provide adequate surface area for microbial action in contrast to larger particles, too small particles could lead to the formation of solid agglomerates, deteriorating microbial respiration and thus microbial growth. Appropriate designated solid matrices including small or larger particles could favor respiration and aeration efficiency of SSF through adequate gas exchanges between the air and the solid surface (Pandey et al., 2000). SSF is an aerobic process and therefore it is highly affected by aeration and agitation. Aeration provides the fermenter's endo-environment with oxygen as well as it eliminates carbon dioxide, volatile metabolites and heat, generated by the microorganism's metabolic activity. Agitation is crucial for the uniform diffusion of temperature and the equilibrium maintenance of mass and heat transfer between the gas-liquid-solid interfacial area of SSF (Krishna 2005). Agitation is adversely related to the porosity of the solid matrix due to shear forces created in SSF systems. Intense agitation forces, may lead to compacting of the substrate particles, inability of fungal attachment to the solids, and damage of fungal mycelia (Lonsane et al., 1992), affecting in some cases microorganisms' performance e.g growth of *Aspergillus oryzae* (Stuart et al., 1999). Generally, the application of agitation, leads to enhanced bio-product yields e.g enzyme production (Krishna 2005). Application of intermittent rather than continuous agitation is more favorable for the prevention of the aforementioned disadvantages.

Different types of bioreactor systems are employed in SSF from laboratory to industrial scale. SSF bioreactors can be classified according to their mixing system e.g static (fixed bed, trays) or stirred ones (horizontal drum or stirred drum) and type of aeration (forced aeration or by diffusion). Durand, (2003) has extensively reviewed various reactor designs, highlighting the main designs that have already been developed over the last years. Successful SSF implementation especially in an industrial level, is fundamentally dependent on the bioreactor configuration. Peculiarities of fermentative substrates e.g chemical structure, size, porosity and water holding capacity, fungus morphology that is critical for the choice of agitation and its frequency, and the aeration.

SSF is a well-established fermentation mode utilising agro-industrial renewable resources for the biotechnological transformation of low to high value biomolecules. Recent developments on SSF offer a great diversity of applications with potential for large-scale production coupled

with techno-economic feasibility specially in the area of enzyme production (Thomas et al., 2013).

2.7.1. Solid state fermentation for enzyme production - The proteases case

Enzymes are macromolecular biological catalysts, accelerating chemical reactions by lowering their activation energy. The market for enzymes is dominated by protease, lipases, phytases, carbohydrases, polymerases and nucleases. They are indispensable part of emerging and dynamic industries e.g food and beverages, animal feed, biofuels, textile, pulp and paper, personal care and cosmetics, nutraceuticals etc. due to their selectivity, efficiency and their environmentally friendly nature. Enzymes market size was worth over USD 5 billion in 2016 and is foreseen to surpass 400×10^3 tons by 2024 growing at a CAGR of 6.8%. Proteases will worth over USD 2 billion by 2024. Carbohydrases exceeded USD 2.5 billion in 2016. Lipases are expected to witness gains at over 6.8% up to 2024. The industrial enzyme market is driven by increasing diversity in enzyme applications and niche products, and stringent environmental norms constraining the use of chemicals.

Microbial proteases are secreted extracellularly, simplifying downstream processes of enzyme recovery as compared to proteases obtained from plants and animals (Sevitha et al., 2011). It is well established that enzymes concentrations produced in SSF systems are much higher than in submerged fermentation mode (Couto and Sanromán 2006). Proteases or peptidases, catalyze proteins, hydrolyzing peptide bonds by the detachment of the terminal amino acids from the protein chain or attack internal peptide bonds of a protein. Proteases are among the most studied group of enzymes in the open literature, mostly produced by SSF of the genus *Aspergillus* that poses the largest expanse of hydrolytic genes including 135 protease genes coding for alkaline, acid and neutral proteases (Vishwanatha et al., 2009). Bacterial alkaline proteases, mainly secreted by *Bacillus* strains, have also been explored due to their thermostability (Das and Prasad 2010). Proteases represent around 60% of the global market, with applications in detergents (35% of total industrial enzyme market), leather processing, food and feed manufacturing, pharmaceuticals, chemicals and bioremediation (Feijoo-Siota and Villa 2011). Properties such as high activity, stability in high alkaline range, temperature, metal ions, compatibility with detergent compounds e.g surfactants and organic solvents are drivers for their excessive industrial demand, stimulating their role in bioengineering and biotechnological sectors.

Table 2.4 includes representative studies, utilising a plethora of agro industrial resources for the production of proteases employing various strains of *Aspergillus*.

Table 2.4 Proteolytic enzymes production via solid state fermentation utilising various lignocellulosic residues by *Aspergillus* strains.

Strain	Feedstock	Activity (U/g)	Fermenter type	References
<i>A. niger</i> LBA 02	50% wheat bran + 50 % soybean meal	262.8	250 mL Erlenmeyer flasks	de Castro et al., 2015
<i>A. oryzae</i> CCBP 001	Canola cake	355.5	Reactor (16 columns, 2.5 cm×20 cm)	Freitas et al., 2015
<i>A. oryzae</i>	Sunflower meal	400.0	250 mL Erlenmeyer flasks	Kachrimanidou et al., 2013
<i>A. oryzae</i>	Rapeseed meal	728.0	Petri dishes (9 cm in diameter)	Wang et al., 2010
<i>A. oryzae</i>	Wheat flour	172.8	Petri dishes (9 cm in diameter)	Wang et al., 2009
<i>A. oryzae</i> NRRL 1808	Wheat bran	31.2	250 mL Erlenmeyer flasks	Sandya et al., 2005
<i>A. oryzae</i> MTCC 5341	Wheat bran + 4.4% defatted soy flour	8.5×10^5	500 mL Erlenmeyer flasks	Vishwanatha et al., 2010
Indigenous consortium	Hair wastes and digested sludge	5.2×10^4	10-L air tight reactor	Yazid et al., 2016

Kachrimanidou et al. (2013) proposed a two-step process for the production of crude proteolytic enzymes on sunflower meal (SFM) by *Aspergillus oryzae*, and their subsequent utilisation for the conversion of SFM into a nutrient-rich fermentative substrate, adequate for the bacterial production of polyhydroxyalkanoates when combined with glycerol. The maximum protease activity achieved was 400 U/g after 48 h of fermentation at 65 °C. The same strain was also utilized in SSF with rapeseed meal yielding 728 U/g of protease activity after 67 h (Wang et al. 2010) as well as with wheat flour yielding in 172.8 U/g after 97 h (Wang et al. 2009), for the formulation of a generic or nutrient complete fermentative feedstock. Sandhya et al. (2005) evaluated various agro-industrial by-products for their potential in neutral protease production. SSF performed on wheat bran was proved to be more effective compared to the other raw materials including PKC, with a proteolytic activity of 31.2 U/g after 72 h. de Castro et al., 2015 reported a proteolytic activity of 262.8 U/g emerging by the cultivation of *A. niger* on a

binary system of equal proportions of wheat bran and soybean meal as well as 246.0 U/g on equal blends of wheat bran, soybean meal, cottonseed meal and orange peel after 48 h of fermentation. Freitas et al. (2015) reported an activity of 355.5 U/g when cultivating *A. oryzae* on canola cake and a reactor system with 16 columns.

Enzymatic activities higher than 10^4 and 10^5 U/g have been reported. Yazid et al. (2016) developed a cost effective technology for the tannery industry, producing enzymes and a compost-like material after enzyme extraction from the solid substrate. SSF of indigenous consortium of microorganisms was carried out on hair wastes combined with anaerobically digested sludge. Maximum protease activity of 5.2×10^4 U/g was achieved after 14 days of fermentation. The alkaline protease was purified with 74% of recovery. Vishwanatha et al. (2010) optimized acid protease production using response surface methodology. Protease was purified 17 folds with a yield of 29%. Optimum fermentative factors of 5.4 pH, 31 °C, supplementation of wheat bran with 4.4% defatted soy flour, after 123 h incubation of *A. oryzae*, yielded in a maximum activity of 8.93×10^5 U/g wheat bran. Experimental values of proteolytic activity approached almost 95% the predicted one.

The SSF is a favorable process in terms of financial efficiency as it employs agro-industrial residues of low or zero cost decreasing so initial capital costs and operating costs. Future biotechnologies should focus on the establishment of effective SSF procedures for the industrial production of enzymes with specific emphasis on the development of appropriate hosts' environments and their genetic manipulation for maximum yields and productivities of the desired enzymes.

2.7.2. Solid state fermentation for lignocellulose conversion to microbial oil employing various fungal strains

Zygomycetes have been extensively studied on lignocellulosic renewable resources, for lipid production. Table 2.5 depict SSF studies that have been so far conducted, considering lipids production along with GLA content.

SSF of *Cunninghamella japonica* on pretreated and/or not supplemented cereal substrates resulted in the maximum lipid production and GLA content ever reported in the open literature. The highest lipid production and GLA content were obtained on soaked rice with respective values 371.3 mg/g of fermented solids (gfs) and 7.9% (Emelyanova, 1996). Twenty-three strains of the genera *Mortierella*, *Cunninghamella*, *Rhizopus*, *Mucor* and *Thamnidium* were

examined for lipid production on apple pomace and spent malt grains (SMG) impregnated with a nutrient solution. *Cunninghamella* and *Mortierella* strains, produced lipids varying between 150 - 168 mg/gfs during 8 days of cultivation with a GLA content fluctuating between 4.2 - 7.4%. *T. elegans* was the most efficient strain resulting in 212 mg/gfs of lipids with a GLA content of 9.1% when cultivated on a mixture of apple pomace and spent SMG impregnated with peanut oil and a nutrient solution under forced aeration (Stredansky et al., 2000). Conti et al., 2001 screened several fungal strains of the order *Mucorales* for lipid production on moistened cereals, focusing on GLA distribution. *C. elegans* was the most efficient strain resulting in 172 mg/g of lipids and 11.6% GLA when cultivated on flasks with 15:5:1 barley – SMG - peanut oil and nutrient solution for 7 days. Lipids production of the other fungal strains varied between 106 - 156 mg/g after 7 days of fermentation with a GLA content ranging between 5.3 - 8%. One hundred and forty-one endophytic fungi were isolated from seven oleaginous plants in order to be explored as potent oil accumulators. The most promising isolates resulted on microbial oil production ranging between 19 - 42 mg/gfs, when cultivated on 20% w/w steam-exploded wheat straw and 5% w/w wheat bran (Peng and Chen 2007). SSF for microbial oil production with the fungal strain *Microsphaeropsis* sp. was optimized in terms of steam exploded wheat straw to wheat bran ratio (9:1), initial moisture content (75%), and incubation temperature (30 °C) with cellulase loading of 10 FPU/g dry solids. Microbial oil was 79 mg/gfs (Peng and Chen 2008). *A. oryzae* was cultivated on a mixture of pretreated wheat straw and bran to produce lipids. Under the optimized conditions from Plackett–Burman design including inoculum size, temperature, ratio of bran to straw, ratio of substrates to mineral salt solution, initial pH and pretreatment strategy, lipid production reached 62.9 mg/gfs after 6 days of fermentation (Hui et al. 2010). Zhang and Hu (2012) demonstrated the effective lipid production by *M. isabellina* utilising soybean hull and its further application for biodiesel production. Final lipids of 47.9 mg/gfs were produced with inoculums size of 10⁴/g soybean hull, 75% initial humidity, 7-day spores without nutrient addition (Zhang and Hu, 2012).

Table 2.5 Lignocellulose conversion to microbial oil by solid state fermentation, employing various fungal strains.

Strain	Feedstock	Days	Bioreactor system	Lipid Yield (mg/gfs ⁴)	GLA on fs (% w/w)	Productivity (g/gfs ⁴ /d)	References
	Soaked rice			371.3	7.9	0.053	
<i>C. japonica</i> IBFM-2	Soaked millet and peptone	7	Petri dishes or cylindrical glass vessels, 850 mm	307.1	7.0	0.043	Emelyanova, 1996
	Soaked peeled barley			294.4	5.5	0.042	
	Apple pomace, SMG ³ and nutrients		250 mL Erlenmeyer flasks	172.0	7.5	0.021	
<i>T. elegans</i> CCF 1456			250 mL Erlenmeyer flasks	210.0	7.6	0.026	
	Apple pomace, SMG ³ , nutrients, peanut oil	8	Plastic bags 500×300 mm ² , area 600 cm ² , 100 mL/min air rate	212.0	9.1	0.026	Stredansky et al., 2000
<i>M. isabelina</i> CCF14				168.0	4.2	0.021	
<i>C. elegans</i> CCF1318	Apple pomace, SMG ³ and nutrients		250 ml Erlenmeyer flasks	166.0	7.4	0.020	
<i>C. echinulata</i> CCF103				150.0	5.8	0.019	
	Pearl barley			158.0	9.3	0.022	
<i>C. elegans</i> CCF 1318	Barley, SMG ³ , peanut oil (15:5:1) and nutrients			172.0	11.6	0.024	
<i>T. elegans</i> CCF 1456		7	500 mL Erlenmeyer flasks	156.0	8.0	0.022	Conti et al., 2001
<i>M. isabellina</i> CCF14	Pearl barley			121.0	5.3	0.017	
<i>M. ramanniana</i> MUCL 8691				113.0	5.3	0.016	

<i>C. echinulata</i> CCF 103				141	6.7	0.020	
<i>M. circinelloides</i> MUCL 15438				106	6.5	0.015	
<i>Microsphaeropsis</i> sp.				42	-	0.004	
<i>Sclerocystis</i> sp.				35	-	0.003	
<i>Phomopsis</i> sp.	Steam-exploded wheat straw mixed with wheat bran	10	Petri dishes (9 cm)	27	-	0.003	Peng and Chen 2007
<i>Nigrospora</i> sp.				23	-	0.002	
<i>Cephalosporium</i> sp.				34	-	0.003	
<i>Microsphaeropsis</i> sp.	Steam exploded wheat straw and wheat bran ¹	10	Petri dishes (9 cm)	79	-	0.008	Peng and Chen 2008
<i>A. oryzae</i> A-4	Wheat straw and bran	6	Petri dishes (9 cm)	62.9	-	0.010	Hui et al., 2010
<i>M. isabellina</i> IFO 7884	Soybean hull	8 ²	250 mL Erlenmeyer flasks	47.9	-	-	Zhang and Hu, 2012

¹ Optimized conditions; ² weeks; ³ spent malt grain (SMG); ⁴ grams of fermented solids (gfs)

2.8. Downstream for microbial oil recovery

2.8.1. Microbial biomass recovery

The downstream process for microbial oil recovery initiates with biomass harvesting from the fermentation broth. Harvesting method contributes significantly on the economics of bioprocesses that utilize microbial oil as the raw material for the production of value-added products. So far, several harvesting methods have been reported including centrifugation, coagulation, filtration, and flotation, thus any of them has been characterized as cost-effective. Water removal from the microbial strains by centrifugation accounts normally for 20 - 30% of the total production cost (Dickinson et al., 2017). Direct microbial oil recovery from wet biomass is a promising practice due to the elimination of the drying stage which is cost intensive. Thus, this process needs extensive research to be applicable at an industrial scale.

2.8.2. Cell wall disruption

The extraction process for lipids recovery is highly dependent on the type of lipid fraction i.e neutral or polar lipids, and their interaction with a membrane protein. The extraction of lipids from microbial biomass is carried out in two steps. Initially physical, chemical or enzymatic disruptions of the cell wall are performed followed by the use of a solvent for lipids recovery. Cell disruption is critical as it has a direct impact on subsequent downstream efficiency (Senanayake & Fichtali, 2006). Conventional lipids extraction is based on methods developed by Folch et al. (1957) and Bligh & Dyer (1959). These methods require a mixture of a non-polar solvent i.e chloroform and a polar solvent i.e methanol to extract the lipids from the dry biological material.

2.8.2.1. Mechanical cell disruption methods

Mechanical methods for cell disruption include the application of shear stress abrasion and they have great industrial potential. Among them, bead milling is a widely applied and effective technic for various algal species, i.e *Chlorella* sp., (Lee et al., 2010), yeasts i.e *Rhodotorula gracilis* (Channi et al., 2016), bacteria i.e *Bacillus cereus*, and fungi i.e *Penicillium citrinum*, (Klimek-Ochab et al., 2011). Ultrasonication has been so far applied in industrial scale for protein extraction and chemical synthesis. Zhang et al. (2014) used ultrasonication in combination with various solvents for lipid extraction from the yeast *Trichosporon oleaginosus*. Chloroform/methanol resulted in lipids efficiency of 100%.

2.8.2.2. Non-Mechanical Cell Disruption Methods

Non-mechanical cell disruption includes physical and chemical methods. Microwave-assisted lipid extraction is an efficient process in terms of yield and cost, operating on non-ionizing electromagnetic oscillating waves, in the range of 300 MHz to 300 GHz. Heat is generated in the polar material and the reorientation of the molecules results in friction and cell rupture (Kumar et al., 2017). Limitations of the methods are related to heat and free radical creation, damaging eventually PUFAs (Günerken et al., 2015). Electroporation consists of two electrodes connected with an electrical power supply in a pulsed electric field (PEF) and the fermentation media is passed between the electrodes. The PEF causes fracture of the cell wall, maintaining the cells viable for further extraction of added-value molecules (Reep & Green, 2012). PEF has been applied for lipid extraction from cyanobacteria i.e. *Synechocystis* PCC 6803) and microalgae i.e. *Chlorella vulgaris*. Respective lipids recovery of 25-75% (Liu et al., 2011) and 50% (Flisar et al., 2014) was obtained. PEF has high potential to be applicable in a large scale due to low energy requirements.

Biocompatible solvents have been used for β -carotene extraction from *Dunaliella salina* using a biphasic reactor (Jackson et al., 2017). The solvents create pores in the cell membrane permitting the secretion of lipids outside the cells. The partition coefficient of a solvent should be high for highly efficient extraction (Dong et al., 2016). Surfactant-assisted lipid extraction is non-toxic, uses biodegradable chemicals and normally is applied on wet biomass. Surfactants are distinguished by hydrophobic and hydrophilic moieties. Cell membranes possess negative charges due to functional groups and they can be disrupted by hydrophobic parts (Kumar et al., 2017). Several surfactants i.e. myristyltrimethylammonium bromide (Lai et al., 2016), N-lauryl sarcosine (Yellapu et al., 2016), and sodium dodecyl sulphate (Wu et al., 2017) have been investigated for lipid recovery from different algal species with efficiencies varying between 82 - 100%, demonstrating their potential for industrial practice. Supercritical fluid extraction (SFE) deals with chemicals that behave as both liquid and gas in their critical temperature and pressure. CO₂ is the most suitable molecule due to properties such as low viscosity, high diffusivity and proper critical temperature and pressure. Biomass contacts with supercritical CO₂ for a specific period of time and lipids are subsequently solubilized in CO₂ and extracted. Thereafter, CO₂ is depressurized. SFE has been applied for lipids extraction from the yeast *Rhodotorula glutinis* with an efficiency of 99% thus resulting in high energy requirements (Duarte et al., 2017).

2.8.2.3. Green recovery of lipid

Traditional lipids extraction employs organic solvents that have a heavy environmental impact, leading research toward eco-friendly and green recovery systems such as PEF, enzyme degradation, simultaneous distillation and extraction process and solvent-free extraction via non-woven fabric (Yellapu et al., 2018). The method of solvent-free extraction via non-woven fabric was used for lipids extraction from the yeast *Rhodotorula glutinis* after concentration and homogenization of the cells, yielding in 10.4 g of lipids per gram of fabric (Shang et al., 2015). This technique is characterized as recyclable and environment-friendly due to the employment of the non-woven fabric. Another green and economically viable technic for lipids recovery constitute ionic liquids, which are non-volatile compounds with good thermal stability. Cooney and Benjamin (2016) reported low extraction efficiency when applying ionic liquids for lipids recovery from algal biomass.

An efficient extraction process should provide maximum product's recovery, reduction of the product contamination, eco-friendly nature, enhanced mass transfer phenomena and simplification of the downstream processing. Consequently, research should focus on aspects including scalability, extraction efficiency, and energy requirements.

2.9. Phytochemicals - The case of phenolic compounds

Phytochemical refer to a wide variety of compounds that occur naturally in plants. They are classified into six main categories based on their chemical structures and characteristics including carbohydrate, lipids, phenolics, terpenoids and alkaloids, and other nitrogen-containing compounds (Huang et al., 2015). Phytochemicals constitute a very promising aspect taking into consideration that the global herbal supplement and remedy market is predicted to reach 8.8 billion US\$ in 2023 at a CAGR of about 4.7% (Anonymous 2018 a).

Palm based phytochemicals mainly include phenolic compounds, terpenes, and sterols (Ofori-Boateng and Lee 2013). The largest and more attractive group encompasses the phenolic compounds mostly phenolic acids, flavonoids, tannins and lignans. Phenolic compounds, widely distributed as secondary metabolic products, appear in either free or in bound forms. They possess remarkable antioxidant activity related to their ability to act as chelators of divalent cations, scavenge free radicals and break radical chain reactions (Babbar et al., 2011).

Several reports have demonstrated the positive effects of these bioactive compounds on the pharmaceutical, medical, and nutraceutical field (Xavier et al., 2017 a; Pande et al., 2017). More

specifically, 4-hydroxybenzoic acid has already been applied as a preservative in pharmaceuticals, foods and cosmetics (Chakraborty et al., 2006). The conventional synthesis of 4-hydroxybenzoic acid involves the chemical reaction of phenol, potassium hydroxide and carbon dioxide under high-pressure conditions. This process bears a high environmental impact not only due to the raw materials but also due to the production of toxic liquid wastes. Finding renewable sources of this phenolic compound is an interesting alternative towards a more sustainable economy. Kawamura et al. (2014) proposed that low molecular weight phenolic compounds could be used for the production of 2-pyrone-4,6-dicarboxylic acid, which is a precursor for the production of various polymers such as polyamide, polyester and polyurethane.

Pyrogallol and catechol are synthesized in plants through the shikimate pathway. Catechol has been found in extracts from the leaves and needles of various deciduous and coniferous trees, whereas pyrogallol is considered as a product of the degradation of plant debris. Moreover, these phenolic compounds are typically found in soils, and also they are precursors of humic-like polymer synthesis (Kocaçalışkan et al., 2006). Pyrogallol finds a broad range of applications due to its antioxidant and antibacterial properties including food sector, cosmetics, chemical and pharmaceutical industries as well as in photography, printing and to dyes production (Wang et al., 2018). A pyrogallol-based oxygen scavenging system with sodium carbonate was proven to be effective for food packaging applications with low water activity (Gaikwad and Lee, 2016). Pyrogallol was also tested for cotton fabric treatments, leading to a final product with >99.9% antibacterial ability and >90 % antioxidant activity (Hong et al., 2015). Depending on the application purposes and its purity, the price for pyrogallol varies from \$20-100 per kilogram, depending on the supplier.

Ferulic acid is a precursor of vanillin, a well-known flavoring agent widely used in food, beverages and cosmetics (Kumar and Pruthi, 2014). Food grade or pharmaceutical grade ferulic acid can cost from US \$100-1000, again depending on its purity, origin and the supplier. Ferulic acid has been reported to increase shelf life of linseed oil and to enhance the stability of lard and soybean oil. A mixture of ferulic acid and glycine was proven to completely inhibit the oxidation of biscuits.

The prospect of using phenolic compounds, deriving from plant biomass, as natural food antioxidants is of paramount industrial interest. Agro-industrial by-products are promising sources of phenolic compounds and they have been explored as a source of natural antioxidants

for the maintenance of nutritional quality through the stabilization or shelf-life extension of lipids and lipid-containing food products. However, several issues associated with efficiency of phenolic extraction, availability of feedstock and safety considerations among others should be considered for the development of products supplemented with appropriate phenolic formulations. Rosemary extracts at a concentration of 500 ppm have been reported as very effective antioxidants in bulk corn oil and significantly more active than carnosol (Frankel et al., 1996). According to Wanasundara and Shahidi (1994) the induction time of canola oil at 65 °C, supplemented with 0.1% ethanol extracts from canola meal, was prolonged in comparison to 0.02% BHA and BHT. Goli et al. (2005) demonstrated that pistachio hull extracts (0.06%) were as effective as BHA and BHT (0.02%), in preventing oxidation of soybean oil at 60 °C. Phenolic compounds obtained from grape pomace, exerted similar antioxidant effect with propyl gallate in fish oil-in-water emulsions (Pazos et al., 2005).

2.10. Oleochemicals

Edible and non-edible vegetable oils including mostly sunflower oil, palm oil, soybean oil, rapeseed oil and coconut oil, have been applied for the production of bioesters for many years. However, the wide availability and low cost of mineral oils established their use as the basis for esters synthesis during the 19th and 20th century. To date, the development of bio-based esters from renewable and sustainable resources has gained significant attention due to the severe impact of petroleum-based oil on several environmental issues. Global market of fatty acid esters was valued at USD 1.8 billion in 2014 and is expected to reach USD 2.4 billion by 2022, with a CAGR of 4.6% (Anonymous, 2016c). Short and middle chain fatty esters, which hold the largest market share (34.5%), are applied as surfactants or biolucricant base oil, detergents, in personal care and cosmetics as emollients, fragrance and skin-conditioning agents and make-up products, in food processing as flavoring agents and in pharmaceuticals (Anonymous, 2018b). It is estimated that around 35 million t of lubricants are used annually with approximately 55% ending up into the environment (Syahir et al., 2017) through evaporation, spillage and total-loss lubrication (Willing 2001). Over 95% of these lubricants are petroleum-based and so, toxic and non-renewable. Lubricants can be used in a wide range of applications including lubrication for the reduction of wear and saving energy, transfer power as hydraulic fluids, cooling due to good thermal conductivity and cleaning for the removal of internally generated debris and external contaminants. Consequently, they are used as engine,

compressor, chainsaw and gear oils as well as hydraulic and insulation fluids (Zainal et al., 2018).

Conventional chemical practice for esters synthesis using vegetable oils, is transesterification or esterification of the oil with various lower and higher chain length alcohols catalyzed by acids or alkali under elevated temperatures (100 - 300 °C), resulting in fatty acid alkyl esters (Panchal et al., 2017). The fatty ester industry is constantly driven towards biocatalytic pathways for the synthesis of esters, to meet global requirements for ecological balance, sustainability issues, and 'green' products. Lipases (triacylglycerol ester hydrolases, E.C. 3.1.1.3–IUPAC) have attracted remarkable attention as catalysts for the production of oleochemicals, due to their stereo-, chemo- and region-selectivity (Fernandes et al., 2018). Biocatalysis is more promising for the production of fatty esters than conventional chemical practice, due to process simplification, high quality end-products, elimination of side-products formation and product degradation, and mild operative conditions. Evonik Industries AG and Eastman Company are two representative manufacturers that commercialized lipases-catalyzed esters in a multiton scale. The latter demonstrated the effective contribution of biocatalysis on diminution of CO₂ emissions, waste generation, and energy consumption (Ansorge-Schumacher and Thum, 2013).

Khan and Rathod (2015) have provided a thorough review on enzymatic synthesis of cosmetic esters. Biocatalysis is the most suitable practice providing mandatory specifications for cosmetic application. Fatty acid esters with surfactant properties have been so far produced using C12 - C20 fatty acids with various C5 - C6 sugars or polyols as acyl acceptors. Phenolic esters with antioxidant properties are synthesized with various hydroxycinnamic acids and alcohols such as 1-octanol, 1-butanol, and 1-propanol. L-ascorbic esters with antioxidant properties include reactions between palm oil, palmitic acid, vinyl and methyl palmitate or C12 - C14 saturated fatty acids with L-ascorbic acid. Esters with fragrance and flavor properties derive from reactions between alcohols including geraniol, cinnamyl alcohol, isoamyl alcohol, ethanol, n-propanol and n-butanol with acyl donors such as acetic, butyric, lauric and myristic acids. All the aforementioned reactions are carried out in a system catalyzed by lipases mostly produced from strains of *Candida* sp., *Rhizomucor* sp. and *Rhizopus* sp. The solvents usually applied include ionic liquids, ethanol, tert-butanol, isooctane, heptane, hexane, toluene, 2-methyl-2-butanol or ether and pyridine based solvents. The enzymatic process is conducted at low temperatures varying between 30 - 70 °C, the formation of ultrapure, colorless, and odorless products (Khan and Rathod, 2015).

Lubricants derived from vegetable oils show properties such as low toxicity, high biodegradability, high lubricity, high flash point, good viscosity index, and low friction and wear features compared to mineral oil-based lubricants. Thus, usage of bio-based lubricants covers a narrow segment of the market due to issues regarding their performance and production scale. In general, biolubricants derived from crude vegetable oils show poor cold flow behaviour and oxidation stability, leading to polymerization and degradation. Such deficiencies could be minimized by chemical modification of vegetable oils for the elimination of β -hydrogen atoms in glycerol. Additionally, to fulfil requirements for targeted applications and improve lubricants properties several additives are used accounting for up to 5% of the lubricants' mass. Esters with biodegradable additives are superior to pure oils or vegetable oil mixtures enhancing resistance to corrosion (Balamurugan et al., 2010). Bio-based lubricants possess more enhanced lubrication properties than mineral and synthetic oils combined with high renewability, biodegradability and non- or low toxicity, rendering them as unique candidates for highly sensitive applications such as forestry and marine. Along with this, the adoption of stringent laws and regulations for greener products ("European Ecolabel for Lubricants") and the protection of the environment and human health from waste oils (Waste Framework Directive 2008/98/EC) is eventually leading to increased demands for bio-based lubricants (Papadaki et al., 2018). Marketability of bio-lubricants is highly dependent on the establishment of efficient large scale production achieving adequate yield rates and economic sustainability.

So far, microbial oil exploitation for esters formulation is in a preliminary stage. Microbial oil rich in polyunsaturated fatty acids i.e GLA could provide a promising feedstock for the cosmetic industry. Bandhu et al. (2017) reported the efficient microbial oil production cultivating *Rhodotorula mucilaginosa* on sugarcane bagasse hydrolysates. The microbial oil showed high viscosity index and a low pour point demonstrating good quality lubricant properties rendering it as an alternative to the conventional mineral lube oil-based lubricants. Papadaki et al., 2017 studied the production of wax esters using microbial oils produced from food waste and by-product streams by three oleaginous yeasts. Enzymatic transesterification of microbial oils to behenyl esters resulted in conversion yield up to 87% and the determination of their physicochemical properties showed that behenyl esters were compared well with natural waxes. The same group (Papadaki et al., 2018) utilized microbial oil produced from confectionery and wheat milling side streams for biolubricants production. Conversion yields up to 88% were achieved when microbial oil produced from *Rhodospiridium toruloides* was

enzymatically esterified with neopentyl glycol (NPG). Physicochemical characterization of the produced biolubricants demonstrated their potential as substitutes for conventional lubricants.

CHAPTER 3

Objectives

This PhD thesis deals with the exploitation of various industrial solid and liquid waste streams for the production of added-value metabolites with applications in food and chemical industry. Specifically, by-products from palm oil processing were used for the production of hydrolysates which served as the nitrogen sources in microbial oil fermentation carried out with *L. starkeyi*. The same by-product streams were subsequently used for phenolic compounds extraction which were further applied as promoters of oxidative stability in sunflower oil. Next, protein rich cakes derived after oil extraction were used as the solid matrices, providing carbon and nitrogen sources for solid state production of fungal lipids which were used as the raw material for the synthesis of esters with lubricant properties. Spent sulphite liquor, the major waste stream of pulp and paper industry was used as the sole carbon sources for microbial oil production with *L. starkeyi* and various fungal strains under batch fermentation mode and fed-batch bioreactor cultivation. Lipids produced by *C. echinulata* were applied as substrate for the production of esters with potent application in the cosmetic industry. The main objectives of the experimental work are presented as follows:

- ✓ Valorisation of by-products from palm oil processing for the production of generic fermentation media for microbial oil synthesis
- ✓ Extraction of phenolic compounds from palm oil processing residues and their application as antioxidants in sunflower oil
- ✓ Synthesis of fatty acid esters using γ -linolenic acid rich microbial oil produced via fungal fermentation of spent sulphite liquor
- ✓ Bioprocess development for biolubricant production using microbial lipids derived via solid state fermentation of protein rich side streams
- ✓ Spent sulphite liquor utilisation for the production of microbial oil under batch and fed-batch fermentation mode

CHAPTER 4

Materials and methods

4.1. General practise

All materials, utensils and solutions involved in fermentations processes, were previously autoclaved at 120 °C for 20 min. Fermentative procedures were performed under sterile conditions using a laminar flow cabinet (Telstar, Bio II Advance).

4.2. Renewable feedstock

Palm kernel cake (PKC), pressed palm fiber (PPF), palm kernel shells (PKS) and empty fruit bunches (EFB) obtained after the palm oil extraction process, were kindly supplied by the company Agropalma (Pará, Brazil). Cotton seed cake (CoSC) and castor seed cake (CaSC) were provided by Petrobras R&D Center (Rio de Janeiro, Brazil). The aforementioned solid residues were utilized as substrate for solid state fermentation (SSF). Crude molasses (46.7%, w/w purity) and glycerol (78.1%, w/w purity) were kindly provided by Petrobras (Rio de Janeiro, Brazil).

The spent sulphite liquor (SSL) utilized in this study was generated by the acidic sulphite pulping process of *Eucalyptus globulus*. SSL and the extracted lignosulphonates were kindly provided by Sniace S.A. (Torrelavega, Spain). SSL was pretreated for lignosulphonates removal. Pretreatment of SSL was conducted by nanofiltration using a vibratory shear-enhanced processing filtration unit (V-SEP, New Logic Research, Emeryville, CA). The nanofiltrated SSL was provided by the company AVECOM NV (Belgium). The membranes used in the V-SEP filter had molecular weight cut-offs (MWCO) of 800 Da (polyethersulfone, NF-PES-10) and surface area of 1.5 m². Filtration using the V-SEP equipment was carried out with 3.5 diluted SSL. The total filtration volume used was 560 L of diluted SSL. Flushing with hot water during operation was employed in order to increase the membrane flux. Periodical caustic cleaning recovered entirely the initial membrane flux during operation. The temperature and pressure used during filtration were respectively 70 °C and 400 psi. The filtration flux was modified during operation to achieve an acceptable flux during filtration in the range of 40.7 - 10 L m⁻² h⁻¹.

4.3. Microbial strains, pre-culture conditions and preservation

The fungal strains *Aspergillus oryzae* was kindly provided by Professor Colin Webb (University of Manchester, UK) and it was used for the production of crude enzyme consortia (mainly proteolytic) through solid state fermentation, which were subsequently used in

hydrolytic experiments. Spores were preserved in silica sand and their reconditioning was carried out using PBS solution containing 8 g/L NaCl, 0.2 g/L KCl, 1.4 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄. *A. oryzae* was sporulated and stored at 4 °C in agar slopes, containing 30 g/L palm kernel cake, 20 g/L wheat milling by-products and 20 g/L agar. The slopes were applied for the inoculation of flasks with the same solid material, which were further used as inoculum in solid state fermentations. More specifically 10 mL of deionized water and Tween 80 (0.01 %, v/v) (Sigma-Aldrich) were added into each slope and the surface was scratched with a wire loop. Subsequently, 1 mL of the spore suspension was added on each flask. Incubation was performed at 30 °C in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) for 4 days. Subsequently, 50 mL of deionized water, supplemented with Tween 80 were added in flasks with the grown fungus and after vigorous shaking using glass beads of 4 mm diameter, a spore suspension was obtained (3×10^7 spores/mL) which was used as inoculum for SSF implementation.

The oleaginous yeast strain *Lipomyces starkeyi* DSM 70296 utilized in the fermentative production of microbial oil was purchased from DSMZ culture collection. The strain was maintained on slopes containing 15 g/L glucose, 15 g/L yeast extract, 15 g/L peptone and 20 g/L agar at 4 °C. A liquid pre-culture with the same composition was prepared as inoculum and incubated at 30 °C in an orbital shaker, at agitation rate of 180 rpm for 40 h.

The fungal strains, *Cunninghamella echinulata* ATHUM 4411, *Mortierella ramanniana* MUCL 2935, *Mortierella ramanniana* ATHUM 2922, *Mortierella isabellina* ATHUM 2935 and *Thamnidium elegans*, used for the production of microbial oil, under submerged and solid state fermentations, were maintained on potato dextrose agar (PDA) slopes and petri dishes at 4 °C. All fungal strains were fully sporulated prior to each experiment in Erlenmeyer flasks containing PDA. The inoculum preparation was similar to that of *A. oryzae* with some modifications. The water volume applied for the PDA flasks after fungal growth for 5 - 7 days, was 50 - 120 mL, resulting in a final spore suspension of $3 - 6 \times 10^6$ spores/mL.

4.4. Evaluation of palm oil processing residues for phenolic compounds

Preliminary extraction experiments were conducted to evaluate PCK, PPF, PKS and EFB for their total phenolic content and antioxidant activity. The extraction parameters were fixed as follows: 20 min extraction time and 30:1 liquid-to-solid ratio.

Subsequently, three different extraction times (10 min, 20 min, 40 min) and four liquid-to-solid ratios (5:1, 20:1, 30:1, 40:1) were studied to identify the best extraction conditions for phenolics from the selected side stream.

4.5. Solid state fermentation for the production of crude enzyme consortia and subsequent production of nutrient-rich hydrolysate

This part deals with the evaluation of PKC as potent solid matrix for the production of crude enzyme consortia, mainly proteases, in tray reactors and rotating drum bioreactor. Subsequent hydrolysis of PKC employing the produced crude enzymes was evaluated for the formulation of a nutrient rich supplement that could substitute commercial nitrogen sources applied in fermentative processes.

4.5.1. Tray reactors

SSF in tray bioreactors were conducted in 250 mL Erlenmeyer flasks at 30 °C. Two different ratios of PKC and PPF (at 100:0 and 70:30 ratios) were assessed as substrates. In each case flasks were filled with 5 g of and were inoculated with an appropriate quantity of fungal spore suspension, as described in Section 4.3, in order to adjust the moisture content of the substrate. Five initial moisture contents (50, 55, 60, 65 and 70 %, w/w) were evaluated for the production of proteases during SSF.

Fermented solids were macerated using a conventional kitchen blender and subsequently mixed with 500 mL sterilized deionized water in 1 L Duran bottles containing a specific amount of PKC. Mixing of the suspension was achieved with magnetic stirrers. The effect of temperature (45, 50, 55, 60, 65 °C), initial concentration of PKC (48.7, 73.7, 98.7 g/L on a dry basis) and two initial proteolytic activities (6.0, 11.9 U/g) were evaluated in order to optimize the hydrolysis process. Samples were withdrawn at regular intervals and mixed with equal quantity of trichloroacetic acid (5%, w/v) to stop enzymatic reactions. The supernatant was used for the analysis of free amino nitrogen (FAN) and inorganic phosphorus (IP). At the end of enzymatic hydrolysis, PKC hydrolysates were centrifuged (9000 × g, 4 °C, 15 min) and the supernatant was filter-sterilized through a 0.22 µm filter unit (Polycap AS, Whatman™ Ltd., Buckinghamshire, UK) for its subsequent use in the fermentative production of microbial oil with *L. starkeyi*.

4.5.2. Rotating drum bioreactor

The SSF for enzyme production, was performed in 15 L Terrafors-IS (Infors, Switzerland) bioreactor at 30 °C for 48 h with an aeration rate in the range of 2.4 - 3 l air/min/kg and intermittent agitation of 0.1 rpm that was applied every 30 min. A quantity of 1000 g of a mixture of PKC and PPF (at 70:30 ratio) was added in the drum bioreactor. The ratio of 70:30 of PKC to PPF solids was used because using only PKC solids resulted in an inefficient fermentation due to cohesion of particles during fermentation, which most probably occurred due to protein hydrolysis that increases the adhesive properties of the mixture. The pre-inoculum was prepared in a tray bioreactor as mentioned in Section 4.3. In this case, a quantity of 250 g of the same substrate mixture was used and fermentation was conducted for 24 h at 30°C. Subsequently, fermented solids were inoculated in the rotating drum bioreactor in a 4:1 ratio of total solid substrate to inoculum ratio. Deionized water was used to adjust the initial moisture content to the optimum condition observed in the fermentations carried out in the tray bioreactor (55%, w/v).

After the end of the SSF, 5 L sterilized deionized water and 500 g of fresh PKC were added in the drum bioreactor to initiate the hydrolysis process. The hydrolysis was conducted for 48 h at 50 °C and intermittent agitation of 10 rpm. Samples were withdrawn at regular intervals and mixed with equal quantity of trichloroacetic acid (5%, w/v) to stop enzymatic reactions. The supernatant was used for the analysis of free amino nitrogen (FAN) and inorganic phosphorus (IP).

4.6. Microbial fermentations for lipids production

With respect to batch submerged fermentations, all experiments were conducted in 250 mL Erlenmeyer flasks with a working volume of 50 mL. Flasks were inoculated with 10% (v/v) yeast pre-culture or 2% (v/v) fungal spore suspension as mentioned in Section 4.3 and incubated at 30 °C in an orbital shaker, at agitation rate of 180 rpm. The fermentation media was enriched with a mineral solution containing (in g/L): KH_2PO_4 , 7.0; Na_2HPO_4 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.15; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.06; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.15. The pH value was adjusted to 6.0 during fermentation using 5 M NaOH. Samples were taken at regular intervals to assess sugar and nitrogen consumption along with biomass and lipid concentration. Lignosulphonates and phenolic compounds determination was carried out for microbial fermentations carried out in Section 4.6.2.1.

4.6.1. Batch submerged fermentations with *L. starkeyi* using palm kernel cake hydrolysates

The potential of microbial oil production utilising PKC hydrolysates as nitrogen source was evaluated by conducting shake flask fermentations with *L. starkeyi*. Various commercial carbon sources including glucose, xylose, mannose, galactose and arabinose were combined with PKC hydrolysates to formulate the fermentation feedstock for yeast proliferation and microbial oil accumulation. In the case of the reference fermentation with commercial nitrogen sources, 3 g/L yeast extract and 1 g/L $(\text{NH}_4)_2\text{SO}_4$ were included.

4.6.2. Batch submerged fermentations utilising spent sulphite liquor

The yeast strain *L. starkeyi* and five fungal strains (Section 4.3) of the division *Zycomycota* were initially evaluated for lipids production under shake flask fermentations, utilising properly diluted nanofiltrated SSL (30 g/L total sugars). Yeast extract (0.5 g/L) and inorganic $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/L) were used as the nitrogen sources.

After the screening step, the selected fungi namely, *C. echinulata* and *M. isabellina* as well as *L. starkeyi* were submitted to various regimes to assess microbial oil production and microbial behaviour as follows. Inorganic nitrogen source (0.5 g/L $(\text{NH}_4)_2\text{SO}_4$) was used in all cases.

4.6.2.1. Carbon to nitrogen ratio effect

The effect of carbon to nitrogen ratio, calculated and expressed as carbon to free amino nitrogen ratio (C/FAN ratio), was evaluated for all microbial cultures using nanofiltrated SSL (5 g/L lignosulphonates). This ratio was altered by applying different yeast extract concentrations (0.5 - 4 g/L) while carbon concentration was kept stable at 30 g/L.

4.6.2.2. Lignosulphonates concentration effect

Continuing with the best C/FAN ratio of the previous Section (3.6.2.1), various LS concentrations (10 - 120 g/L) were applied in a fermentative media simulated according to the sugar composition of nanofiltrated SSL, for all the selected oleaginous microorganisms.

4.6.3. Fed-batch submerged fermentations utilising spent sulphite liquor

Fed batch fermentations with *L. starkeyi* and *C. echinulata* were carried out in a bench-top bioreactor (Labfors 4, Infors HT) with working volume of 1 L at constant temperature of 30 °C. The pH was maintained at 6 using 5 M NaOH. Each experiment was initiated in batch mode

with nanofiltrated SSL (both microorganisms) and sugar-simulated SSL containing 90 g/L of lignosulphonates (only *C. echinulata*) as fermentation media, supplemented with mineral solution and inorganic nitrogen source as aforementioned in Section 4.6. Continual feeding supply, at varying pump rates, determined by the catabolic activity of each microorganism was applied with a total sugar concentration of 450 - 550 g/L. Feeding solution was simulated to the sugars and lignosulphonates percentage contained in nanofiltrated SSL.

In the case of *L. starkeyi*, four sets of experiments were conducted at C/FAN ratios of 173, 51, 33 and 26. Cascade agitation rate of 200 - 500 rpm was applied for the C/FAN ratios of 173 and 51 while 200 - 800 rpm were applied for the C/FAN ratios of 33 and 26. Cascade agitation rates in combination with a continuous air supply increased in several steps from 1 - 2 vvm, were used to maintain the dissolved oxygen concentration at 20%. 10% (v/v) inoculum was employed using a 40 h pre-culture (Section 4.3). Continual feeding was initiated when sugars concentration reached up to 16 g/L.

In the case of *C. echinulata*, two sets of experiments (5 g/L and 90 g/L lignosulphonates) were conducted at C/FAN ratio of 101. Cascade agitation rate of 150 - 300 rpm and a continuous air supply of 0.5 vvm were used to maintain the dissolved oxygen concentration at 20%. 2% (v/v) of spores' suspension according to the methodology described in Section 4.3, was employed as inoculum. Continual feeding was initiated after 48 hours of fermentation when sugars concentration reached up to 13.5 g/L into the fermentation broth.

Samples were taken periodically throughout fermentation and concentrations of sugars, nitrogen, biomass and microbial oil were determined.

4.6.4. Fungal solid state fermentation in tray reactors

SSF were conducted in tray bioreactors using a non-humidified chamber. Erlenmeyer flasks of 250 mL were filled with 5 g of substrate and were inoculated with appropriate quantity of fungal spore suspension, as described in Section 4.3, in order to adjust the moisture content of the substrate. Initially, a screening of five fungal strains was implemented using PKC as substrate. Their ability to grow and produce lipids was studied at 30 °C and 65% (w/w) initial moisture content for up to 15 days. In sequent experiments, *C. echinulata* and *M. ramanniana* MUCL 9235 were chosen for further assessment of fermentative conditions. Lipid production was evaluated in terms of temperature (25, 27, 30, 33, 35 °C), initial moisture content (55, 60, 65, 70 and 75%) and PKC to PPF ratios (1:1 and 3:1).

The best conditions (temperature, initial moisture content and PKC to PPF ratio) determined in previous experiments for *C. echinulata* and *M. ramanniana* MUCL 9235 were repeated in tray bioreactors using an air humidified chambers controlled at 90% air saturation. In this case, 600 mL beakers were used filled with 12 g of substrate. For further optimisation of SSF in air humidified chamber, *C. echinulate* was selected using the best fermentation conditions (65% initial moisture content, 30 °C and PKC as the sole substrate). A set of experiments were conducted using different substrate supplementations, such as: a) glycerol (5%, w/w), b) molasses (5%, w/w), c) mineral solution (g/L: KH₂PO₄, 7.0; Na₂HPO₄, 2.5; MgSO₄·7H₂O, 1.5; FeCl₃, 0.09; ZnSO₄·7H₂O, 0.02; MnSO₄·H₂O, 0.06; CaCl₂·2H₂O, 0.15) and d) nitrogen sources (yeast extract 0.5%, w/w and (NH₄)₂SO₄ 0.16%, w/w). Thereinafter, two agro-industrial residues, richer in protein content than PKC, namely CoSC and CaSC were assessed for lipid production through SSF.

Samples were taken at regular intervals to evaluate lipid, biomass, proteases and lipases production along with water activity and humidity loss during fermentation.

4.6.5. Solid state fermentation in packed bed bioreactors with *C. echinulata*

SSF of *C. echinulata* was performed in water-jacketed PBR - Raimbault columns (height, 20 cm; diameter, 4 cm) containing 30 g of CoSC for 10 days. Temperature (30 °C) was controlled with circulating coolant water. Aeration via saturated air, was pumped through the columns, and controlled at 10 L air/min/kg with a flowmeter attached to the column air outlet. The inoculum was prepared in a tray bioreactor as mentioned in Section 4.3. Samples were taken at regular intervals to evaluate lipid, biomass, proteases and lipases production along with water activity and humidity loss during fermentation.

4.7. Production of esters

4.7.1. Raw material

Reactions for the production of esters were catalyzed by commercial lipases, employing a solvent-free system. The process was conducted in water jacketed closed vials, agitated on a hotplate stirrer, at 165 rpm. Samples of 100 µl were taken at regular intervals and centrifuged (9000 × g, 10 min) to separate the enzyme from the reaction mixture.

Microbial oil deriving from submerged batch cultivations of *C. echinulata* on SSL fermentation media, was utilized as raw material for the production of bio-esters. Microbial oil extracted

from SSF of *C. echinulata* on CoSC was applied for the production of polyol esters with lubrication properties. The average molecular weight of the applied lipids was calculated based on their fatty acid profile, according to the methodology given by Shrestha and Gerpen (2010).

4.7.2. Lipases and alcohols applied

The commercial lipase Novozyme 435 (lipase B from *Candida antarctica*, immobilized on a macroporous acrylic resin) was purchased from Sigma-Aldrich and it was involved in the biocatalysis for cosmetic esters synthesis. In this case, reactions were performed with isopropyl alcohol (ISA) and 2-ethylhexyl alcohol (EHA) which were purchased from Penta and Sigma-Aldrich chemicals, respectively.

The commercial lipase Lipomod 34MDP (from *Candida rugosa*), was kindly provided by Professor Denise M.G. Freire (Federal University of Rio de Janeiro, Brazil). Lipomod 34MDP was applied as biocatalyst for the hydrolysis of microbial oil as well as for the synthesis of esters with lubrication properties. Esterification reactions were carried out using neopentyl glycol (NPG) polyol (Sigma-Aldrich).

4.7.3. Production of bio-esters

Microbial oil, fractionated lipid classes and hydrolyzed microbial oil were utilized for the enzymatic production of fatty acid esters. Conversion yields of palmitate (PE), oleate (OE), as well as sum of linoleate and linolenate (LAE & LNE) esters, derived from reactions with both ISA and EHA were monitored. The sum of PE, OE and LAE & LNE indicates the total conversion yield (TCY).

4.7.3.1. Temperature effect

Transesterification reactions with microbial oil and the two alcohols were studied at five different temperatures (30, 40, 50, 60 and 70 °C) using an enzyme loading of 10% (w/w), 1:3 microbial oil to alcohol molar ratio and a total reaction time of 24 h.

4.7.3.2. Substrate molar ratio effect

Microbial oil was transesterified with both alcohols at various lipid to alcohol molar ratios (1:2.84 - 1:4). Reactions were carried out using an enzyme loading of 10% (w/w) at 60 °C, for 24 h.

4.7.3.3. Enzyme amount effect

Transesterification reactions of microbial oil with both alcohols were also performed applying different enzyme amounts in the range of 5 - 20% (w/w), using a 1:3 microbial oil to alcohol molar ratio, at 60 °C for 24 h.

4.7.3.4. Kinetic profile of esters production via transesterification of microbial oil

Conversion yield of esters was monitored over 48 h under the optimized conditions (temperature, substrate molar ratio and enzyme amount), by taking samples at regular intervals.

4.7.3.5. Effect of individual lipids fractions on esters conversion yield

Transesterification reactions using the individual lipid fractions were carried out to evaluate their impact on esters conversion yield. After the fractionation of microbial oil, which is described in the Section 4.8.9 neutral lipids (NL) were used in transesterification reactions with ISA and EHA using different molar ratios (1:3; 1:3.35 and 1:3.6), at 60 °C for 24 h. Moreover, the effect of glycolipids plus sphingolipids (GL+SL) and phospholipids (PL) fractions on TCY of isopropyl esters was evaluated in mixtures with NL. The ratios between lipid fractions used in these experiments were equivalent with their percentage in total lipids (TL). The effect of lipid classes on TCY was also evaluated using higher quantities of GL+SL and PL in mixtures of NL (NL+2(GL+SL) and NL+2PL).

4.7.3.6. Esterification reactions with hydrolyzed microbial oil

The microbial oil was enzymatically hydrolyzed, as described in Section 4.8.9, for subsequent esterification reactions with both alcohols. The reactions were carried out using a stoichiometric molar ratio of 1:1 (w/w), 10% (w/w) biocatalyst, at 60 °C, for 24 h.

4.7.3.7. Enzyme reuse and stability determination

Enzyme stability was assessed throughout six sequential esterification reactions. Reactions were carried out using 10% (w/w) of Novozyme 435, with an initial enzyme activity of 27.0 U/g of substrate, 1:1 lipids to alcohol molar ratio at 60 °C. The reaction time of each batch was set at 4 h. After each batch reaction lipase was removed by filtration, followed by triple washing with 6 mL of n-hexane (Sigma-Aldrich) each time, drying at room temperature and then reused in the next reaction. The enzyme activity was also monitored after specific batch reactions.

4.7.4. Production of neopentyl glycol esters

Esterification conditions for reactions between hydrolyzed microbial oil and NPG were taken from Papadaki et al. (2018). Specifically, a substrate to alcohol molar ratio of 2:1 was employed and reaction was carried out at 45 °C with an enzyme loading of 4% w/w (based on the reaction quantity) and 1% deionized water.

4.8. Analytical methods

4.8.1. Determination of free amino nitrogen and inorganic phosphorus

Free amino nitrogen (FAN) concentration in hydrolysis and fermentations was determined according to the ninhydrin colorimetric method (Lie, 1973). The concentration of FAN refers to the nitrogen contained in free amino groups of amino acids and peptides. The reagents used for this analysis were:

Color reagent: 49.7 g/L Na₂HPO₄·2H₂O, 5 g/L ninhydrin and 3 g/L fructose were dissolved in deionized water and mixed. Around 60 g/L KH₂PO₄ were gradually added to the mixture until a pH of 6.6-6.8 was achieved.

Dilution reagent: 2 g of KIO₃ were dissolved in 616 mL of deionised water and subsequently 384 mL of absolute ethanol (99% v/v) were added.

Glycine stock solution: 0.1072 g of glycine were dissolved in deionised water until a final volume of 100 mL.

Glycine standard solution: 1 mL of glycine stock solution was diluted in 100 mL final deionized water to obtain a final concentration of FAN 2 mg/L.

All the aforementioned reagents were stored at 4 °C and were regularly prepared.

The protocol applied was as follows: 1 mL of 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 sample. All tubes were boiled for exactly 16 min and subsequently, cooled down for 20 min. Then 2.5 mL of dilution reagent were added. Samples were vigorously shaken with vortex and then absorbance was read in a spectrophotometer (U-2000, Spectrophotometer, Hitachi) at 570 nm, against the blank sample. All samples were analyzed in duplicate.

FAN content (mg/L) was quantified based on a calibration curve ($y=6.4941x+0.0762$, $R^2=0.9994$) was determined by diluting glycine standard solution to obtain different concentrations of FAN (0.5, 1, 1.5 and 2 mg/L).

Inorganic phosphorus (IP) was assayed by the ammonium molybdate spectrophotometric method described by Harland and Harland (1980), based on the oxidation of organically combined phosphorus with perchloric acid to produce orthophosphate.

For IP (mg/L) determination, the following protocol was applied: Samples were properly diluted to 5 mL final volume into glass test tubes. In each tube, 0.4 mL of perchloric acid (60%, v/v), 0.3 mL of freshly prepared ascorbic acid (1%, w/v) and 0.4 mL of ammonium molybdate (4%, w/v) were successively added. After each addition, samples were vortexed for 10 sec. The tubes were left for 10 min, allowing the color to be developed and then absorbance was read at 730 nm using a spectrophotometer against water as a blank sample. All the samples were carried out in duplicate.

A 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. The solution was used to prepare the calibration curve ($y=12.156x+0.1666$, $R^2=0.9975$) after dilutions to obtain different IP concentrations (2, 4, 6, 8 and 10 mg/L).

4.8.2. Determination of total Kjeldahl nitrogen

Total Kjeldahl nitrogen (TKN) content was measured using a Kjeltex TM 8100 distillation Unit (Foss, Denmark). The method is based on total conversion of the initial forms of nitrogen into ammonium salts. Specifically, 0.5 g of dry sample were inserted into a digestion tube together with 25 mL H_2SO_4 and a tablet catalyst containing 3.5 g K_2SO_4 and Cu_2SO_4 . A blank sample was prepared as aforementioned. Digestion of the samples was conducted at 430 °C for 1 h and subsequently the tubes were cooled to room temperature. A distillation step followed by the automatic addition of 30 mL H_2O and 100 mL NaOH (40% w/v).

TKN was calculated as follows:

$$N (\%) = \frac{(V_s - V_b) \times N \times 14.007 \times 100}{W_s}$$

Where, V_s = volume (mL) of 0.1N HCl consumed for the sample

V_b = volume (mL) of 0.1N HCl consumed for the blank

N = normality of HCl

W_s = weight (mg) of sample

Protein content was calculated as follows:

$$P (\%) = N (\%) \times 6.25$$

Where, 6.25: coefficient based on nitrogen percentage in protein (16%).

4.8.3. Determination of ash, lipid content and structural components of lignocellulosic biomass

Ash and oil content were determined according to the AACC Approved Methods 08-01, and 30-25, respectively.

The determination of acid-detergent fiber (ADF), acid-detergent lignin (ADL) and neutral detergent fiber (NDF) refers to AOAC Official Method 973.18, Fiber (Acid Detergent) and Lignin (H₂SO₄) in Animal Feed and was based to Gerhardt application fiber bag-system. Concentration of cellulose was obtained by subtracting ADL from ADF, hemicellulose was calculated by subtraction of ADF from NDF, while lignin corresponded to ADL.

4.8.4. Determination of carbon sources

The concentration of carbon sources in fermentation broth was determined by High Performance Liquid Chromatography (Prominence, Shimadzu, Kyoto, Japan) equipped with an Rezex ROA-organic acid H⁺ column (300 mm length x 7.8 mm internal diameter, Phenomenex), coupled to a differential refractometer (RID-10A, Shimadzu, Kyoto, Japan). The mobile phase was a 10 mM H₂SO₄ aqueous solution with 0.6 mL/min flow rate at 65 °C. Monosaccharides were also determined with a Shodex SPO810 column at 60 °C and 1 mL/min flow rate of pure water as the mobile phase.

4.8.5. Assay of enzymes activity in solid state fermentations

Lipases were determined as previously described by Freire et al., 1997 with slight modifications. Briefly, 0.4 g of fermented solids were added to a 20 mL mixture prepared as follows and incubated for 20 minutes at 35 °C and 200 rpm. Soybean oil (5% w/v) was emulsified with arabic gum (5% w/v) in 100 mM sodium phosphate buffer at pH 7.0, homogenized for 3 minutes prior to each batch of enzymatic reactions. Afterwards, 20 mL of 1:1 ethanol-aceton (v/v) were added to the aforementioned solution and incubated for 10

minutes under the same conditions. The free fatty acids produced, were titrated with 0.04 N NaOH until a pH 11 was achieved, using an automatic titration apparatus (Mettler DL21). One lipase activity unit (U) was defined as the amount of enzymes that releases 1 μmol FFA per 1 minute.

Proteases activity was determined as follows. The enzymes were extracted from the fermented solids (2.5 g) with a solution of 200 mM phosphate buffer (50 mL) at a pH value of 6. Protease activity was quantified by the FAN production during the hydrolysis of the solid substrate (5 mL) with 15 g/L casein solution (5 mL) at 55 °C within 30 min. One unit (U) of proteolytic activity was defined as the amount of protease that releases 1 μg FAN in 1 minute.

4.8.6. Enzymatic activity assay of commercial Novozyme 435

Enzymatic assay of lipase was carried out according to ACS Specifications (1993). More specifically, 2.5 mL deionized water, 1 mL 2 M Tris HCl Buffer (pH 7.7) and 3 mL olive oil were magnetically swirled and equilibrated to 37 °C using water jacketed glass vials on a hotplate stirrer (165 rpm). A specific amount of enzyme was added to the reaction mixture and a 30-minute incubation followed. Subsequently 3 mL 95% ethanol was added. Then the mixture was titrated with 50 mM NaOH in the presence of thymolphthalein indicator to a light blue color. A blank sample was prepared by following the same procedure, but without the addition of enzyme. One unit of lipase activity was defined as the amount of enzyme that hydrolyses 1.0 μmole of fatty acid from a triglyceride per minute, at pH 7.7 and 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).

4.8.7. Determination of fungal biomass in solid state fermentations

Fungal biomass quantification was carried out under a two-step methodology. Initially, dried fermented solids (0.5 g) and pure fungal biomass (various quantities, 20 - 140 mg) were hydrolyzed with 6 N HCl and the released N-acetyl glucosamine was determined using Ehrlick's reagent according to Aidoo et al. (1981). Optical density was read at 530 nm against a blank reagent and after fitted to a standard curve of N-acetyl glucosamine ($y=30.87x+0.0202$, $R^2=0.9917$). N-acetyl glucosamine was calculated for all samples and it was correlated with the pure fungal biomass ($y=20.856x-0.4416$, $R^2=0.9764$).

4.8.8. Determination of microbial biomass and oil in submerged fermentations

The total dry weight (TDW) was determined by repeatedly washing the produced microbial biomass, drying at 85 °C for 24 h and cooling in a desiccator. Prior to oil extraction, TDW was grinded to obtain fine solids. Subsequent chemical disruption of yeast cells was carried out by heat treatment with HCl according to Tapia et al. (2012). Specifically, dry biomass was immersed in 4 M HCl at a ratio of 1.5:125 w/v and the mixture was heated at 80 °C for 30-45 min. Cellular debris was removed by centrifugation (9000 × g, 4 °C, 10 min) and the lipids were extracted with a chloroform/methanol solution (2:1, v/v) as proposed by Folch et al. (1957). In the case of fungal biomass, the step of HCl treatment was omitted.

After the evaporation of the Folch solution, lipid content was determined gravimetrically in pre-weighed round bottom flasks.

4.8.9. Fractionation and hydrolysis of microbial oil

Microbial oil was washed with a 3:1 (v/v) mixture of Folch solution (2:1, v/v chloroform - methanol) and KCl (0.88% w/v) (Renta), following centrifugation (10000 × g, 10 °C for 10 min). The organic phase was collected and vacuum evaporated to obtain the clarified microbial oil for its subsequent fractionation. Lipids fractionation was carried out using a column (1.7 × 37.5 cm) of silica gel 60 silanized (0.063 - 0.2 mm, Merck) that was previously activated overnight at 120 °C. Five bed volumes of chloroform, acetone and methanol were applied for the sequential elution of neutral lipids (NL), glycolipids plus sphingolipids (GL+SL) and phospholipids (PL), respectively. The elution procedure was controlled at a flow rate of 0.5-1.0 mL/min. The three isolated fractions were vacuum evaporated for subsequent use.

Microbial oil was hydrolyzed using the lipase Lipomod 34MDP (2%, w/w, calculated by oil weight) and 0.1 M Tris-HCl buffer at pH 8 at a ratio of 1:1 (v/v). The hydrolysis was carried out in round bottom flasks that were temperature controlled at 30 °C for 24 h, using a hotplate stirrer (Witeg, MSH 20D). The reaction was stopped by the addition of hexane and the mixture was transferred in a separating funnel. The lower layer containing the buffer, glycerol and enzyme was discarded and the upper organic layer was vacuum evaporated to obtain the free fatty acids. The acidity of the free fatty acid fraction was measured by titration against 0.04 M NaOH using an autotitrator.

4.8.10. Determination of fatty acid composition of microbial oil

The analysis of fatty acid composition in microbial oil was conducted with the production of fatty acid methyl esters (FAMES) employing transesterification with sodium methoxide followed by esterification with methanol using HCl as catalyst. The latter stage was followed in order to esterify free fatty acids into FAMES that may have been produced due to microbial oil hydrolysis by intracellular lipases. The determination of FAMES was carried out by Gas Chromatography (GC) Fisons 8060 equipped with a chrompack column (60 m × 0.32 mm) and a flame ionization detector (FID) using helium as carrier gas (2 mL/min). The oven program was initiated at 50 °C, heated to 200 °C with a ratio of 25 °C/min (1 min), then increased with ratio of 3 °C/min up to 240 °C and finally increased to 250 °C with a ratio of 25 °C/min and maintained for 3 min. Detector temperature was set at 250 °C. FAMES were identified by reference to a standard (Supelco® 37 Component FAME Mix, 10 mg/mL in CH₂Cl₂, 47885-U, Merck).

4.8.11. Quantification of bio-esters

The determination of 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, using helium as the carrier gas at a flow rate of 1 mL/min. Separations of the compounds was performed on a ZB-5HT inferno capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness, Phenomenex). The oven program was initiated at 80 °C, heated to 240 °C with a ratio of 20 °C/min, then increased with ratio of 15 °C/min up to 290 °C and finally increased to 400 °C and maintained for 6 min. Detector temperature was set at 400 °C.

The quantification of palmitic and oleic esters was based on calibration curves of reference standards. Isopropyl and 2-ethylhexyl esters of linoleic and γ-linolenic acids were determined based on reference standards of oleic acid esters due to the lack of commercial standards. Methyl palmitate was used as the internal standard.

Conversion yield was calculated based on the fact that 1 mole of microbial oil produces 3 moles of esters as follows (Keng et al., 2009):

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

4.8.12. Quantification of polyol esters

Conversion yield of the produced neopentyl glycol esters was calculated based on the difference of the acidity between the free fatty acid fraction that was used as substrate and the final product. A specific amount of sample was diluted in 40 mL acetone:ethanol solution (1:1, v/v) followed by autotitration with 0.04 M NaOH. The acidity was calculated as follows:

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

Where, V = volume (mL) of NaOH used for titration

M = molarity of NaOH

FA = molecular weight of fatty acids

w = weight (g) of sample used for titration

Conversion yield was determined as follows:

$$\text{Conversion yield (\%)} = \frac{(A_i - A_f)}{A_i} \times 100$$

Where, A_i = initial acidity

A_f = final acidity

4.8.13. Properties determination of bio-esters

Acid, iodine and saponification values for the produced esters, were determined according to U.S. Pharmacopeia methods. The acid value was expressed as mg of KOH required to neutralize the free acids per g of sample. The saponification value was expressed as mg of KOH required to neutralize the free acids and saponify the esters contained per g of sample. The iodine value was expressed as g of I_2 absorbed per 100 g of sample. Refractive index and density were calculated according to Annual American National Standards (1998).

4.8.14. Properties determination of polyol ester

Structure and purity analysis of biolubricants were performed by 1H , ^{13}C , and gHSQCAD Nuclear Magnetic Resonance (NMR) on a Bruker Avance III 500 equipment (11.75 Tesla of magnetic field) at 28 °C. The sample was dissolved in $CDCl_3$ containing 0.05% v/v TMS as THE internal reference ($\delta = 0$ ppm). Experiments were recorded in a 5 mm direct detection probe. The 1H NMR spectrum of 8% v/v solution was acquired with the observation frequency

of 500.13 MHz, using 30° rf pulses, relaxation delay of 15.0 s, acquisition time of 3.2 s, spectral width of 10.0 KHz and 64 transients were accumulated. The ¹H NMR spectrum was processed using a line broadening of 0.3 Hz. The ¹³C NMR spectrum of 25% v/v solution was acquired with the observation frequency of 125.8 MHz, using 90° rf pulses, relaxation delay of 20 s, acquisition time of 1.1 s, spectral width of 29.8 Hz and 2048 transients were accumulated. The ¹H broad band decoupler was set in the inverted-gated mode to avoid NOE (zgig pulse sequence). The ¹³C spectrum was processed using a line broadening of 1.0 Hz. For the ¹H-¹³C gHSQC spectrum of 25% v/v solution, the one-bon ¹J_{CH} coupling constant filter of 145 Hz was used to set delays in the gHSQCedetgpsisq 2.3 pulse sequence. gHSQC spectrum was recorded using 128 F2 increments, 64 scans per increment, relaxation delay of 1.5 s, acquisition time of 0.220 s, spectral window of 9.4 KHz (F2) and 188.5 KHz (F1) filled with 4096 x 1024 data points. A QSINE function using 1.0 Hz x 0.3 Hz (F1 x F2) was applied to both F1 and F2 dimensions before zero filling F2 to 4 K points and Fourier transformation.

Viscosity was determined with a calibrated viscometer tube (Cannon-Fenske) at two different temperatures (40 °C and 100 °C). Viscosity index (cSt) was calculated according to the ASTM D445 international viscosity index table.

Acid number was determined according to ASTM D664 and it was expressed as mg of KOH required to neutralize the free fatty acids per g of sample.

4.8.15. Phenolic compounds determination, characterization and incorporation in sunflower oil

4.8.14.1. *Extraction of phenolic compounds*

The extraction procedure was carried out in an ultrasonic bath using 5 g dry sample and an aqueous solution of ethanol (70%, v/v) acidified with HCl (0.01%, v/v) as a solvent. After extraction, the samples were filtered and extracts were evaporated at 40 °C under reduced pressure (Rotary evaporator, BUCHI, R-114). The residue was re-dissolved in 50 mL acetone/water (1:1, v/v), washed four times with an equal volume of hexane, followed by ethyl acetate extraction (50 mL, four times). The ethyl acetate extracts were combined, and the solvent was evaporated. The residue was re-dissolved in 10 mL methanol and kept at -20 °C until further analysis.

4.8.14.2. *Total phenolic compounds determination*

The TPC of the methanolic extracts was determined using the Folin-Ciocalteu method, as described by Faustino et al. (2010) and Papageorgiou et al. (2008). Briefly, 50 μL extract, 450 mL distilled water and 2.5 mL 0.2 N Folin-Ciocalteu reagent were transferred into a test tube, vortexed for 1 min and allowed to stand for 8 min. Subsequently, 2 mL of an aqueous solution of Na_2CO_3 (75 g/L) were added and the mixture was left to react in the dark for 90 min at room temperature. The absorbance (relative to that of a blank prepared using methanol instead of extract) was measured at 765 nm using a double-beam UV-Vis spectrophotometer (Jasco V-530) and compared to a gallic acid calibration curve ($y=0.9174x-0.0231$, $R^2=0.9986$). The results were expressed as mg of gallic acid equivalents (GAE) per g of dry mass.

4.8.14.3. Determination of the antioxidant activity

The antioxidant activity of the methanolic extracts was assayed using the DPPH-free radical scavenging method as proposed by Scherer and Godoy (2009). Briefly, 100 μL of properly diluted methanolic extract were added to 3.9 mL of DPPH solution (80.1 μM in methanol) and left in the dark at room temperature for 90 min. The absorbance was measured at 517 nm against methanol as a blank, using a double-beam UV-Vis spectrophotometer (Jasco V-530, Tokyo, Japan). A mixture consisted of 100 μL methanol and 3.9 mL of DPPH solution was used as a control. The antioxidant activity index (AAI) was calculated as follows according to Scherer and Godoy (2009):

$$AAI = \frac{\text{Final concentration of DPPH } \left(\frac{\mu\text{g}}{\text{mL}}\right)}{IC_{50}\left(\frac{\mu\text{g}}{\text{mL}}\right)}$$

Where, IC_{50} : concentration providing 50% inhibition.

4.8.14.4. Identification of individual phenolic compounds

Chromatographic analysis was carried out on a Prominence HPLC system (Shimadzu, Kyoto, Japan), equipped with an on-line degassing unit (DGU-20A), a quaternary pump (LC-20A), an auto sampler (SIL-20A), a column-oven (CTO-20A) and a photo-diode array detector (SPD-M20A). Separation was performed on a Waters Nova-Pack C18 column (150 mm x 3.9 mm, 4 μm particle size, Milford) at ambient temperature (25 $^{\circ}\text{C}$) with gradient elution (solution A: 0.1% by volume formic acid in 5% methanol (Merck,) and solution B: 0.1% by volume formic acid (Merck) in 100% methanol). The gradient program was the following: 0, 100% A; 15 min, 75% A; 40 min, 65% A; 60 min, 55% A; 65 min, 50% A; 90-95 min, 0% A and 110-120 min,

100% A. The flow rate of the mobile phase was 1 mL/min and the injection volume was 20 μ L (Cai et al., 2004).

Identification of the individual phenolic compounds was based on comparison of the retention times and the UV spectrum of unknown peaks to those of authentic compounds. Quantitative analysis was based on calibration curves constructed at specific wavelengths of reference compounds using the external standard method.

4.8.14.5. Phenolic extracts incorporation in sunflower oil

A commercial sunflower oil was supplemented with specific amounts of PKC and PPF phenolic extracts. A known volume of methanolic extract isolated as described in Section 4.8.15.1, was transferred into a test tube and the solvent was evaporated under nitrogen stream. The residue was re-dissolved by addition of 0.8 mL propylene glycol. Subsequently, 5 g of sunflower oil were added, and the mixture was shaken vigorously for 15 min at room temperature. Propylene glycol was employed as an aid for phenolics dissolution in the oily phase (Salta et al., 2007). The synthetic antioxidant butylated hydroxyanisole (BHA, Sigma-Aldrich) was also used for comparison at a concentration of 0.02%, which is the maximum allowed.

4.8.14.6. Accelerated oxidation stability test

A Metrohm Rancimat model 679 (Herisau, Switzerland) was used to measure the oxidative stability index (OSI) before and after the enrichment of the sunflower oil with the PKC and PPF extracts. The tests were carried out with 3 g oil samples at 120 °C and at an airflow rate of 20 L/h. OSI was expressed as induction time (h).

4.9. Statistical analysis

JMP 8 software was used for statistical analysis (Section 4.8.15). The data were compared using analysis of variance (ANOVA) and Pearson's linear correlation at 5% significance level. Significant differences between means were determined by Honest Significant Difference (HSD-Tukey test) at levels of $p < 0.05$ and 0.01. All values were reported as means of three independent determinations and the results were expressed as mean values \pm standard deviation.

CHAPTER 5

Valorisation of by-products from palm oil processing for the production of generic fermentation media for microbial oil synthesis

5.1. Introduction

The establishment of consolidated bioprocesses in conventional industrial plants is dependent on the exploitation of all residual streams for the production of energy, chemicals, biopolymers and various value-added products. Development of solid state fermentation (SSF) constitutes a feasible process to be integrated in biorefinery concepts focusing on the valorisation of various agro-industrial waste and by-product streams (Thomas et al., 2013).

Oil palm tree (*Elaeis guineensis*) is the principal source of palm oil and palm kernel oil. It is estimated that roughly 10% of the palm tree yields in palm oil while the rest 90% corresponds to palm biomass, particularly to empty fruit bunches (EFB), palm pressed fiber (PPF), palm kernel cake (PKC), palm kernel shells (PKS,) oil palm fronds, trunks, leaves and roots and finally palm oil mill effluent (POME). These streams that amount to 428 million tons per year globally (Anonymous, 2017) are considered as by-products with restricted applications and a low economic value.

Industrial large-scale implementation of SSF processes encounters difficulties in separation and refining of the final product, aeration, inadequate heat removal and substrate distribution among others (Couto and Sanromán 2006). The aforementioned bottlenecks along with the necessity to establish SSF processes achieving high productivities and minimum operational problems indicate the need for the optimisation of solid-state bioreactor systems with enhanced enzyme production efficiency.

In order to overcome scale-up limitations of conventional tray bioreactors, instrumented rotating drum bioreactor constitutes a promising model to elucidate and control SSF processes. A 15 L Terrafors bioreactor was designed for thermophilic xylanase production process (Mienda et al., 2011) aiming to undertake many of the current SSF industrial production. Tippkötter et al. (2014) employed the same bioreactor to scale up enzymatic hydrolysis of lignocellulose deriving from beech wood, followed by its further utilisation for the production of biobutanol and dicarboxylic acids.

SSF bioprocesses could be implemented as individual processing strategies for the production of enzymes, biopolymers or microbial oil (Chutmanop et al., 2008; Oliveira et al., 2004; Cheirsilp and Kitcha, 2015). Alternatively, SSF could be integrated in multiple stage steps coupled with enzymatic hydrolysis to generate nutrient rich supplements utilized in yeast bioconversions for microbial oil production (Tsakona et al. 2014)

The capability of the oleaginous yeast *Lipomyces starkeyi* to assimilate a broad range of feedstock resulting in the accumulation of high intracellular lipids has been widely evaluated in several studies employing various fermentation modes. Lin et al. (2011) achieved a total dry weight of 104.6 g/L with a lipid content of 64.9 % (w/w) using glucose-based mineral medium. Research is currently focused on the utilisation of renewable resources, including wheat straw hydrolysates, flour-rich waste hydrolysates, yeast spent mass and sugarcane bagasse (Yu et al., 2011; Tsakona et al., 2014; Yang et al., 2014; Anschau et al., 2014).

The target of the present study is to exploit the nutrient content of PKC in order to formulate a generic feedstock for fermentation processes. The PKC was initially used as substrate for the production of enzymes by the fungal strain *Aspergillus oryzae* via SSF carried out in tray bioreactors and a rotating drum bioreactor. The optimisation of SSF in tray bioreactors was used as the basis for technology transfer to a fully controlled rotating drum bioreactor. The crude enzyme consortia produced during SSF were subsequently used to hydrolyze PKC macromolecules to provide fermentation nutrient supplement that could replace commercial nutrient sources. The capability of the produced feedstock was assessed by conducting shake flask fermentations with the oleaginous yeast *L. starkeyi* using different carbon sources.

5.2. Composition of palm kernel cake and palm pressed fiber

The composition of PKC and PPF is presented in Table 5.1. The protein content of PKC was 12.8% while in the case of PPF, it was 3 folds lower. Other oilseed meals, such as sunflower, rapeseed and soybean meals contain more protein than PKC, ranging between 26 - 52% (Kachrimanidou et al., 2013; Koutinas et al., 2014).

Hydrolysis of the lignocellulose fraction in PKC into C5 and C6 sugars provides carbon sources for the production fermentative production of ethanol (Jorgensen et al., 2010) and butanol (Shukor et al., 2016). PKC has been evaluated as the sole carbon source for the production of β -mannanase by *Aspergillus niger* in column bioreactors (Abdeshahian et al., 2010) or supplemented with salt solution and nitrogen and carbon sources for tannase production (Sabu et al., 2005). Enzymatic hydrolysis of the protein contained in PKC lead to the release of amino acids and peptides thus facilitating their uptake by microbial strains for cell growth and metabolite synthesis.

Table 5.1 Composition of palm kernel cake and palm pressed fiber.

Composition	Palm kernel cake	Palm pressed fiber
Ash	3.1±0.1	3.0±0.2
Moisture	4.1±0.2	3.9±0.1
Protein	12.8±0.6	4.2±0.2
C/N ratio	25.2±0.9	69.5±2.7
Lipids	9.2±1.3	4.4±0.2
Acid Detergent Fiber	50.7±2.3	62.4±3.0
Acid Detergent Lignin	13.3±0.3	26.9±1.1
Neutral Detergent Fiber	66.9±3.5	79.0±4.0
Cellulose	37.5±1.5	35.5±1.2
Hemicellulose	16.2±0.4	16.6±0.3

^a Results were expressed as g per 100 g of dry solids

5.3. Solid state fermentation in tray bioreactors

The main goal of this study was the production of a nutrient-rich supplement from PKC and PPF for fermentative production of microbial oil. A two-stage process was implemented where PKC and PPF were evaluated as sole substrates in SSF to produce crude enzyme consortia. The fungal strain *A. oryzae* was employed as a highly efficient protease producer. After the protease activity reached the highest value, the fermented mass was combined with unprocessed PKC in enzymatic hydrolytic reactions to formulate eventually a generic feedstock for bioconversion.

Moisture content is one of the most important parameters for the optimisation of SSF. The micronutrients tend to be more accessible when water is adequate in the solid substrate although in the case of excessive concentrations of water, oxygen diffusion is restricted (Chutmanop et al., 2008). Optimisation of SSF focused on maximizing the production of protease. Thus, five sets of experiments were carried out evaluating different initial moisture contents during SSF of PKC used as the sole substrate. Figure 5.1 presents the profile change of protease activity at different initial moisture contents (50 - 70%). A similar protease production pattern was observed in all cases that increased up to 48 h followed by steady declining. The highest proteolytic activity (319.3 U/g) was obtained after 48 h of fermentation. This value is lower than the maximum protease activity achieved by Kachrimanidou et al. (2013) via SSF with the

same fungal strain cultivated on sunflower meal as substrate (400 U/g). The same strain was also utilized in SSF with rapeseed meal leading to a protease activity of 728 U/g after 67 h (Wang et al. 2010) as well as with wheat flour leading to production of a protease activity of 172.8 U/g after 97 h (Wang et al. 2009). Sandhya et al. (2005) evaluated various agro-industrial by-products for their potential to promote neutral protease production. It was demonstrated that SSF performed on wheat bran with *A. oryzae* NRRL 1808 was more effective compared to the other raw materials including PKC, with a proteolytic activity of 31.2 U/g after 72 h.

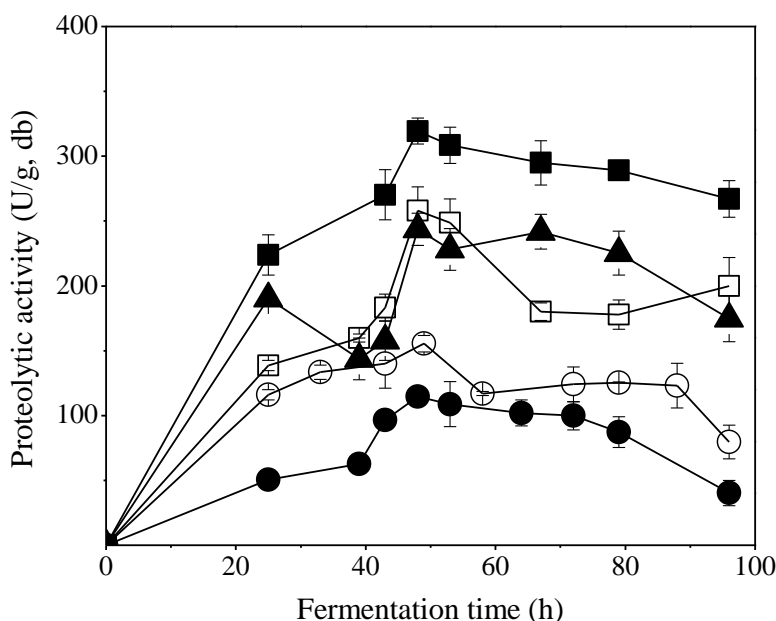


Figure 5.1 Protease production during solid state fermentation with *A. oryzae* cultivated on palm kernel cake at different initial moisture contents: 50% (○), 55% (■), 60% (▲), 65% (□), and 70% (●).

According to the results presented in Figure 5.1, the protease activity was maximized at an initial moisture content of 55%. Other studies employing *A. oryzae* strains for protease production have reported optimal moisture contents of 43.6 % in the case of wheat bran (Sandhya et al., 2005), 50% in the case of wheat bran and rice bran at a ratio of 0.33 (Chutmanop et al., 2008) and 65% in the case of sunflower meal (Kachrimanidou et al., 2013). Moisture contents lower than 55 % lead to diminished water activity, to levels unfavorable for fungal growth. Moisture contents higher than 55% cause a decrease in substrate porosity, leading to the adhesion of solid particles, thereof impeding the oxygen intake by the fungus.

Subsequently, profile change of protease production during SSF of 70:30 PKC to PPF ratio was investigated at 55% and 65% initial moisture content (Figure 5.2). The maximum proteolytic activity (208.4 U/g, db) was achieved at 55% initial moisture content after 52 h. The obtained value is approximately 35% lower than the proteolytic activity obtained by utilising only PKC as the solid substrate in SSF. For this reason, subsequent experiments for the optimisation of PKC hydrolysis were performed implementing solely PKC as the substrate in SSF carried out for 48 h with 55% moisture content. The hydrolysis of PKC was also evaluated using crude enzymes produced via SSF carried out with a PKC to PPF ratio of 70:30.

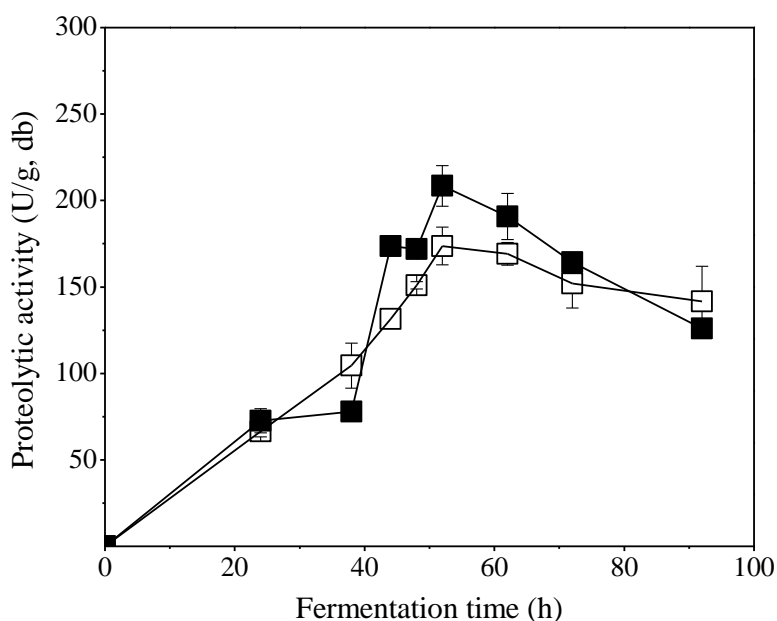


Figure 5.2 Protease production during solid state fermentation with *A. oryzae* cultivated in a solid substrate containing a palm kernel cake to palm pressed fiber ratio of 70:30 and two initial moisture contents: 55% (■) and 65% (□).

5.4. Enzymatic hydrolysis of palm kernel cake

The formulation of a generic fermentation feedstock from PKC was evaluated regarding the hydrolysis of protein to amino acids and peptides along with the release of IP from the hydrolysis of phytate. Enzymatic hydrolysis of renewable resources is attributed to the synergistic action of various enzymes and occurs simultaneously with autolysis of fungal mass arising from oxygen exhaustion in Duran bottles leading to the production of various nutrients (Koutinas et al., 2005).

The effect of temperature, initial solids concentration and initial enzymatic activity on free amino nitrogen (FAN) and inorganic phosphorus (IP) production was assessed during hydrolysis of PKC. Figure 5.3 presents the production of FAN and IP performed at 40 - 65 °C with an initial PKC concentration of 66.7 g/L, uncontrolled pH conditions and after 48 h of enzymatic hydrolysis. Hydrolysis at 50 °C showed the highest FAN production (5.6 mg/g) combined with maximum IP production of 1.7 mg/g. Lower FAN concentrations were obtained at 40 °C (5.3 mg/g). Temperatures higher than 50 °C resulted in a decrease for both FAN and IP production. The conversion yield of Total Kjeldahl Nitrogen (TKN) to FAN (Figure 5.3) reached 27.2% at the best temperature (50 °C).

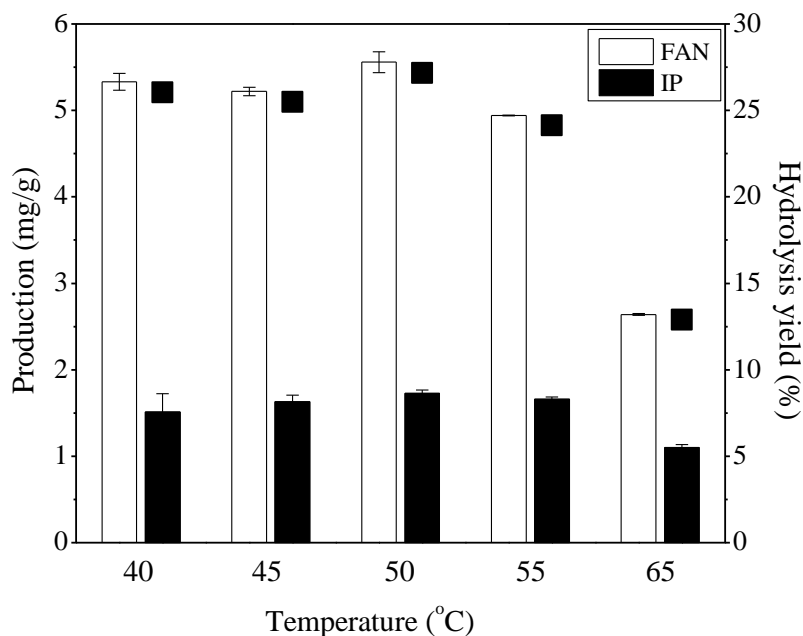


Figure 5.3 Production of free amino nitrogen (FAN) and inorganic phosphorus (IP) and hydrolysis yield (■) during the hydrolysis of palm kernel cake (66.7 g/L, db) at five different temperatures (40 - 65 °C) using initial proteolytic activity of 6 U/mL.

Figure 5.4 illustrates the profile change of the produced FAN and IP during hydrolysis performed with three different initial PKC concentrations (48.7, 73.7, and 98.7 g/L, db) at 50 °C using an initial proteolytic activity of 6 U/mL. The reported concentrations of solids include all solids utilized in SSF together with the unprocessed PKC added in hydrolysis experiments. The initial FAN and IP concentrations were subtracted from initial values to assess FAN and IP generation deriving from hydrolysis of PKC. Increasing PKC solid concentrations resulted in increased FAN and IP production, yielding respectively in 451.6 mg/L and 161.3 mg/L when

an initial solid concentration of 98.7 g/L was utilized after 48 h of hydrolysis. Conversion yield of TKN to FAN was calculated including the total quantity for PKC used both in SSF and in hydrolysis, reaching 27.9% when employing an initial PKC concentration of 48.7 g/L. Increasing concentrations of PKC led to decreased TKN to FAN conversion yields varying between 21.4 - 22.3% for the higher initial solid loadings. Thereof subsequent experiments were performed at 48.7 g/L.

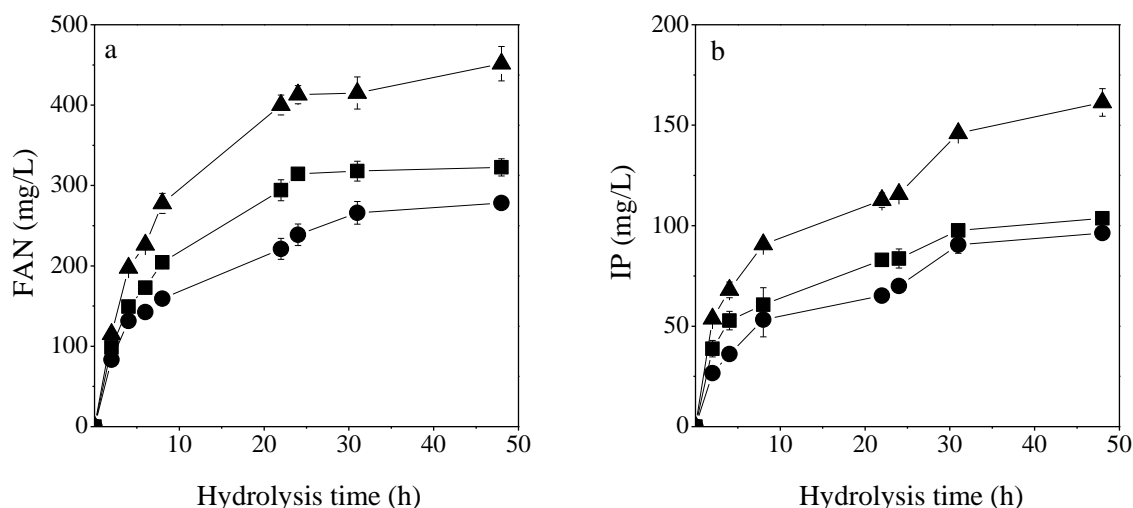


Figure 5.4 Production of free amino nitrogen (FAN) (a) and phosphorus (IP) (b) during the enzymatic hydrolysis of palm kernel cake at three different initial substrate concentrations (48.7 g/L (●), 73.7 g/L (■), 98.7 g/L (▲), db) using initial proteolytic activity of 6 U/mL.

The effect of initial proteolytic activity was evaluated at 48.7 g/L initial PKC concentration (Figure 5.5). Increasing initial proteolytic activity (11.9 U/mL) led to similar FAN and IP production compared to the initial proteolytic activity of 6 U/mL. The maximum FAN production was 262.0 mg/L and 278.3 mg/L, whereas IP production was 96.3 and 101.6 mg/L, for proteolytic activities of 6 and 11.9 U/mL, respectively. Since the increase of proteolytic activity did not demonstrate an apparent effect in PKC hydrolysis and further increase of FAN production, PKC hydrolysis was subsequently carried out using 48.7 g/L PKC concentration and 6 U/mL proteolytic activity at 50 °C.

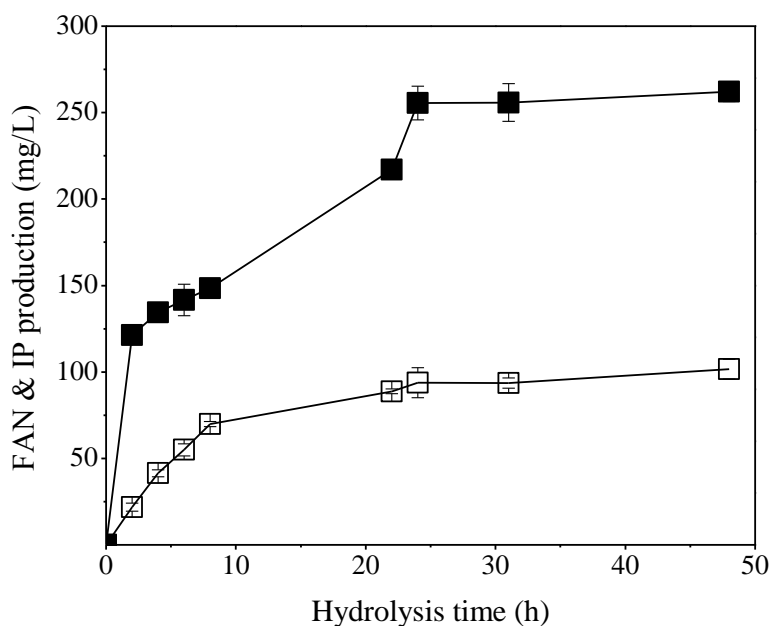


Figure 5.5 Production of FAN (■) and IP (□) during the hydrolysis of palm kernel cake using the initial proteolytic activity of 11.9 U/mL.

5.5. Solid state fermentation and production of nutrient-rich hydrolysates in a rotating drum bioreactor

After evaluating the SSF in tray bioreactors and hydrolysis of PKC for the production of a generic fermentation feedstock, the production of enzymes and PKC hydrolysates was scaled up in a rotating drum solid-state bioreactor (Terrafors). Two fermentations were carried out using 1000 g of a mixture of PKC and PPF at a ratio of 70:30. The fermentations were performed at 50 °C, 55% moisture content, and uncontrolled pH, following the best conditions identified in the SSFs carried out in tray bioreactors (Figure 5.6). It should be stressed that, initially, fermentations in the rotating drum bioreactor were carried out with PKC as the sole substrate, but the fermentation efficiency was rather low due to operational limitations occurred by the agglomeration of PKC particles during fermentation. This could be attributed to the enhanced adhesive properties of the hydrolyzed protein of PKC. The optimum ratio of 70% PKC and 30% PPF was identified by conducting several fermentations in the rotating drum bioreactor.

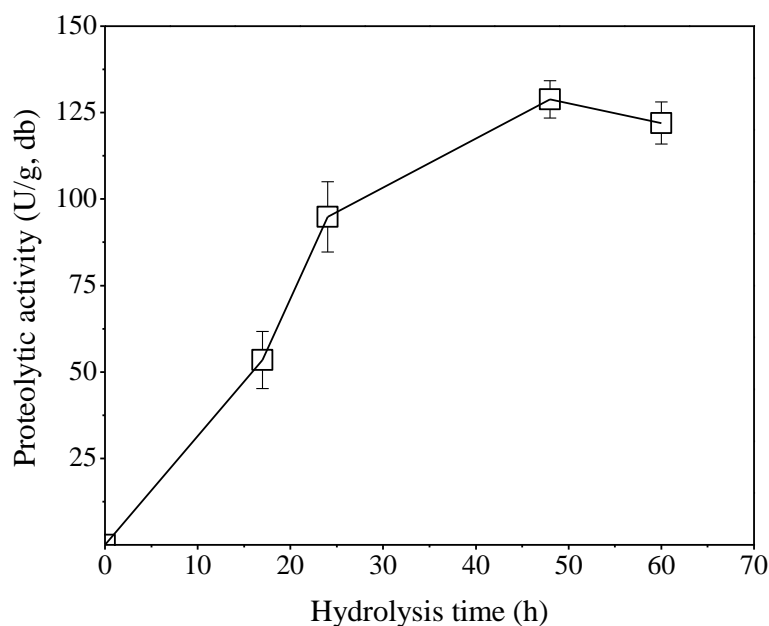


Figure 5.6 Protease production during solid state fermentations carried out (55% moisture content, 50 °C, and uncontrolled pH) in a rotating drum bioreactor using *A. oryzae* and a palm kernel cake to palm pressed fiber ratio of 70:30.

As depicted in Figure 5.6, the highest proteolytic activities of 128.8 U/g was obtained at 48 h. These values are lower compared to the maximum protease activity (208.4 U/g, db) achieved in tray bioreactors using 5 g of the same substrate.

After SSF, distilled water was aseptically added in the same Terrafors bioreactor to initiate hydrolytic reactions. In this context, it is important to mention that the Terrafors was only opened at the end of the hydrolysis step, in order to prevent contamination. The FAN production after 48 h of hydrolysis was 201.5 ± 5.1 mg/L. The FAN concentration achieved was lower compared to the maximum FAN production achieved in tray bioreactors. This could be attributed to the lower protease activities obtained during the SSF in the rotating drum bioreactor. Furthermore, the Terrafors has low maximum mixing speed of 10 rpm, which does not provide adequate mixing during enzymatic hydrolysis at high solid matter contents (Roche et al., 2009).

5.6. Shake flask cultures of *L. starkeyi* for microbial oil synthesis

Utilisation of hydrolysates deriving from renewable resources as fermentation nutrient supplements has been previously reported as beneficial for the cultivation of the oleaginous

strain used in this study (Tsakona et al., 2014; Leiva et al., 2015). The ability of *L. starkeyi* to grow and produce microbial oil on PKC hydrolysates was evaluated. Table 5.2 presents the results of shake flask experiments carried out with an initial sugar concentration of around 60 g/L, while the initial FAN concentration in the hydrolysate was 231.5 ± 13.4 mg/L. Arabinose, xylose, galactose, mannose, and glucose were employed as the C5 and C6 sugars in view of their prevalence in hydrolysate deriving from lignocellulosic biomass. All fermentations lasted from 120 to 192 h. The C/N ratio for each case examined, was 84.5 ± 3.5 calculated by the carbon content of the synthetic hexose or pentose employed and the initial FAN concentration in the PKC hydrolysate. Moreover, a shake flask fermentation was carried out on glucose supplemented with commercial nutrient sources and a shake flask fermentation was carried out using only PKC hydrolysate.

Table 5.2 Fermentation efficiency achieved by *L. starkeyi* when cultivated in shake flasks on different carbon sources with an initial sugar concentration of 60 g/L using either palm kernel cake hydrolysates or commercial nutrient supplements.

Substrate	C/N ratio	TDW ² (g/L)	Microbial oil (g/L)	Lipid content (%)	Yield (g/g)	Productivity (g/L/h)
Arabinose	82	12.7	3.7	29.1	0.078	0.019
Xylose	81	20.7	5.7	27.5	0.116	0.047
Galactose	88	21.2	7.0	33.1	0.121	0.049
Mannose	89	22.4	7.1	31.7	0.143	0.059
Glucose	80	20.6	7.3	35.5	0.127	0.076
Glucose ¹	85	22.2	8.2	36.8	0.153	0.068

¹ Commercial nutrient supplements: yeast extract, peptone, and minerals; ² total dry weight

When glucose, xylose, mannose, and galactose were supplemented with PKC hydrolysate, the final total dry weight (TDW) ranged from 20.6 to 22.4 g/L, which was similar or slightly lower than the TDW (22.2 g/L) obtained with glucose and commercial nutrient supplements (yeast extract and peptone). Only in the case of arabinose, the TDW achieved (12.7 g/L) was significantly lower than any other fermentation carried out with *L. starkeyi* in this study. The concentration of lipids achieved was in the range of 5.7 - 7.3 g/L when glucose, xylose, mannose, and galactose were used in combination with PKC hydrolysates. The lipid concentration (8.2 g/L) and intracellular content (36.8 %) were higher when glucose and commercial nutrient supplements were used. In all fermentations, lipid accumulation was

initiated after 48 h of inoculation when FAN was almost depleted from the medium. When PKC hydrolysate was used without carbon source as fermentation medium, no lipid accumulation was observed and the TDW was only 0.7 g/L. As a conclusion, the results obtained with PKC hydrolysate show that *L. starkeyi* can be cultivated in this complex nutrient source, leading to microbial oil production.

Some literature-cited studies have focused on batch production of microbial oil from *L. starkeyi* when cultivated on lignocellulosic hydrolysates and industrial waste streams including olive mill wastewater (Yousuf et al., 2010), potato starch (Wild et al., 2010), and several hydrolysates of wheat straw (Yu et al., 2011), rice (Probst 2014), *Arundo donax* (Pirozzi et al., 2014) and sugarcane bagasse (Xavier et al., 2017 b). In all cases TDW ranged from 6.3 g/L to 14.7 g/L with microbial oil concentrations varying between 1.3 - 4.6 g/L while productivities were quite low up to 0.08 g/L/h. Other studies dealing with fermentation of corn (Huang et al., 2014; Probst & Vadlani 2015; Calvey et al., 2016), wheat bran (Probst & Vadlani 2015) and spent yeast mass (Yang et al., 2014) hydrolysates have been proven more efficient reaching TDW of 17.1 - 24.6 g/L and microbial oil production of 6.1 g/L - 9.4 g/L. Intracellular lipids contents higher than 50% have been achieved when detoxified rice bran hydrolysate was utilized as carbon and nitrogen sources by *L. starkeyi* resulting in TDW of 13.5 g/L (Sutanto et al., 2017) while lipids titers up to 12 g/L have been reported when flour-rich waste hydrolysates were used as the sole media in shake flask fermentations (Tsakona et al., 2014).

Limited literature-cited studies have reported microbial oil production through supplementation with oilseed meal hydrolysates. Kiran et al. (2013) developed a medium rich in nitrogen for lipid production by hydrolyzing rapeseed meal using the enzymes produced by *A. oryzae*. Rapeseed meal hydrolysates and glucose were used in shake flask fermentations with *Rhodospiridium toruloides* Y4 resulting in 10 g/L of TDW. Leiva et al. (2015) utilized sunflower meal hydrolysates combined with crude glycerol during cultivation of *L. starkeyi* in shake flasks achieving 17.4 g/L of TDW and 5.1 g/L microbial oil.

Table 5.3 shows the fatty acid profile of microbial lipids produced during the cultivation of *L. starkeyi* in PKC hydrolysates. Microbial oil contained mainly oleic acid (C18:1) and palmitic acid (C16:0) followed by stearic acid (C18:0). The percentage of saturated fatty acids was higher than 35% (w/w) of the total fatty acids. Xavier and Franco (2014) reported that C18:1 (48.2%), C16:0 (27.8 %), C18:2 (10.1 %), and C18:0 (6%) were the main fatty acids determined in the microbial oil produced by *L. starkeyi* when cultivated on hemicellulose hydrolysate from

sugarcane bagasse in batch fermentations. Tapia et al. (2012) reported the production of C18:1 (45.7%), C16:0 (36.2%), C18:0 (12.1 %), and C18:2 (3.5%) using the same strain of *L. starkeyi* in batch cultures. Anschau and Franco (2015) demonstrated that C16:0 (42.3%) and C18:1 (38.6%) were the predominant lipids when *L. starkeyi* was cultivated in xylose and urea under fed-batch mode.

Table 5.3 Fatty acid profile (% , w/w) of intracellular lipids accumulated at the end of shake flask fermentations of *L. starkeyi* cultivated on various carbon sources with an initial sugar concentration of 60 g/L using either palm kernel cake hydrolysates or commercial nutrient supplements.

Substrate	C14:0	C14:1 Δ9	C16:0	C16:1 Δ9	C18:0	C18:1 Δ9	C18:2 Δ9,12	C18:3 Δ9,12,15	C20:4 Δ5,8,11,14	Others
Arabinose	0.6		30.7	6.2	7.3	40.4	8.6			6.1
Xylose	0.5	1	32.4	5.2	7	44.9	2.9			6.1
Glucose	0.8		33.7	4.9	7.5	45.3	3.9			3.9
Mannose	0.9	4.7	24.6	4.4	9.9	38.3	5.9		5.2	6.1
Galactose	2.3	4	19.6	3.2	13.7	31	6.1	7.7	5.9	6.5
Glucose ^a	1	0.8	36.1	4.3	6.8	43	4.1			4.1

^aCommercial nutrient supplements: yeast extract, peptone, and minerals

5.7. Concluding remarks

The main advantage of the process developed in this study is the utilisation of a crude renewable resource for the production of fermentation supplements, leading to the replacement of yeast extract. It is expected that the cost of on-site production nutrient supplements will be cheaper than the supply of commercial nutrient supplements such as yeast extract due to the development of an integrated process. Koutinas et al. (2004) showed that the production of crude fermentation media from wheat could be more cost competitive than commercial feedstocks.

CHAPTER 6

Extraction of phenolic compounds from palm oil processing residues and their application as antioxidants

6.1. Introduction

Oil palm mills pose significant environmental concerns through the generation of solid and liquid waste streams during the manufacturing process combined with the utilisation of excess quantities of water and energy. To overcome arising sustainability issues in combination with the financial viability of the process, efficient exploitation of residual palm biomass should focus on the production of high value-added products such as phytochemicals.

The largest and more attractive group of phytochemicals encompasses the phenolic compounds mostly phenolic acids, flavonoids, tannins and lignans. Several reports have demonstrated the positive effects of these bioactive compounds on the pharmaceutical, medical, and nutraceutical field (Xavier et al., 2017 a; Pande et al., 2017). Studies on the extraction, recovery and quantification of phenolics from oil palm biomass are rather limited in the literature. Indicatively, several phenolic fractions deriving from oil palm fruits (Neo et al., 2010), empty fruit bunches (EFB) (Han and May 2012), palm pressed fiber (PPF) (Nang et al., 2007) and palm kernel cake (PKC) (Kua et al., 2015) have been extracted and evaluated, with *p*-hydroxybenzoic acid, *p*-coumaric acid and ferulic acid being the predominant phenolics.

The prospect of using phenolic compounds, deriving from agro-industrial by-products, as natural food antioxidants is of paramount industrial interest. Besides, individual phenolic compounds i.e 4-hydroxybenzoic acid, pyrogallol and ferulic acid could be applied in various sectors including pharmaceuticals, cosmetics, chemical industry, resins manufacturing and food processing and preservation (Chakraborty et al., 2006; Wang et al., 2018, Kumar and Pruthi 2014; Kawamura et al., 2014; Kim, 2015).

The aim of the current study was initially to evaluate the phenolic content of various side streams deriving from the palm oil production process and subsequently to choose the stream with the highest phenolics concentration and antioxidant activity. HPLC-DAD analysis was employed for the identification and quantification of the main phenolic compounds present in the extracts of the solid residues. Parameters including, extraction time and liquid to solid ratio were assessed for the efficient extraction of phenolic compounds from PKC, which were further incorporated into sunflower oil targeting to the extension of its shelf life.

6.2. Assessment of total phenolic content and antioxidant activity of oil palm residues

Preliminary experiments were conducted using oil palm solid wastes in order to select the most efficient in terms of total phenolic content (TPC) and antioxidant activity index (AAI). Figure

6.1 depicts the TPC and the AAI of PKC, PPF, palm kernel shells (PKS) and EFB extracts from two consecutive extraction cycles. Based on ANOVA's results, a statistically significant difference on the concentration of total phenolics for the aforementioned waste streams ($p < 0.01$) was detected. Proceeding with Tukey test (Figure 6.1), comparison between TPC of PPF and PKS did not show any statistically significant difference with respective values of 3.6 mg GAE/gds of dry sample (gds) and 3.7 mg GAE/gds. EFB showed the lowest TPC (1.8 mg GAE/gds) whereas PKC exhibited the highest TPC equal to 5.2 mg GAE/gds. Statistically significant differences were demonstrated between the TPC of PKC and PPF or EFB or PKS, of PPF and EFB and finally of EFB and PKS. PKC extract exposed a relatively higher TPC than the ethanolic extract of sunflower meal (4.4 mg GAE/gds), a by-product from the biodiesel industry (Kachrimanidou et al., 2015) and the sesame cake extracts (0.8 mg GAE/gds) (Mohdaly et al., 2013).

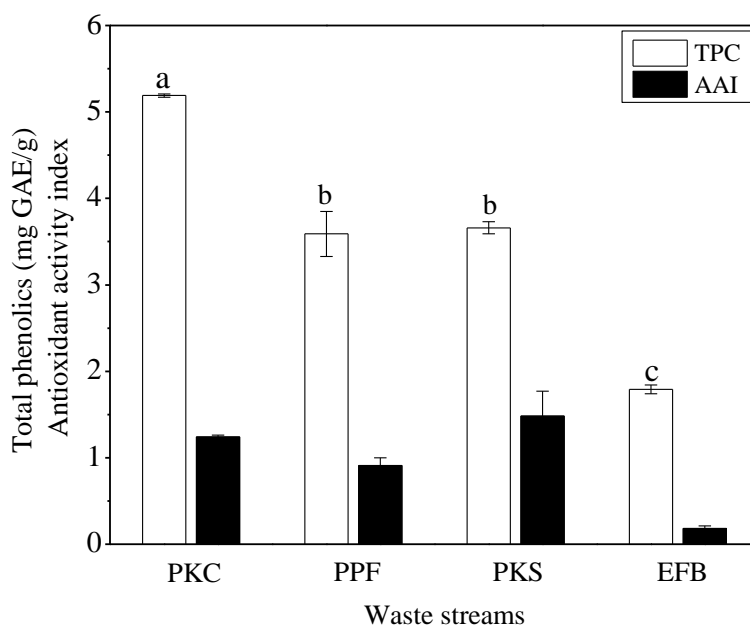


Figure 6.1 Total phenolic content (mg gallic acid equivalents/g dry matter) and antioxidant activity index of palm kernel cake (PCK), palm pressed fiber (PPF), palm kernel shells (PKS) and empty fruit bunches (EFB) extracts from two extraction cycles using a liquid-to-solid ratio of 30:1 and 20 min extraction time. Data are presented as mean \pm standard deviation of three independent replicates ($p < 0.01$, 95%). Columns with different letter are significantly different ($p < 0.05$ and $p < 0.01$).

As estimated by the 2,2 diphenyl-1-picrylhydrazyl radical (DPPH) assay, the AAI of the extracts, varied between 0.18 and 1.48, following the order of EFB < PPF < PKC < PKS. According to Scherer and Godoy (2009) classification, extracts of PPF belong to the moderate antioxidants, whereas the extracts of PKC and PKS belong to the strong ones. A significant positive correlation was obtained between TPC and AAI ($R^2 = 0.80$) regarding all waste streams. Similar data were obtained by Velioglu et al. (1998) that reported TPC and antioxidant activity of 28 plants, including several fruits, vegetables, medicinal plants as well as sunflower seeds and hull, flaxseeds and wheat germ. They demonstrated a significant relationship between these two factors for all plant materials except for the anthocyanin-rich materials and medicinal plants.

As the PKC extract had the highest TPC as well as strong antioxidant activity, it was selected for further investigation. Moreover, PKC is one of the main side streams of the palm fruit processing industry.

6.3. Effect of extraction time and liquid-to-solid ratio on total phenolic content and antioxidant activity of palm kernel cake

The extraction time and liquid to solid ratio were investigated to establish the best conditions for maximum TPC and AAI regarding PKC extracts. Three different extraction times were tested (10, 20 and 40 min) and the data obtained are presented in Figure 6.2. ANOVA analysis showed a statistically significant effect of the extraction time on TPC of PKC ($p < 0.01$). Increasing extraction time until a threshold and based on total phenolic potential of the solid matrix, lead to an increment of total extracted phenolics. It was observed that the lowest TPC (2.8 mg GAE/gdb) was obtained with the shortest extraction time (10 minutes), whereas the highest TPC (6.1 mg GAE/gdb) pertained to 40 minutes. TPC of PKC obtained from the three different extraction times presented statistically significant differences among all possible combined pairs. When 20 minutes were applied for extraction, TPC was reduced almost 15% in comparison to 40 minutes. Concluding with Pearson's linear correlation, a significant negative correlation between TPC and AAI was reported ($R^2 = - 0.77$). AAI was not significantly affected by any applied regime.

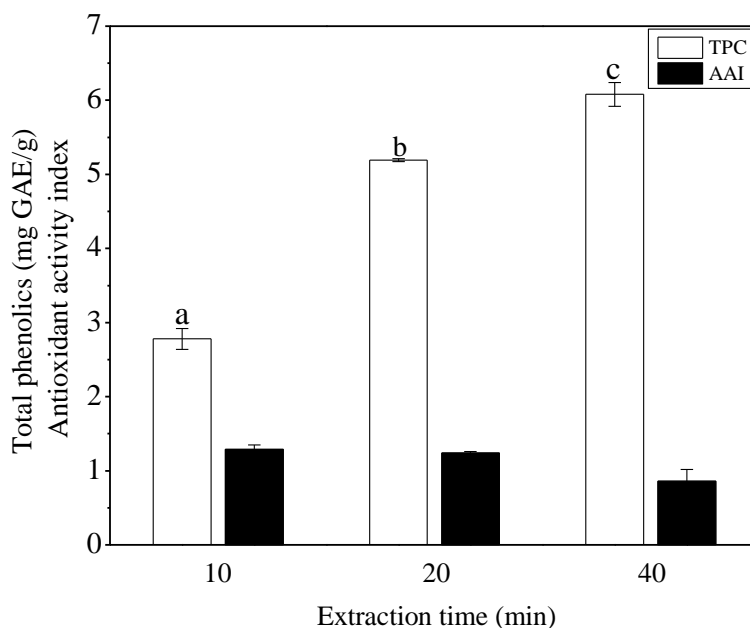


Figure 6.2 Effect of extraction time on total phenolic content of palm kernel cake (mg gallic acid equivalents/g dry matter) and its antioxidant activity index using a liquid to solid ratio of 30:1. Data are presented as mean \pm standard deviation of three independent replicates ($p < 0.01$, 95%). Columns with different letter are significantly different ($p < 0.05$ and $p < 0.01$).

Subsequently, the liquid to solid ratios of 5:1, 20:1, 30:1 and 40:1 were investigated and their effect on TPC and AAI is presented in Figure 6.3. Increasing the liquid-to-solid ratio, both TPC and AAI were improved, revealing statistically significant differences as far as TPC was concerned ($p < 0.01$). These results could be attributed to mass transfer phenomena and more specifically to the higher penetration of solvent into plant cells leading to the release of more phenolic compounds and their easier diffusion into the solvent (Radojković et al., 2012). Tukey test demonstrated that solely the ratio of 5:1 differs significantly from the other ratios (20:1, or 30:1, or 40:1). Finally, a significant positive correlation between TPC and AAI was demonstrated ($R^2 = 0.99$).

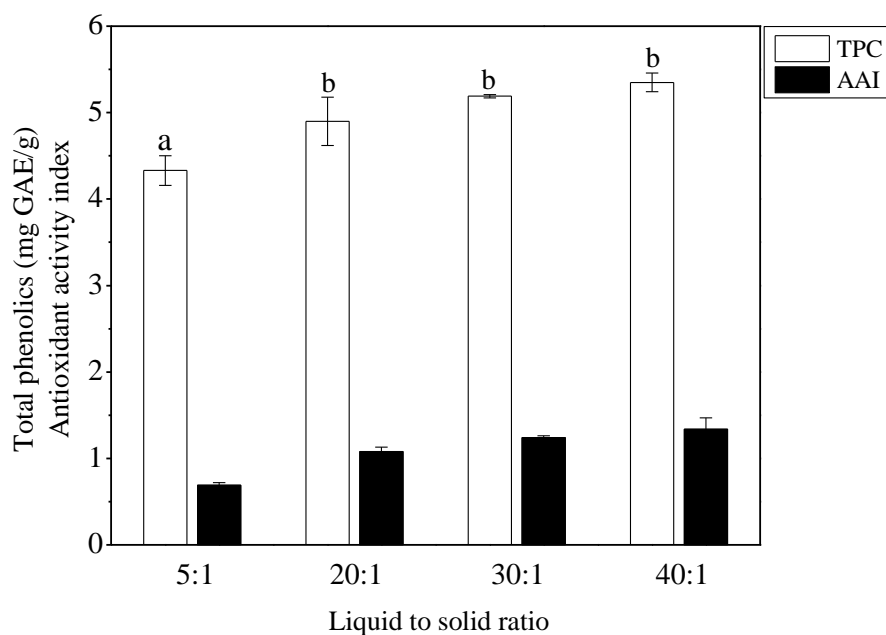


Figure 6.3 Effect of liquid-to-solid ratio on total phenolic content (TPC) of palm kernel cake (mg gallic acid equivalents/g dry matter) and its antioxidant activity index (AAI) using 20 min extraction time. Data are presented as mean \pm standard deviation of three independent replicates ($p < 0.01$, 95%). Columns with different letter are significantly different ($p < 0.05$ and $p < 0.01$).

Wong et al. (2015) studied pH (2 - 6), temperature (30 - 80 °C), liquid to solid ratio (10:1-100:1), ethanol concentration (20 - 80 %) and extraction time (30 - 300 min) for the extraction of phenolic compounds from PKC. It was demonstrated that TPC and antioxidant activity increased when the liquid to solid ratio was increased from 10:1 to 100:1. However, the effect of extraction time on TPC content was not found statistically significant, which is in contrast to our results. Additionally, Bucić-Kojić et al. (2007) reported a significant difference of polyphenols recovery yield from grape seeds depending on liquid to solid ratio, with the highest polyphenols concentration obtained using a ratio of 40:1. Furthermore, Prasad et al. (2012) employed a factorial design approach to identify the significant factors contributing to enhanced phenolics recovery and antioxidant capacity in extracts of *Mangifera pajang* pericarp. Ethanol concentration and liquid to solid ratio were reported as highly significant contributors.

6.4. Determination of individual phenolic compounds

The main phenolic compounds derived from the extraction of the different palm oil wastes were identified by HPLC analysis (Table 6.1). Eleven compounds were identified in the PKC extract with pyrogallol being the predominant one (1550 µg/gds), followed by 4-hydroxybenzoic acid (980 µg/gds), gallic acid (590 µg/gds) and ferulic acid (560 µg/gds). Catechol, homovanillyl alcohol and catechin were also detected in significant amounts. In EFB, five compounds were identified with 4- hydroxybenzoic acid being the major one (550 µg/gds). The main phenolic compound in PPF extract was 4- hydroxybenzoic acid (760 µg/gds), whereas pyrogallol dominated in PKS extract (370 µg/gds). Catechin and 4-hydroxybenzoic acid were present in all residues.

Table 6.1 Concentration of phenolic compounds found in the extracts of palm kernel cake (PKC), empty fruit bunches (EFB), palm pressed fiber (PPF) and palm kernel shells (PKS).

Compound ^a	Phenolics (µg/g)			
	PKC	EFB	PPF	PKS
Pyrogallol (270)	1550	-	-	370
Gallic acid (270)	590	100	-	-
Catechol (275)	270	-	-	-
Homovanillic alcohol (280)	150	-	110	-
Catechin (280)	130	50	60	110
Vanillin (280)	30	-	-	20
Lariciresinol (280)	40	-	-	-
4-Hydroxybenzoic acid (250)	980	550	760	230
Myricetin (370)	50	-	-	-
Ferulic acid (325)	560	-	-	-
Syringaldehyde (306)	30	40	40	
Guaiacol (270)	-	150	-	-
Sinapinic acid (325)	-	-	-	160
Total	4380	890	970	890

^aThe wavelength (nm) used for quantitative purposes is presented in the parentheses following the name of each compound

Similar results have been reported by other studies. Phenolic extracts obtained from oil palm fruits mainly consisted of 4-hydroxybenzoic acid, p-coumaric acid and ferulic acid in concentrations of 376 µg/gds, 55 µg/gds and 114 µg/gds, respectively (Neo et al., 2010). Ferulic acid and 4-hydroxybenzoic acid were also detected in various genera of the Palm family (Chakraborty et al., 2006) suggesting that the latter could be considered as a possible taxonomy marker for palms.

6.5. Estimation of sunflower oil oxidative stability

The Rancimat method is widely applied to determine the oxidative stability of edible oils. In this section, phenolic extracts from PKC and PPF were used to enrich sunflower oil and subsequently, its stability against oxidation was evaluated. PKC extracts were selected since they exhibited the highest TPC value, and the highest total phenolics as measured via HPLC. PPF extracts were also tested, mainly for comparison, since it showed considerable total phenolics concentration based on HPLC analysis. In both cases, the entire extract was redissolved in propylene glycol and added into 5 g of sunflower oil. After extraction, dry weight of PKC crude extract was 42 mg, whilst the corresponding value for PPF was 23.2 mg corresponding to an extract addition of 0.8% and 0.5% respectively.

Butylated hydroxyanisole (BHA) is a very efficient synthetic antioxidant and it was tested at its legal limit (0.02%) as a comparative agent. Sunflower oil without the addition of any antioxidant was also used as control agent. Figure 6.4 depicts the results of Rancimat test. Relying on ANOVA's statistics, the effect of the extract type on the induction time of sunflower oil was statistically significant ($p < 0.01$). Tukey test, revealed statistically significant differences between all the possible combined pairs (Figure 6.4) except for the comparison between BHA and 0.8% PKC extract. The induction time of the control agent was only 2.6 h. Supplementation of sunflower oil with 0.46% PPF extract, contributed to 28% improvement of its induction time while PKC extract resulted in almost 60% increment, a result very similar to the one obtained with BHA. The results are very promising, especially taking into account that BHA is a pure compound, whilst the PKC extract contains also other substances, of non-phenolic nature that could act as pro-oxidants.

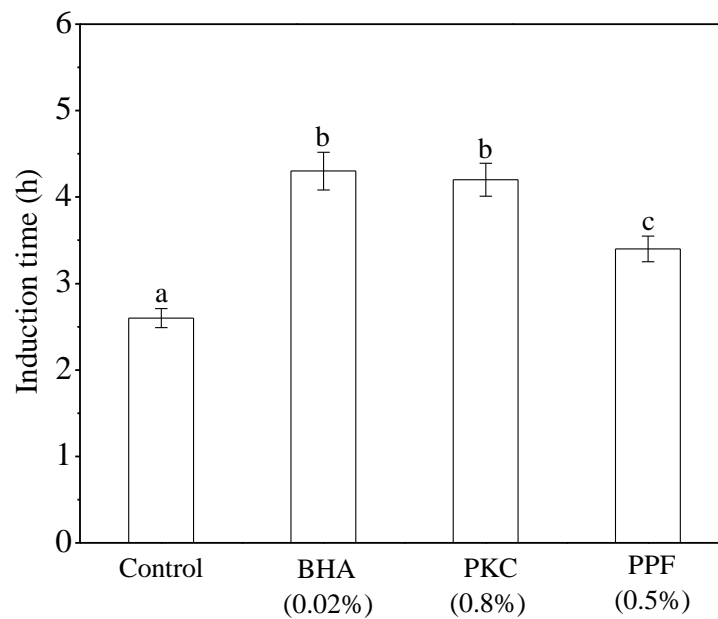


Figure 6.4 Oxidative stability index (expressed as induction time) of sunflower oil, enriched with 0.8% palm kernel cake (PKC) extract and 0.5% palm pressed fiber (PPF) extract. The induction times of butylated hydroxyanisole (BHA-0.02%) as well as a control are presented for comparison.

Several studies have reported the addition of extracts from various plant sources in sunflower oil. Gamel and Kiritsakis (1999) reported that 0.02% rosemary methanolic extracts increased the oxidative stability of sunflower oil at 63 °C and 120 °C. Yanishlieva et al. (1997) demonstrated that ethanol extracts of savory (0.1 - 0.5%) improved the oxidative stability of sunflower oil after 50 h at 180 °C. Finally, the antioxidative activities of six plant extracts (catnip, hyssop, lemon balm, oregano, sage, and thyme) in sunflower oil were evaluated. Sage extracts (600 and 1200 mg/L) effectively inhibited the oxidation process in sunflower oil and exposed the highest antioxidative activity compared to 300 mg/L butylated hydroxytoluene (BHT) (Abdalla and Roozen, 1999).

6.6. Concluding remarks

This experimental part, deals with the extraction of phenolic compounds from palm oil residues, including PKC, PPF, PKS and EFB. Phenolic extracts were incorporated in sunflower oil aiming to prolong its shelf life. The main indication of this study was the exploitation of by-product streams deriving from natural sources as antioxidants, with potent application in food industry.

CHAPTER 7

Polyol esters production using microbial lipids derived via solid state fermentation of protein rich side streams

7.1. Introduction

Solid state fermentation (SSF), the oldest known fermentation strategy, offers great potential for production of various value-added products i.e enzymes, biopesticides, biofuels, carotenoids, pigments, and biosurfactants. Cereals, waste streams from vegetable oil milling industry, edible starchy roots, sugarcane bagasse, soybean and sugar beet pulp constitute challenging sources for biotransformation (Arora et al., 2018). Cakes from palm kernel (PKC), cotton seed (CoSC), and castor seed (CaSC), generated after the oil extraction process, are low-cost materials conventionally used as animal feed, steam and electricity generation or they are disposed in landfills. Efficient valorisation of these streams should focus on the development of integrated biorefinery concepts including the production of high value added metabolites (de Castro et al., 2011; Aguiéiras et al., 2019).

Selection of an efficient microbial strain, cost-effective, nutrient rich and abundant feedstock as well as optimisation of process parameters including bioreactors design, are considered critical factors for a successive and cost-feasible implementation of SSF (Thomas et al., 2013). Packed bed reactors (PBR) are closed systems with forced aeration offering advantages, such as limited bacterial contamination as well as CO₂ and heat removal. Nevertheless, reduction of bed porosity during fermentation deteriorates its efficiency (Arora et al. 2018).

Several fungal phylums and mostly *Zygomycota*, have been studied for lipid production giving specific insight to the γ -linolenic acid (GLA) content, using lignocellulosic renewable resources in SSF cultures. For instance, the genus *Cunninghamella* may reach lipid production up to 394.4 mg/g of fermented solids (Emelyanova, 1996) and GLA contents up to 11.6% (w/w) (Conti et al., 2001) using rice and barley as substrates respectively.

Environmental concerns have recently encouraged research on biolubricants production. Biolubricants derived from vegetable oils and animal fats are superior to petroleum-based lubricants in terms of biodegradability, viscosity indices and lubricity (Salimon et al., 2010). In the last years, a more sustainable alternative has been developed for biolubricants production using non-food lipid resources, such as castor seed oil (Greco-Duarte et al., 2017), microbial oil from oleaginous yeasts using confectionery industry wastes (Papadaki et al., 2018) and palm fatty acid distillate (Fernandes et al., 2018).

The main objective of this study was the evaluation of microbial oil produced by oleaginous fungal strains for the production of esters with lubricant properties. The fungal strains

Cunninghamella echinulata, *Mortierella ramanniana* sp., *Mortierella isabellina* and *Thamnidium elegans* were initially used for microbial oil production via SSF on palm kernel cake. Further optimisation of fermentative conditions with *C. echinulata* was used as the basis for technology transfer to a packed bed bioreactor with cotton seed cake as substrate. Microbial oil was extracted from the fermented solids and subsequently used for enzymatic ester production with Lipomod 34-MDP in a solvent free system using neopentyl glycol. The potential utilisation of the produced esters as biolubricants was evaluated through the determination of their physicochemical properties.

7.2. Screening of fungal strains for lipid production

Fungal strains were initially screened on PKC for their ability to grow and accumulate intracellular lipids through SSF during a 15-day cultivation period. Figure 7.1 illustrates their macroscopic growth.

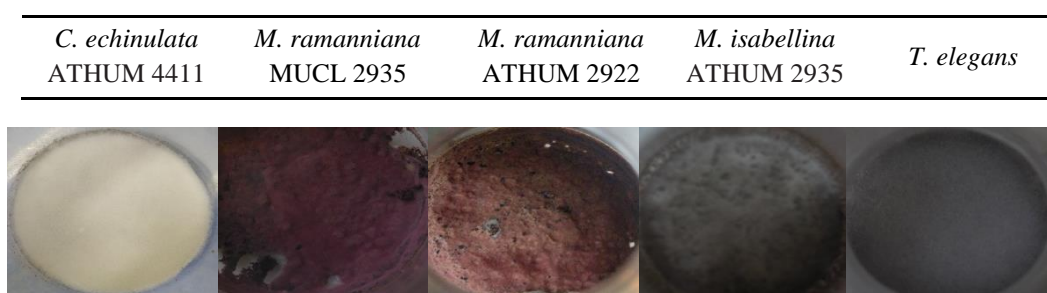


Figure 7.1 Lower filamentous fungi cultivation in 250 mL shake flasks, using palm kernel cake for microbial oil production.

Figure 7.2 presents the lipid production of *C. echinulata*, *M. ramanniana* MUCL and *M. ramanniana* ATHUM obtained in SSF with 65% initial moisture content in a non-humidified chamber. Lipids accumulation for *C. echinulata* reached 129.1 mg/g of fermented solids (gfs) at 11 days. After 15-days, cultures of *C. echinulata* sporulated, due to nutrient depletion, and lipid concentration was reduced to 35.5 mg/gfs. *M. ramanniana* MUCL and ATHUM strains presented lower lipid production, up to 33.8 mg/gfs and 25.0 mg/gfs at 5 and 15 days respectively. *T. elegans* and *M. isabellina* displayed a lipolytic tendency depleting the initial oil content of PKC, by 94.2% (15 days) and 62.2% (11 days), respectively. In these cases, biotransformation of initial PKC oil to fungal lipids could not be determined. In SSF of *M. isabellina*, moisture losses were more than 50% whereas the minimum moisture loss (14%) was determined in SSF of *C. echinulata* (Figure 8.2). The results showed that the higher lipid

production was related to lower moisture losses. This may be attributed to the higher mycelium growth and therefore lipid accumulation, of *C. echinulata*, *M. ramanniana* MUCL and *M. ramanniana* ATHUM than the other fungal strains, which was based on a macroscopic observation at this stage of the study.

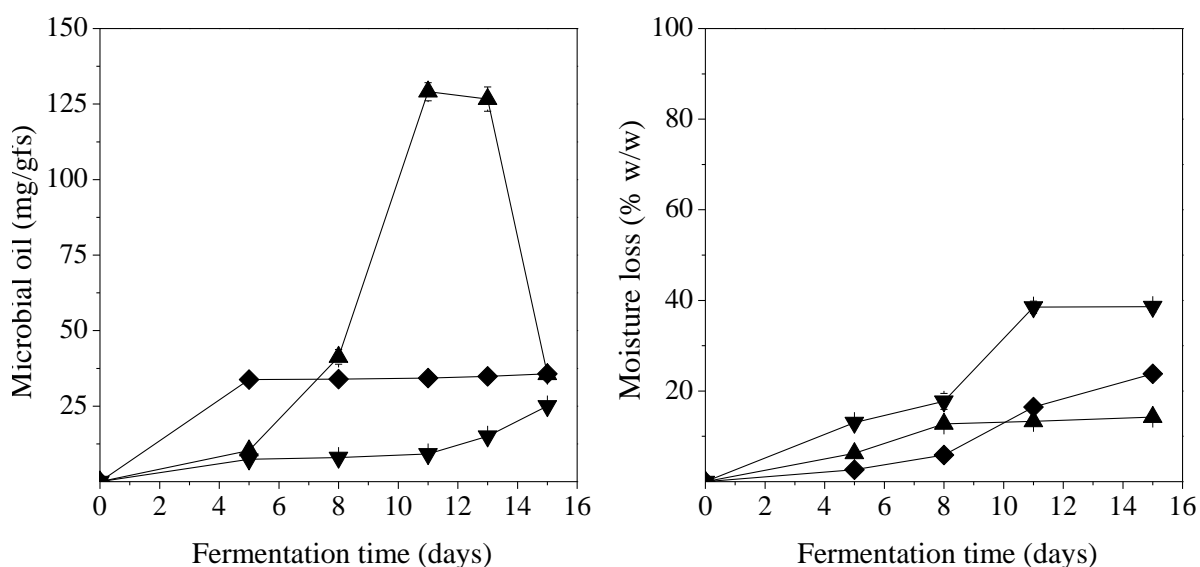


Figure 7.2 Microbial oil production and moisture loss during solid state fermentation of (▲) *C. echinulata*, (◆) *M. ramanniana* MUCL, and (▼) *M. ramanniana* ATHUM, using palm kernel cake as substrate at 30 °C and initial moisture content of 65%.

Table 7.1 depicts the fatty acid composition of unfermented PKC oil as well as fermented PKC after SSF by the aforementioned fungal strains. PKC oil mainly consists of lauric acid (C12:0) (47.7%), myristic acid (C14:0) (15.3%), palmitic acid (C16:0) (9.3%) and oleic acid (C18:1) (15.1%) with a ratio of saturated to unsaturated lipids (RSU) equal to 4.7. At the end of SSF, all fungal strains were able to transform the fatty acid composition of unfermented PKC oil, reducing the RSU to the range of 0.8 - 1.5. In particular, C12:0 was reduced to 13.1 - 27.7%, while C18:1 and C16:0 were increased to 27.6 - 34.9% and 12.2 - 17.3%, respectively. Low quantities of GLA (C18:3) were also detected (0.7 - 5.3%) for all fungal strains, except for *T. elegans*. Evidently, the increased production of C18:1 fatty acid may be attributed mostly to *ex novo* lipogenesis rather than the partial saturation of linoleic acid (C18:2), since C18:2 fatty acid was also increased throughout the fermentation. Lower filamentous fungi are able to follow versatile metabolic pathways, leading to fatty acid biosynthesis and bioconversion processes (Abu et al., 2000).

Table 7.1 Fatty acid profile of oil derived from unfermented palm kernel cake (PKC) and from 15-days solid state fermentation on PKC by five fungal strains.

Oil source	Fatty acid methyl esters (%)										RSU ^a
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	Others	
Unfermented PKC	3.5	3.6	47.7	15.3	9.3	3.0	15.1	2.5	-	-	4.7
<i>C. echinulata</i>	1.5	1.9	27.7	10.8	12.2	3.0	27.6	9.9	5.3	-	1.3
<i>M. ramanniana</i> MUCL	0.6	0.7	13.1	8.4	17.3	4.2	34.9	18.0	2.7	-	0.8
<i>M. ramanniana</i> ATHUM 2922	1.5	2.0	24.7	9.4	15.9	6.0	34.2	5.6	0.7	0.1	1.5
<i>M. isabellina</i>	-	1.2	22.5	11.9	14.5	5.0	33.7	9.4	1.7	-	1.2
<i>T. elegans</i>	-	0.9	19.2	10.5	16.0	5.3	32.8	9.0	-	6.2	1.2

^a RSU: ratio of saturated to unsaturated lipids

7.3. Effect of temperature

The fungi *C. echinulata* and *M. ramanniana* MUCL were the most efficient strains for lipid production among all fungal strains and thus were selected for further evaluation of the fermentation conditions. Results concerning the effect of temperature on lipid production are presented in Figure 7.3. Low lipid production was achieved at 25 °C (75.5 mg/gfs) and 35 °C (40 mg/gfs) with *C. echinulata* after 11 days. The most effective temperature for *C. echinulata* was 30 °C yielding a maximum lipid production of 129.1 mg/gfs after 11 days, while at 27 °C and 33 °C lipid production was up to 111 mg/gfs after 11 days (Figure 8.3a). In the case of *M. ramanniana* MUCL, the most efficient temperature was 27 °C resulting in 100 mg/gfs of lipids after 7 days (Figure 8.3c). Lipid production varied between 31.4 - 35.7 mg/g at 25 °C, 30 °C and 33 °C, whereas at 35 °C only a 30.4% depletion of PKC oil content was observed. It should be stressed that an increase in temperature, led eventually to increased moisture loss for both fungal strains (Figure 7.3b, d).

Temperature constitutes an important variable for SSF performance as it directly affects the growth of microorganism and metabolites production. This is related to phenomena like protein denaturation, enzyme inhibition, and cell death. Temperature control is complicated when dealing with SSF due to its static nature and poor thermal conductivity of solids matrices. The results obtained in this study showed that temperature affected lipid production mainly due to

its impact on the moisture content of the substrate, which may be related to the use of non-humidified chambers.

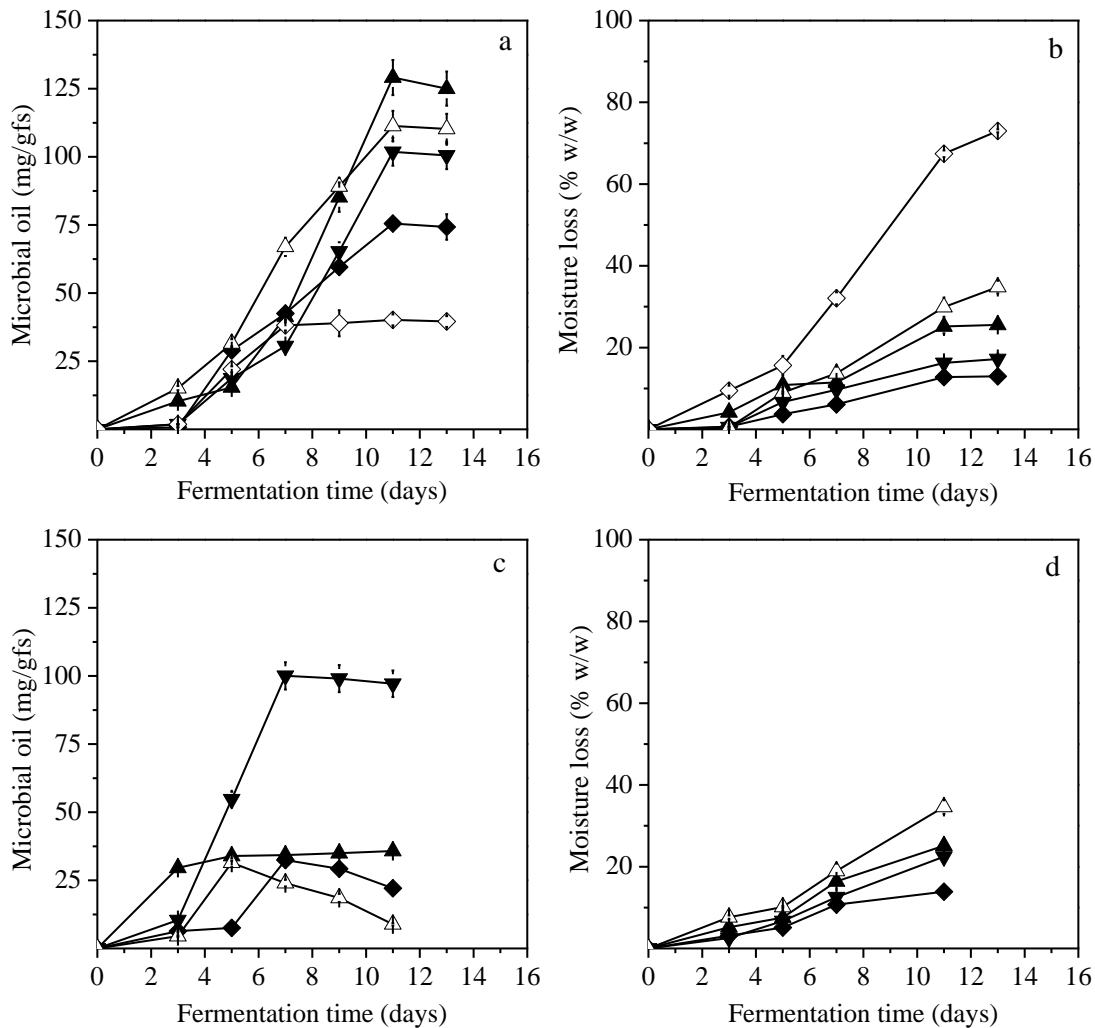


Figure 7.3 Microbial oil production and moisture loss during solid state fermentation of *C. echinulata* (a, b) and *M. ramanniana* MUCL (c, d) at five different temperatures (◆: 25 °C, ▼: 27 °C, ▲: 30 °C, △: 33 °C, ◇: 35 °C), using palm kernel cake as substrate at initial moisture content of 65%.

Fatty acids, are considered to be regulators of membrane fluidity and permeability (Russell, 1990). Investigating the correlation between temperature and lipid unsaturation, it can be concluded an inverse relationship between these two factors (Théberge et al., 1996). This was verified by the fatty acid analysis (Table 7.2), that showed an increasing RSU from 27 °C to 35 °C for both fungal strains. The lowest temperature regime, displayed the highest RSU (3.2 - 3.6) and the lowest fatty acid transformation.

GLA formation varied between 0.5 - 4.5% in the case of *C. echinulata*. C12:0 was substantially decreased by 41.1% at 27 °C and 46.3% at 30 °C. C16:0, C18:1 and C18:2 were increased in all temperature regimes. In the case of *M. ramanniana* MUCL, the highest fatty acid transformation was achieved at 27 °C, resulting in 2.7% GLA, 34.9% C18:1 and 18.0% C18:2, whereas C12:0 was reduced by 72.5%.

Table 7.2 Effect of temperature on fatty acid profile of oil derived from 11-days solid state fermentation on palm kernel cake (PKC) by *C. echinulata* and *M. ramanniana* MUCL.

Temperature (°C)	Fatty acid methyl esters (%)									RSU ^a
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	
<i>C. echinulata</i>										
25	1.5	2.4	41.5	15.5	12.9	4.5	18.0	3.2	0.5	3.6
27	1.3	2.1	28.1	11.1	12.5	3.6	26.9	9.9	4.5	1.4
30	1.8	1.9	25.6	11	17.1	5.7	23.5	7.5	3.8	1.8
33	0.9	2.6	39.3	16.3	10.6	3.5	20.7	5.7	0.5	2.7
35	1	2.0	40.1	15.5	12.7	5.3	18.3	3.9	1.2	3.3
<i>M. ramanniana</i> MUCL										
25	3.2	3.3	42.9	14.1	9.4	3.1	19	4.2	0.8	3.2
27	0.6	0.7	13.1	8.4	17.3	4.2	34.9	18.0	2.7	0.8
30	3.1	3.1	40.6	14	10	3.8	21.4	3.7	0.5	2.9
33	3	3.1	40.3	13.5	10.9	4.7	19.6	4.0	0.8	3.1
35	3.2	3.4	42.9	14.4	9.8	3.3	19.7	3.0	0.3	3.4

^a RSU: ratio of saturated to unsaturated lipids

7.4. Effect of initial moisture content

Consequently, the effect of initial moisture content of the substrate was studied in the range of 55 - 75% on lipid production by *C. echinulata* (30 °C) and *M. ramanniana* MUCL (27 °C) (Figure 8.4). The highest lipids production by *C. echinulata* was 129.1 mg/gfs at 65% initial moisture content, followed by 108.8 mg/gfs at initial moisture content of 70%, after 11 days. Moisture levels lower than 65%, were insufficient for the growth of *C. echinulata* resulting in PKC oil content reduction by 71.9% and 79.8% at 55% and 60% initial moisture level, respectively. In these cases, moisture losses were determined between 53 - 76% at the end of the fermentation. The highest initial moisture content of 75% led to the lowest microbial oil

production of 57.3 mg/gfs and to the lowest moisture loss (15.7%) after 11 days of fermentation. Solid state fermentation of *M. ramanniana* MUCL led to the maximum lipid production of 110.1 mg/gfs at 70% initial moisture content at 7 days. Satisfactory lipid production was achieved at initial moisture contents of 65% and 75% reaching respective values of 100.1 mg/gfs and 93.8 mg/gfs at 7 days. Initial moisture content of 60%, led to the lowest lipid production of 26.7 mg/gfs at 11 days. The moisture level of 55% was insufficient for microbial lipids production, since only depletion of PKC oil content (27%) was determined. In all cases, moisture losses were found higher when low initial moisture contents were applied (Figures 7.4 b, d).

This study showed that a moisture content around 65 - 70%, depending on the microorganism, is required for maximum lipid production, since lower moisture levels are related to difficulties in nutrient diffusion and microbial growth, and higher levels (>70%) lead to substrate conglomeration, hindering air transfer phenomena and promoting bacterial contamination (Krishna, 2005).

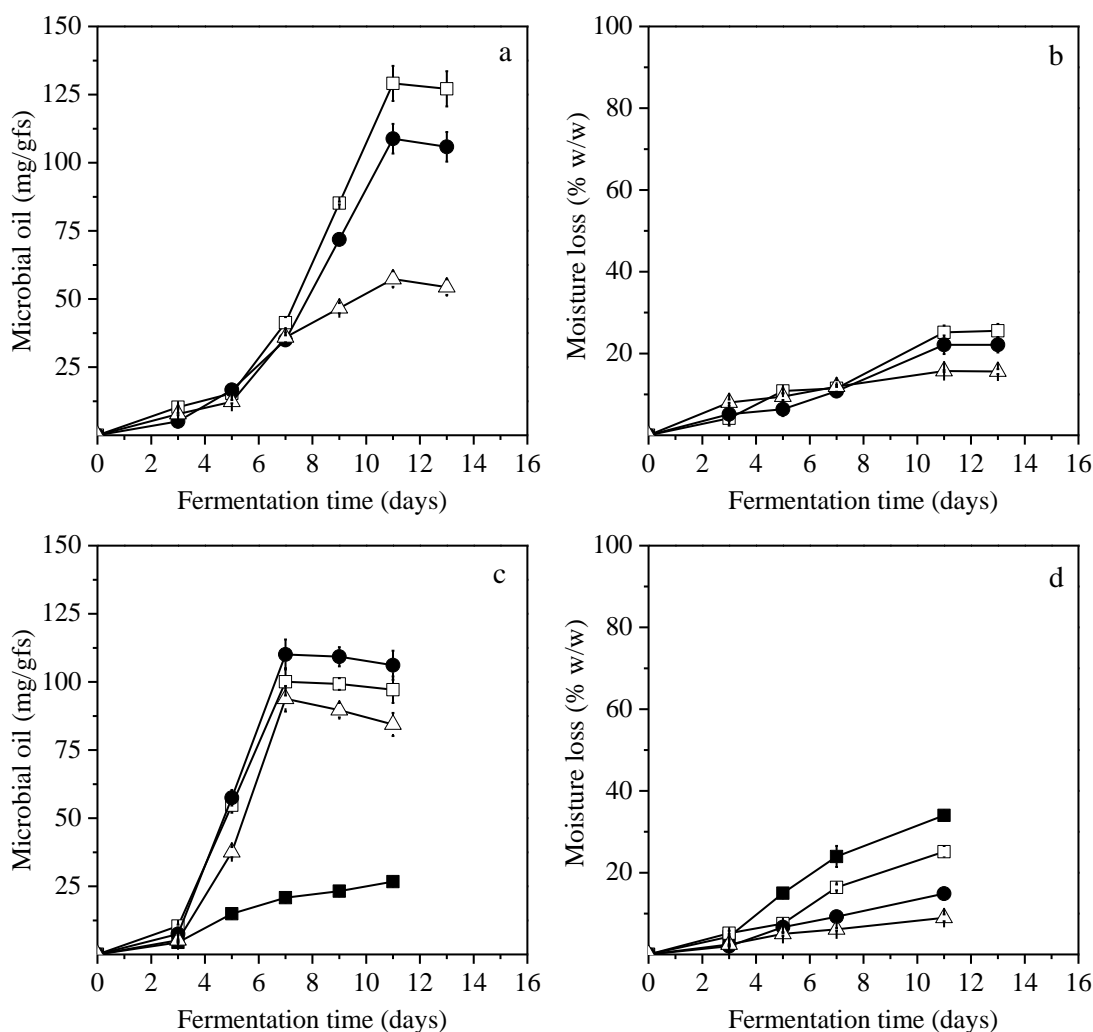


Figure 7.4 Microbial oil production and moisture loss during solid state fermentation of *C. echinulata* at 30 °C (a, b) and *M. ramanniana* MUCL at 27 °C (c, d), using palm kernel cake as substrate at different initial moisture contents (% w/w) (■: 60, □: 65, ●: 70, △: 75).

Fatty acid composition of the 11-day fermented PKC (Table 7.3), presented notable changes in comparison to the fatty acid composition of unfermented PKC for both fungal strains. The best initial moisture level (65%) for *C. echinulata* resulted in the highest mono- and poly-unsaturated fatty acid content with the lowest RSU (1.5). In particular, fermented solids of *C. echinulata* showed increased content of C18:1 (26.2%) and C18:2 (10%). C12:0 was decreased from 47.7% to 28.5%. *C. echinulata* fermented solids contained also GLA up to 4.5%. With respect to *M. ramanniana* MUCL, initial moisture contents of 55% and 60% did not influence the fatty acid composition while the produced GLA was low (0.5% - 0.7%). At higher initial moisture levels, C16:0 and C18:1 contents were increased while C12:0 was significantly

decreased. GLA formation reached its maximum (3.3%) at the best initial moisture contents (70%) for lipid accumulation.

Table 7.3 Effect of initial moisture content on fatty acid profile of oil derived from 11-days solid state fermentation on palm kernel cake (PKC) by *C. echinulata* and *M. ramanniana* MUCL.

Moisture content (%)	Fatty acid methyl esters (%)									RSU ^a
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	
<i>C. echinulata</i>										
55	2.4	2.6	34.6	11.3	13.7	6.2	22.9	6.3	-	2.5
60	2.6	2.2	31.2	11.8	14.8	6.4	22.6	6.0	2.4	2.3
65	1.5	1.9	28.5	11.8	12.2	3.4	26.2	10.0	4.5	1.5
70	0.9	1.7	38.9	16.5	11.7	3.2	20.7	4.6	1.9	2.7
75	0.5	0.7	34.3	18.4	13.6	4.7	22.4	4.2	1.2	2.6
<i>M. ramanniana</i> MUCL										
55	3.3	3.2	43.9	14.8	9.9	3.2	17.9	3.3	0.5	3.6
60	3.3	3.2	43.7	14.7	10.1	3.3	17.7	3.3	0.7	3.6
65	0.6	0.7	13.1	8.4	17.3	4.2	34.9	18.0	2.7	0.8
70	1.1	1.2	16.8	10.6	14.9	6.5	34.6	10.9	3.3	1.0
75	1.7	2.0	26.6	11.6	13.4	6.0	39.4	6.9	2.5	1.6

^a RSU: ratio of saturated to unsaturated lipids

7.5. Effect of different palm kernel cake to palm pressed fiber ratios

In an attempt to increase the surface area of SSF and its capacity to interchange with microbial growth and heat and mass transfer phenomena, two different ratios of PKC to PPF were studied (1:1 and 3:1, w/w). PPF is normally utilized for the prevention of the solids packing and porosity increase, serving mostly as a supporting matrix or a texturizer rather than a nutrient source (de Castro et al., 2016). In the case of 1:1 ratio, the produced microbial oil did not exceed the initial oil content of the unfermented substrate. Specifically, it was observed a depletion of PKC oil by 62.9% and 23.9% for *C. echinulata* and *M. ramanniana* MUCL respectively, after 11 days. This was possibly related to the lignin-rich and protein-low content of PPF. The moisture loss was 33.0% for *C. echinulata* and 19.1% for *M. ramanniana* MUCL. *C. echinulata* showed

similar behaviour when cultivated at 3:1 ratio of PKC:PPF. Considering *M. ramanniana* MUCL, it produced 69.9 mg/gfs of lipids after 11 days, when applying the 3:1 solid ratio with a moisture loss equal to 17.1%.

Fatty acid composition of lipids produced by *C. echinulata* under the two different solid ratios was quite different from the initial fatty acid composition of the unfermented solids (Table 7.4). C12:0 and C14:0, were decreased to 17.7% - 18.9% and 6.9% respectively. C16:0 and C18:1 were increased 20.0% - 21.0% and 23.3% - 36.7%, respectively. When *C. echinulata* was cultivated in the 1:1 ratio, the GLA content was slightly higher (3.2%) than in the 3:1 ratio (2.7%). The RSU was importantly decreased in both ratios. In the case of *M. ramanniana* MUCL, the fatty acid profile of 1:1 ratio, was similar to the initial unfermented substrate. At 3:1 ratio, a substantial increase of C18:1 (31.9%) and decrease of C12:0 (22.1%) was monitored. The GLA content of microbial oil was increased up to 1.9%.

Table 7.4 Effect of different palm kernel cake to palm pressed fiber (PKC:PPF) ratios on fatty acid profile of oil derived from unfermented PKC:PPF, after 11-days solid state fermentation by *C. echinulata* and *M. ramanniana* MUCL.

PKC:PPF ratio (w/w)	Fatty acid methyl esters (%)											RSU ^a
	C8:0	C10:0	C12:0	C14:0	Δ^9 C14:1	C16:0	Δ^9 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	
	Unfermented substrate											
1:1	3.0	2.7	37.3	11.3	0.9	15.8	0.8	3.0	21.6	3.6	-	2.8
3:1	3.5	3.2	42.5	13.9	0.7	12.4	0.6	2.6	18.0	2.6	-	3.6
	<i>C. echinulata</i>											
1:1	-	1.5	17.7	6.9	7.4	20.0	5.2	3.8	26.7	7.5	3.2	1.0
3:1	2.1	1.5	18.9	6.9	6.4	21.0	4.1	6.4	23.3	6.8	2.7	1.3
	<i>M. ramanniana</i> MUCL											
1:1	2.1	2.3	32.0	12.0	1.7	13.8	1.3	3.7	25.5	4.6	1.0	1.9
3:1	1.3	1.7	22.1	8.7	1.2	18.4	1.2	4.9	31.9	6.8	1.9	1.3

^a RSU: ratio of saturated to unsaturated lipids

7.6. Effect of nutrient supplementation

Solid state fermentations under the optimized conditions (temperature and initial moisture content) for *C. echinulata* and *M. ramanniana* MUCL were repeated in an air humidified

chamber with 90% air saturation. Similar lipids yields were obtained compared to results of SSF conducted in a non-humidified chamber (data not shown). Experiments with *C. echinulata* demonstrated the highest potential in lipid accumulation and therefore it was selected for further study. In this case, an attempt to monitor the biomass production was conducted via the estimation of fungal glucosamine content. Four sets of fermentations were carried out, with individual supplementation of PKC with 5% molasses, 5% crude glycerol, a mixture of mineral solution or nitrogen sources. Except for nitrogen sources, the other supplements resulted in high moisture losses up to 51.0% after 8 days and low biomass production, varying between 54.0 - 83.5 mg/gfs. The produced intracellular lipids did not exceed the initial oil of the unfermented PKC. The initial oil content of the unfermented solids (Table 5.1) was depleted by 64.4% - 80.8% after 8 days. Enrichment of PKC with nitrogen sources, seemed to trigger lipid production. Maximum lipid production of 91.0 ± 3.9 mg/gfs was achieved at 7 days in the presence of nitrogen source (data not shown), while the maximum lipid production of 129.1 mg/gfs was attained after 11 days without additional nitrogen source (Figure 7.3). This indicated that PKC enrichment with additional nitrogen sources enhanced lipid productivity in SSF. Also, the high biomass formation in this case (144.3 mg/gfs), seemed to deteriorate moisture loss of the substrate to 8.6% after 8 days.

Fatty acid profile of all supplementation treatments showed alterations in comparison to the oil of the unfermented PKC (Table 7.5). C12:0 was reduced in all cases varying between 24.8 - 39.0%, while C18:1 and C16:0 were increased in the range of 23.4 - 39.1% and 10.3 - 18.1%, respectively. C14:0 remained either almost stable (14.8%) as in the case of nitrogen sources enrichment, or decreased (7.7 - 10.4%). Moreover, GLA was not detected in any applied treatment.

Table 7.5 Effect of different supplementation treatments on fatty acid profile of oil derived from unfermented palm kernel cake (PKC), after 10 days of solid state fermentation by *C. echinulata*, using PKC as substrate.

Substrate	Fatty acid methyl esters (%)									RSU ^a
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	
N-sources	-	-	39.0	14.8	15.1	3.8	23.4	3.9	-	2.7
Minerals	-	1.6	33.5	10.4	10.3	2.1	39.1	2.9	-	1.4
Glycerol	-	-	24.8	7.7	18.1	9.6	34.2	5.5	-	1.5
Molasses	-	-	29.8	9.7	17.1	7.6	30.2	5.5	-	1.8

^a RSU: ratio of saturated to unsaturated lipids

7.7. Screening of protein-rich agricultural residues for lipid production

The previous section (7.6) indicated that *C. echinulata* may perform better in solid matrices with higher protein content than PKC. Thus, two agricultural residues (CoSC and CaSC), rich in protein (Table 7.6), were selected for solid state production of intracellular lipids. Their composition is presented in Table 7.6. CaSC showed the highest protein content (35.4%), followed by CoSC (24.4%), PCK (12.8%) and PPF (4.2%) (Table 5.1). The highest oil content (9.2%) was determined for PKC (Table 5.1) and CoSC, while PPF and CaSC presented the lowest oil content values (up to 6.7%). Carbon to nitrogen (C/N) ratios were reversely related to the protein content, with CaSC corresponding to the lowest value (8.0) and PPF to the highest (69.5) (Table 5.1).

Table 7.6 Composition of cotton seed cake (CoSC) and castor seed cake (CaSC).

Composition	CoSC	CaSC
Moisture	4.6±0.2	4.5±0.1
Protein (6.25×N) ^a	24.4±1.3	35.4±1.9
C/N ^b ratio ^a	11.5±0.5	8.0±0.4
Oil ^a	9.2±0.3	6.7±1.8

^a Results were expressed as g per 100 g of dry solids

^b carbon to nitrogen (C/N)

Figure 7.5 presents biomass growth, lipids accumulation, proteases activity and lipases activity during SSF of PKC, CoSC and CaSC. *C.echinulata* was efficiently grown in all renewable resources with CoSC resulting in increased biomass production of 304.1 mg/gfs. Lipid production was 122.2 mg/gfs with an intracellular lipid content of 40.2%. Productivity in PKC, was two folds lower in comparison to CoSC (0.02 g/gfs/day) and biomass production amounted to 234.1 mg/gfs, after 11 days. SSF of CaSC led to biomass formation of 252.1 mg/gfs with low intracellular lipid content (29.9%), which was attributed to the low C/N ratio of the substrate (Table 7.6). Higher C/N ratios favor intracellular lipids production while lower ratios until a particular threshold, have been proven to be beneficial for biomass growth (Freire et al., 1997). As it is depicted in Table 7.6, CaSC had the lowest C/N ratio (8.0), followed by CoSC (11.5) and PKC (25.2). Moreover, the presence of a potential toxin (ricin) and an allergenic protein fraction (CB-1A or 2S albumin isoforms) may also influenced the metabolism for lipid

production since they are limiting factors for the utilization of castor seed waste by living organisms (Godoy et al., 2009).

Lipases activity in all substrates was remarkably low. The highest value was observed at the 3rd day of SSF on CoSC (8.5 U/gfs) and sharply decreased thereafter (0.5 U/gfs). Considering CaSC, lipases activity reached the maximum value of 5.2 U/gfs at the 4th day of fermentation and decreased to 0.1 - 0.4 U/gfs thereafter, whereas for PKC it was in the range of 0.4 - 1.5 U/gfs throughout fermentation.

Proteases production was enhanced in the three solid matrices. In the case of CaSC and CoSC, maximum respective values of 112.8 U/gfs after 7 days and 114.2 U/gfs after 3 days were achieved. Proteolytic activity in PKC was 146.7 U/gfs after 3 days and reached the maximum value of 201.2 U/gfs after 7 days.

Lipases activity has been reported to be negatively affected by proteolysis (Freire et al. 1997) and validated via an unstructured mathematical model that describes time course variations of extracellular lipases and proteases activities for batch fermentations of the fungus *Penicillium restrictum* (Freire et al., 1999). Due to the aforementioned, high proteases activity may have caused lipase inactivation.

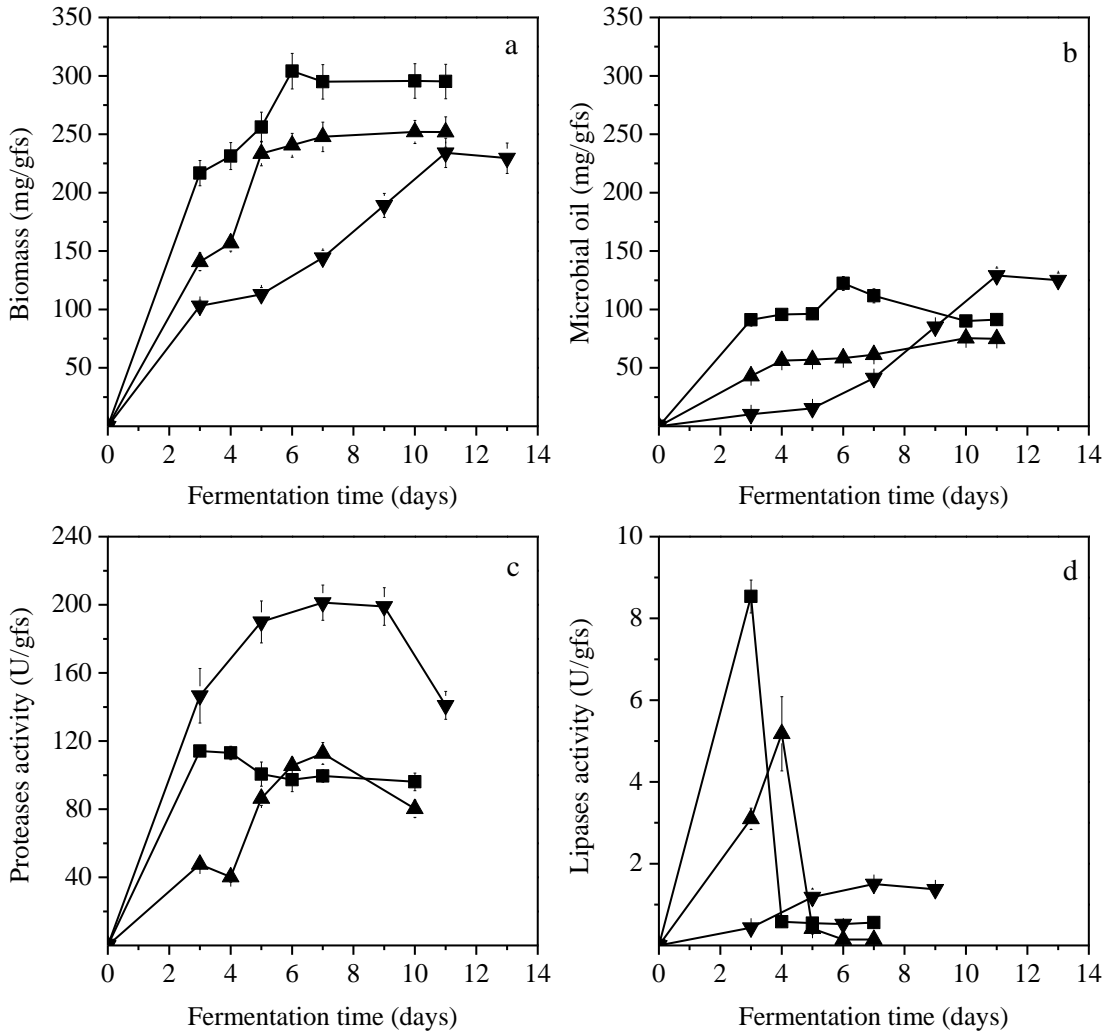


Figure 7.5 Kinetic of biomass production, lipids accumulation, proteases and lipases activities, during solid state fermentation of *C. echinulata* using palm kernel cake (▼), cotton seed cake (■) and castor seed cake (▲) as substrates at 30 °C and 65% initial moisture content.

Moisture losses for CoSC and CaSC respectively, reached 9.6% and 12.8% after 11 days while PKC resulted in 25.5% loss at the same fermentation time (Figure 7.6). Water activity (A_w) of all substrates during fermentation course, is shown in Figure 7.6. A_w constitutes a thermodynamic factor that indicates the available water of the solid substrate, participating in several reactions that take place during the SSF. As it can be seen in Figure 7.6b, A_w decreases during SSF as a result of moisture losses of the solid substrates. The decrease of initial moisture content of PKC was higher than that of the other substrates leading to higher A_w changes. It was finally reduced to 0.96. For CoSC and CaSC, A_w was reduced up to 0.98 at the end of fermentation.

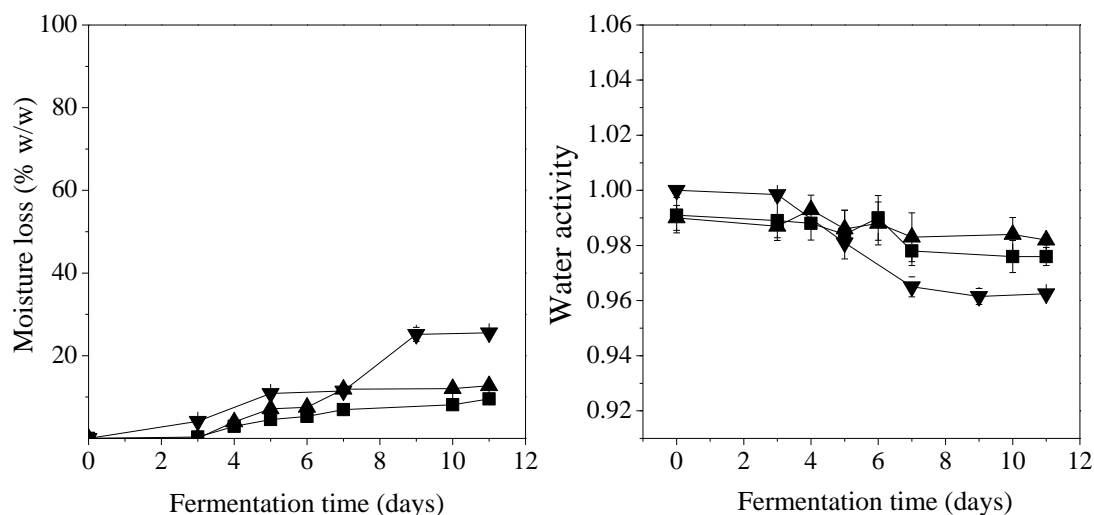


Figure 7.6 Moisture loss and water activity of 11 days solid state fermentation of *C. echinulata*, using palm kernel cake (▼), cotton seed cake (■) and castor seed cake (▲) as substrates at 30 °C and 65% initial moisture content.

Table 7.7 shows the fatty acid profile of microbial lipids produced during the cultivation of *C. echinulata* at the aforementioned substrates. Initial oil of CoSC and lipids extracted from the fermented CoSC presented similar profile, mainly consisted of C18:2 (49.7 - 51.9%) and C16:0 (27.1 - 27.5%) followed by C18:1 (16.6 - 17.1%). Lipids extracted from fermented CaSC showed differences as compared to the initial oil of the substrate. Ricinoleic acid ((12R)C18:1) was the predominant fatty acid and it was decreased to 65.2%. C16:0 and C18:1 were increased to 9.5% and 10.3%, respectively. GLA content of the fermented CoSC and CaSC was quite low (0.8%).

Table 7.7 Fatty acid profile of intercellular lipids produced by *C. echinulata*, in cotton seed cake (CoSC) and castor seed cake (CaSC) after 11 days of fermentation.

Oil source	Fatty acid methyl esters (%)								RSU ^a
	C12:0	C16:0	C18:0	Δ^9 C18:1	(12R) Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	Others	
Unfermented CoSC	-	27.1	4.5	16.6	-	51.9	-	-	0.5
Fermented CoSC	-	27.5	4.2	17.1	-	49.7	0.8	0.8	0.5
Unfermented CaBC	-	5.7	2.7	8.6	74.2	8.8	-	-	0.1
Fermented CaBC	2.2	9.5	3.0	10.3	65.2	8.3	0.8	0.7	0.2

^a RSU: ratio of saturated to unsaturated lipids

7.8. Solid state fermentation in packed bed reactor

Lipid production was scaled up from tray bioreactor to lab-scale packed bed bioreactor (PBR). Maximum biomass growth (320.5 mg/gfs) was observed after 5 days with a lipid content of 37.7% and a productivity of 0.024 g/gfs/d (Figure 7.7). Although biomass and lipids production were lower than those achieved in tray bioreactors, the productivity was higher. Lipases activity was low (0.3 - 1.4 U/gfs). The highest proteases activity was achieved after 2 days (133.1 U/gfs), which then gradually decreased to 62.2 U/gfs at the 7th day. Moisture losses were quite controllable until the 6th day reaching up to 11.5% while increased thereafter (30.9 - 36.5%). This is probably attributed to porosity reduction of the bed that was observed during fermentation, creating a packed cotton matrix (Soccol et al., 2017). The formation of the packed solid column can result in limited aeration during fermentation and consequently lead to reduced fermentation efficiency.

Fatty acid analysis showed that C18:2 (45.8%) was the predominant fatty acid followed by C16:0 (33.9%), C18:1 (16.4%) and C18:2 (3.9%). GLA was not detected in SSF employed in PBR.

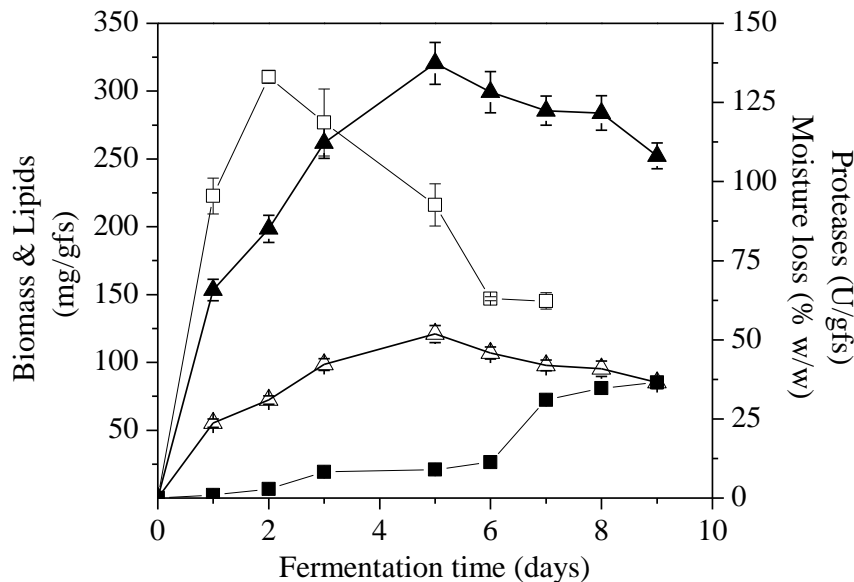


Figure 7.7 Biomass growth (▲), lipids accumulation (Δ), protease activity (□) and moisture loss (■), during solid state fermentation of *C. echinulata* using cotton seed cake as substrate in packed bed reactor at 30 °C and 65% initial moisture content.

SSF for lipids production, carried out in shake flasks, petri dishes or plastic bags has been implemented in several studies. Stredansky et al. (2000) demonstrated the efficient lipid

production varying between 150 - 168 mg/gfs and productivities of 0.019 - 0.026 g/gfs/d, when *Cunninghamella* and *Mortierella* strains were cultivated on apple pomace and spent malt grain (SMG) impregnated with a nutrient solution. GLA content fluctuated between 4.2 - 7.4%. In the same study, *T. elegans* resulted in 212 mg/gfs of lipids with a GLA content of 9.1% when cultivated on a mixture of apple pomace and SMG impregnated with peanut oil and a nutrient solution under forced aeration. In another study, *C. elegans* was the most efficient strain resulting in 172 mg/gfs of lipids, productivity of 0.024 mg/gfs/d and 11.6% GLA when cultivated on 15:5:1 barley – SMG - peanut oil and nutrient solution (Conti et al., 2001). Lower lipids production has been reported by Peng and Chen 2007 (23 - 42 mg/gfs), Peng and Chen 2008 (79.0 mg/gfs), Hui et al. 2010 (62.9 mg/gfs) and Zhang and Hu, 2012 (47.9 mg/gfs) with productivities in the range of 0.002 - 0.01 mg/gfs/d using wheat straw and bran and soybean hulls as substrates. This indicates that the composition of substrate has a key role in lipid production and fatty acid composition.

This is the first report of microbial oil production in PBR. Microbial oil production (121.0 mg/gfs) was lower than the aforementioned studies, but productivity (0.024 g/gfs/d) was similar or even higher. Experiments conducted on tray reactors led to microbial oil production in the range of 75.4 - 129.1 mg/gfs when PKC, CoSC and CaBC were employed. The GLA content was in the range of 0.8 - 4.5% and productivities of 0.008 - 0.02 mg/gfs/d were observed. Further investigation on SSF supplementation, pretreatment and aeration should be investigated in order to achieve sufficient microbial oil production and GLA content that is suitable for industrial implementation.

7.9. Polyol esters production

Microbial lipids with fatty acid composition similar to vegetable oils are suitable for the production of oleochemicals (Kiran et al., 2015), eliminating thus the utilisation of food-grade vegetable oils for this purpose. The microbial oil of *C. echinulata*, which was extracted from fermented CoSC, was initially hydrolysed in order to obtain a free fatty acid-rich fraction. The enzymatic bioconversion of free fatty acids to biolubricants was monitored via the reduction of the acidity, since analytical standards of polyols esters are not commercially available. The initial acidity of microbial oil was 18.0% which was increased to 87.7% after 24 h of enzymatic hydrolysis. A schematic diagram of the process is provided in Figure 7.8.

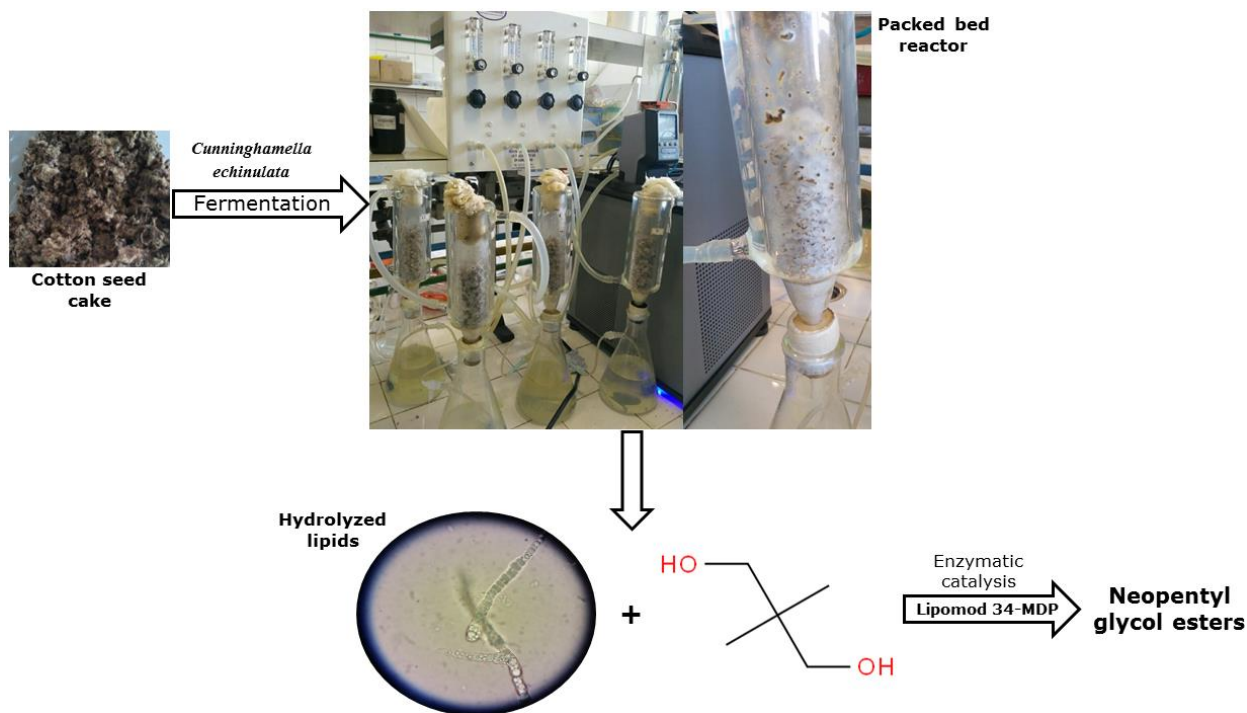


Figure 7.8 Schematic diagram for neopentyl glycol esters production utilising hydrolysed microbial oil.

Figure 7.9 present the results obtained from the enzymatic reaction of hydrolyzed microbial oil with NPG catalyzed by LipoMOD 34MDP. Conversion yield reached up to 74.3% after 30 minutes of reaction while a conversion yield higher than 80% was achieved after 2 h.

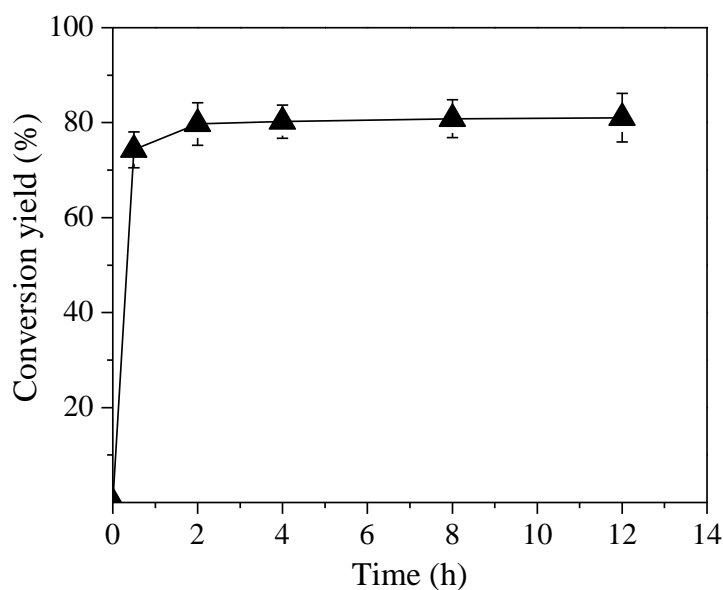


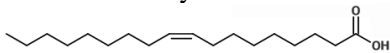

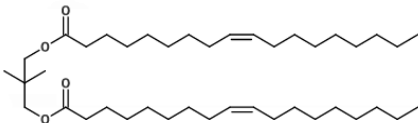
Figure 7.9 Kinetic profile of polyol esters production by enzymatic esterification of the hydrolysed microbial oil of *C. echinulata* with neopentyl glycol.

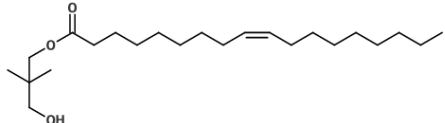
Studies dealing with microbial oil as raw material for biolubricants production are limited in the literature. Papadaki et al. (2018) reported the utilisation of microbial oil produced from oleaginous yeasts for the production of biolubricants, showing a conversion yield of 88% for NPG esters at 72 h. Most of the studies have focused on the enzymatic production of biolubricants from vegetable oils such as rapeseed oil with conversion yield up to 90% after 68 h (Uosukainen et al., 1998), fatty acid distillates from palm oil with conversion yield up to 94% at 24 h (Fernandes et al., 2018), or oleic acid with a conversion yield of 80% after 50 h (Åkerman et al., 2011).

7.9.1. Nuclear magnetic resonance

The composition of NPG esters concerning the content of di- and mono-esters was determined via NMR analysis (Tables 7.8). According to the data obtained, monoester content (29.6%, w/w) was quite lower than the diesters content (56.1%, w/w). The non-esterified content of NPG was 3.5% while fatty acids content was 10.8%. This could be improved by using surplus free fatty acid content during esterification. For instance, Cavalcanti et al. (2018) reported that enzymatic esterification of free fatty acids of soybean oil with NPG resulted in 100% esterification of the OH groups when applying a molar ratio of free fatty acids to polyol equal to 3.75:1.

Table 7.8 NMR analysis of biolubricants produced at 24 h via esterification of neopentyl glycol (NPG) and hydrolysed microbial oil derived from solid state cultivation of *C. echinulata* on cotton seed cake.

Compound	Molar (%)	Mass (%)
Fatty acids 	15.7	10.8
Neopentyl glycol 	13.5	3.5
Neopentyl glycol diesters 	37.6	56.1

Neopentyl glycol monoesters		
	33.2	29.6

7.9.2. Characterization of neopentyl glycol esters

Desirable viscosity values and indices are related to high working efficiencies of mechanical devices and low energy requirements, as a result of favorable friction forces that lead to prevention of collision and rubbing between two contacting surfaces. High viscosity indices ensure that lubricants remain effective even at high temperatures by maintaining the thickness of the oil film (Mobarak et al., 2014). Table 7.9 depicts acid number, kinematic viscosity and viscosity index of polyol esters. The determined viscosity index (161), is quite comparable with esters produced via enzymatic esterification of NPG with free fatty acids derived from yeast lipids (181 - 183) or soybean oil (214) (Cavalcanti et al., 2018). Since microbial oil consists of triglycerides that maintain intermolecular interactions in elevated temperatures, the viscosity index of microbial oil-based lubricant is normally higher than that of mineral oils (Zainal et al., 2018).

Table 7.9 Characterisation of polyol esters deriving from enzymatic reaction between hydrolysed microbial oil and neopentyl glycol catalysed by Lipomod 34MDP.

Characteristic	Results
Acid number (mg KOH/g)	23.2
Kinematic viscosity 40 °C (mm ² /s)	30.1
Kinematic viscosity 100 °C (mm ² /s)	6.2
Viscosity Index	161

7.10. Concluding remarks

This study demonstrated that the valorisation of protein rich substrates led to higher biomass production. Lipid accumulation was not enhanced, since higher proteases activity led to decreased lipases activity. The *ex novo* lipid accumulation includes the biomodification of oil derived from the substrate to microbial lipids, through reactions catalyzed by lipases (Uçkun Kiran et al., 2015). The study of lipase activity proved that low amounts of fungal lipids were produced because of the low lipase activity. The produced microbial lipids were successfully

converted to esters with lubrication properties through a solvent free enzymatic reaction. The present study showed that agriculture residues can be employed as fermentation feedstock for the production of fungal lipids for specialty applications.

CHAPTER 8

Synthesis of fatty acid esters using γ -linolenic acid rich microbial oil produced via fungal fermentation of lignocellulose hydrolysate

8.1. Introduction

The pulp and paper industry produces considerable quantities of spent liquors. Spent sulphite liquor (SSL) is the condensed liquid side stream generated by acid bisulphite pulp manufacture for the production of high quality paper. SSL contains high concentrations of pentoses and hexoses as well as lignin degradation compounds e.g lignosulphonates (LS). Production of high value added metabolites i.e microbial oil, especially rich in polyunsaturated fatty acids (PUFA) i.e γ -linolenic acid (GLA), offers great potential considering that similar studies have not been reported in the literature.

The production of microbial oil rich in GLA is of high importance, mainly because of its unique nutraceutical properties (Bellou et al., 2014). Increasing market demand for GLA-lipids, inadequate supply of GLA from agricultural and animal sources and lack of GLA in humans, has driven research towards the biotechnological production of GLA, employing microorganisms capable of producing it in high concentrations (up to 24%). Specifically, lower filamentous fungi of the division *Zygomycota* are of paramount interest for the cosmetic industry as they can accumulate significant quantities of intracellular lipids rich in GLA (Čertík et al., 2012).

Microbial oil has recently been utilized for the production of high-added value fatty acid esters, such as biolubricants and waxes, within the concept of sustainable utilisation of food by-products (Papadaki et al., 2017; 2018). Short and middle chain fatty esters hold a big share of fatty acid esters market and they are applied as surfactants or biolucricant base oil, in personal care and cosmetics as emollients, fragrance and skin-conditioning agents and make-up products, in food processing as flavoring agents and in pharmaceuticals (Anonymous, 2018b; Khan and Rathod, 2015).

Enzymatic esterifications for the production of esters have been widely reported in the literature (Khan and Rathod, 2015) while transesterification processes, especially utilising microbial oils as acyl donators, have been rather limited. So far, biocatalytic synthesis of isopropyl and 2-ethylhexyl esters have been accomplished utilising high-oleic sunflower oil (Bouaid et al., 2007), palmitic acid (He et al., 2002; Tan et al., 2006), crambe and camelina oil (Steinke et al., 2000) and rapeseed oil (Linko et al., 1994).

This study focused on the valorisation of SSL as fermentative feedstock for the production of a GLA-rich microbial oil using *Mortierella* sp., *Cunninghamella* sp. and *Thamnidium* sp. fungal

strains. This is the first report describing the production of fatty acid esters from microbial oil rich in GLA using isopropyl and 2-ethylhexyl alcohols. The enzymatic synthesis was catalyzed by the commercial lipase Novozyme 435 in a solvent-free system. The reaction conditions evaluated, were temperature, substrate molar ratio and biocatalyst quantity. An insight to the influence of individual lipid classes to ester formation has also been carried out. Physicochemical properties of esters are determined as indispensable variables of their market target for their potent application in the food and cosmetic industry or nutraceuticals.

8.2. Microbial oil production and fatty acid profile

Five fungal strains of the division *Zygomycota* were evaluated for their ability to grow and accumulate intracellular lipids utilising nano-filtrated SSL as carbon source with a carbon to FAN ratio of 248 (Table 8.1). Their effective cultivation was indicated by total dry weight (TDW) ranging between 5.5 - 10.1 g/L and lipid content varying between 36.2 - 54.3%. *M. isabellina* was the most promising strain in terms of microbial oil production (5.5 g/L), intracellular content (54.3%), yield (0.2 g/g) and productivity (0.68 g/L/d).

Table 8.1. Fermentation efficiency achieved by various fungal strains when cultivated in shake flasks using nano-filtrated spent sulphite liquor at an initial sugar concentration of 30 g/L.

Microorganisms	TDW ¹ (g/L)	Microbial oil (g/L)	Lipid content (%)	Yield (g/g)	Productivity (g/L/d)
<i>C. echinulata</i> ATHUM 4411	5.5±0.21	2.0±0.11	36.2±1.52	0.09±0.00	0.25±0.01
<i>M. ramanniana</i> MUCL 2935	8.1±0.32	4.2±0.13	52.4±2.35	0.18±0.01	0.53±0.02
<i>M. ramanniana</i> ATHUM 2922	6.7±0.35	3.2±0.15	47.4±2.16	0.17±0.01	0.40±0.01
<i>M. isabellina</i> ATHUM 2935	10.1±0.50	5.5±0.16	54.3±2.37	0.20±0.01	0.68±0.03
<i>T. elegans</i>	7.5±0.23	3.1±0.07	41.5±1.50	0.13±0.01	0.39±0.02

¹TDW: total dry weight of cell mass

Table 8.2 shows the fatty acid profile of microbial lipids, produced by all strains. Microbial oil mainly contained oleic acid (C18:1) and palmitic acid (C16:0) followed by stearic acid (C18:0). *C. echinulata* presented the most interesting and desirable fatty acid profile, since the GLA content was 12%. The percentage of GLA for the other fungal strains reached up to 6.6%. Thus, lipids deriving from *C. echinulata* were selected as the raw material for bio-esters production.

Table 8.2. Fatty acid profile of microbial oil produced by various fungal strains in shake flasks using nano-filtrated spent sulphite liquor at an initial sugar concentration of 30 g/L.

Microorganisms	Fatty acid methyl esters (%)						
	C16:0	Δ^7 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	Others
<i>C. echinulata</i> ATHUM 4411	21.3	1.4	5.1	41.8	18.0	12.0	0.5
<i>M. ramanniana</i> MUCL 2935	21.9	0.5	5.7	49.7	15.8	5.4	0.9
<i>M. ramanniana</i> ATHUM 2922	20.6	0.6	5.8	49.9	15.7	5.3	2.1
<i>M. isabellina</i> ATHUM 2935	26.1	2.8	3.0	46.9	16.6	3.6	0.9
<i>T. elegans</i>	20.7	0.7	8.6	49.9	12.5	6.6	1.0

8.3. Characterization of lipids classes of microbial oil produced by *C. echinulata*

Lipids produced from *C. echinulata* were fractionated to classes of neutral lipids (NL), glycolipids plus sphingolipids (GL+SL) and phospholipids (PL), which were further characterized concerning their fatty acid composition (Table 8.3). The three lipid fractions mainly consisted of C18:1 (46.1 - 46.8%), followed by C16:0 (16.9 - 21.6%) and linoleic acid (C18:2) (12.1 - 15.0%) and GLA (10.8 - 14.3%). The predominant lipid fraction was NL (88.5%), followed by GL+SL (7.5%) and SL (4.0%). Molecular weight of microbial oil was calculated 868.9 g/mol.

Table 8.3. Characterization of individual lipid classes of microbial oil produced by *C. echinulata* in shake flask fermentation using nano-filtrated spent sulphite liquor at an initial sugar concentration of 30 g/L.

Lipid classes ¹	% (w/w)	Fatty acid methyl esters (%)						
		C16:0	Δ^9 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	Others
NL	88.5±1.7	17.6	1.5	9.6	46.8	12.1	10.8	1.6
GL+SL	7.5±0.9	16.9	0.9	6.9	46.1	15.0	14.3	0.0
PL	4.0±0.8	21.6	4.2	0.5	46.2	13.7	13.8	0.0

¹ Lipid classes refer to total lipids: NL: neutral lipids; GL+SL: glycolipids plus sphingolipids; PL: phospholipids

8.4. Bio-esters production utilising microbial oil

8.4.1. Effect of temperature

Bio-esters production was initially evaluated in terms of temperature (Figure 8.1). Reactions were conducted using a stoichiometric lipid to alcohol molar ratio (1:3) and 10% (w/w) of Novozyme 435. The maximum TCY for isopropyl esters (58.1%) was obtained at 60 °C. In this case, the conversion yields of palmitic, oleic and linoleic plus linolenic esters were 8.1%, 33.5% and 16.5%, respectively. TCY for 2-ethylhexyl esters was found quite similar at 60 °C and 70 °C, reaching up to 56.2% and 57.9%, respectively. For both temperatures the esters production was 9.0 - 10.7% for palmitic, 31.3 - 31.7% for oleic and 15.4 - 16.3% for linoleic plus linolenic acids. The reactions conditions were further studied, as it is described in the following sections, at 60 °C.

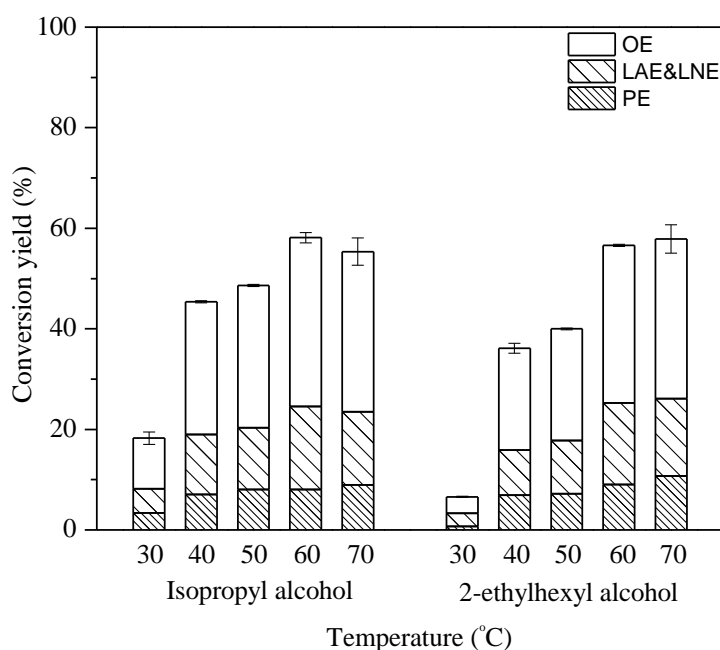


Figure 8.1 Effect of temperature on total conversion yield and individual yields of palmitate (PE), oleate (OE), and sum of linoleate and linolenate esters (LAE & LNE) during transesterifications of microbial oil with isopropanol and 2-ethylhexanol catalyzed by 10% (w/w) of Novozyme 435.

8.4.2. Effect of lipids to alcohol molar ratio

In subsequent transesterifications, various microbial oil to alcohol molar ratios were assessed (Figure 8.2). The TCY was gradually decreased as the molar ratio was increased further than

the stoichiometric molar ratio (1:3, lipid to alcohol), indicating that excess of alcohol has inhibitory effect on the enzyme activity. Specifically, the TCY was decreased to 21.2% and 35.6% for isopropyl and 2-ethylhexyl esters respectively, at a molar ratio of 1:4 (lipid to alcohol). The same trend was observed when the molar ratio was lower (1:2.84) than the stoichiometric molar ratio, since the lower quantity of alcohol was not adequate for the transesterification of triglycerides.

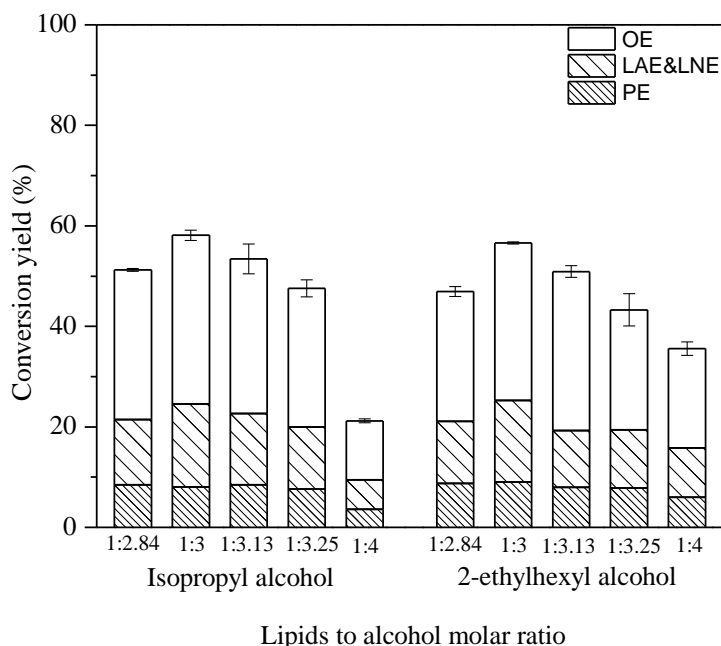


Figure 8.2 Effect of microbial oil to alcohol molar ratios on the total conversion yield and individual yields of palmitate (PE), oleate (OE), and sum of linoleate and linolenate esters (LAE & LNE) during transesterifications of microbial oil with isopropanol and 2-ethylhexanol catalyzed by 10% (w/w) of Novozyme 435 at 60 °C.

8.4.3. Effect of biocatalyst amount

Enzyme concentration significantly affects the economic feasibility of the whole biocatalytic process. Also, high amounts of enzyme could lead to their agglomeration, consequently hindering the substrate's potential for enzyme action. Figure 8.3 presents the effect of biocatalyst quantity on esters conversion yield. It was demonstrated that compared to the addition of 10% enzyme, TCY of isopropyl esters was increased only by 3.7% when 20% of enzyme was added to the reaction while the addition of 5% of enzyme resulted in a decrease of TCY (41.4%). In the case of 2-ethylhexyl esters, the TCY was almost stable when 10% and 20%

of biocatalyst was applied, thus it was substantially decreased (26.5%) when 5% of enzyme was used.

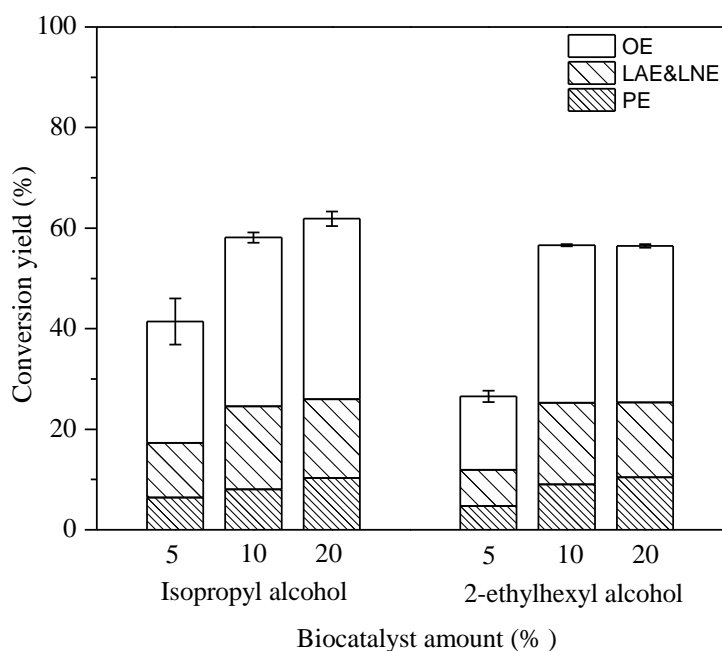


Figure 8.3 Effect of biocatalyst amount on the total conversion yield and individual yields of palmitate (PE), oleate (OE), and sum of linoleate and linolenate esters (LAE & LNE) during transesterifications of microbial oil with isopropanol and 2-ethylhexanol catalyzed by Novozyme 435 using 1:3 lipids to alcohol molar ratio at 60 °C.

8.4.4. Effect of reaction time

The TCY of isopropyl esters was gradually increased from 2 h to 12.5 h reaching a maximum yield of 57.2% (Figure 8.4). At 12.5 h, TCY of 2-ethylhexyl esters was gradually increased up to 41.9%, whereas TCY of 54.3% was achieved at 24 h. Both esters formation was monitored for 48 h, but no further increase of TCY was observed after 24 h (data not shown).

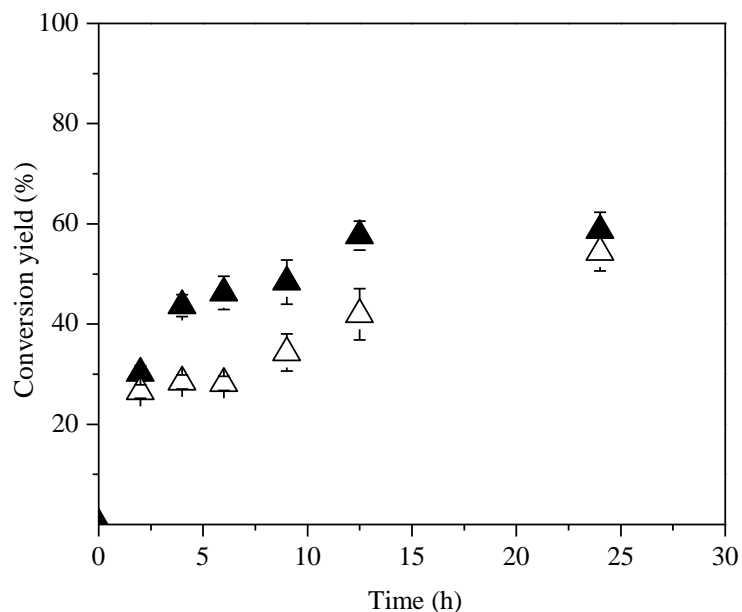


Figure 8.4 Time course of transesterifications of microbial oil with isopropanol (▲) and 2-ethylhexanol (Δ) catalyzed by 10% (w/w) of Novozyme 435 at 60 °C for 24 h.

8.5. Bio-esters production utilising individual lipid classes

Since the highest TCY achieved for isopropyl esters and 2-ethylhexyl esters was not significantly improved, the effect of lipid fractions on TCY was investigated. After fractionation of microbial oil, the NL fraction was utilised in reactions with different NL to alcohol molar ratios (1:3, 1:3.35 and 1:3.6). The results presented in Figure 8.5a demonstrated that the stoichiometric molar ratio (1:3) was insufficient for achieving the highest conversion yield with both alcohols. It was demonstrated that at a ratio of 1:3.35, TCY reached the maximum value of 80.1% for isopropyl esters and 73.8% for 2-ethylhexyl esters (Figure 8.5a). Also, the absence of the GL+SL and PL fractions was found to improve the TCY, as compared to reactions performed with microbial oil.

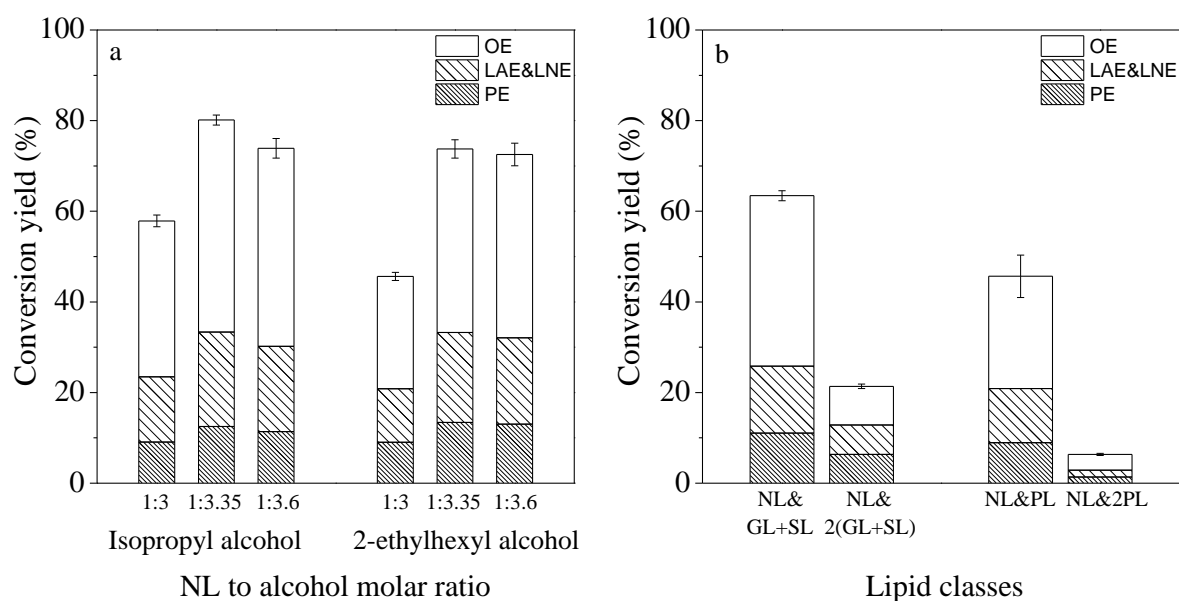


Figure 8.5 Total conversion yield and individual yields of palmitate (PE), oleate (OE), and sum of linoleate and linolenate esters (LAE & LNE) during transesterifications of neutral lipids (NL) with isopropanol and 2-ethylhexanol (a) at different NL to alcohol ratios and (b) mixtures of NL with glycolipids and sphingolipids (GL+SL), and NL with phospholipids (PL). All reactions were catalyzed by 10% (w/w) of Novozyme 435 at 60 °C.

The effect of GL+SL and PL fractions on TPC was further studied in reactions carried out with mixtures of NL with GL+SL, NL with 2(GL+SL), NL with PL, and NL with 2PL (Figure 8.5b). The study focused on isopropyl esters formation since their TCY was higher than the corresponding TCY of 2-ethylhexyl esters. Mixtures of NL with GL+SL resulted in TCY of 63.4%, whereas the utilization of double quantity of GL+SL led to considerably lower TCY (21.4%). TPC was reduced by 20.8% and 73.3% in the presence of GL+SL and 2(GL+SL), respectively, with reference to the yield achieved using the NL fraction (80.1%). Similar results were obtained in reactions containing mixtures of NL with PL and NL with 2PL, that led to TCY with respective values of 45.7% and 6.4%. In the presence of PL, TCY was lower than in the reactions with GL+SL. The reduction in the case of using PL and 2PL was 42.9% and 92.0%, respectively. GL+SL and PL lipid fractions seemed to contribute significantly to the decrease of esters formation. These results are in agreement with previous studies reporting the negative effect of PL on the conversion yield of microbial and algal derived oil (Papadaki et al., 2017; Nagle and Lemke, 1990). Moreover, PL action has been adversely related to the lipolytic activity of Novozyme 435 (Talukder et al., 2009).

8.6. Bio-esters production utilising hydrolyzed microbial oil

Subsequent experiments were conducted with free fatty acids obtained after enzymatic hydrolysis of microbial oil, 1:1 alcohol to free fatty acids molar ratio and 60 °C investigating the time course of esters formation and biocatalyst stability after several repeated batch reactions.

8.5.1. Effect of reaction time

Esters yield derived from ISA and EHA was monitored for 24 h. After 2 h of reaction, TCY reached 90.8% for isopropyl esters while the maximum value of around 95% was obtained after 4 h. TCY of 2-ethylhexyl esters reached 77.6% at 2 h, with a maximum value of around 80.0% after 6 h (Figure 8.6). The utilization of free fatty acids fractions derived from microbial oil led to higher conversion yields at shorter reaction time, as compared to the transesterification reactions using either microbial oil or individual lipid fractions. This demonstrates that Novozyme 435 has better performance during esterification than in the hydrolysis of triglycerides, which is the first reaction step of transesterification.

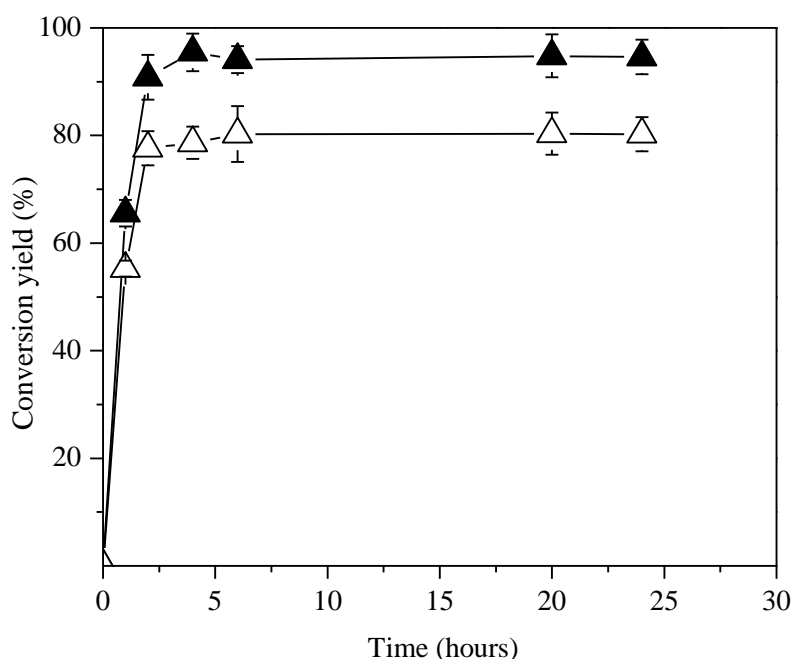


Figure 8.6 Time course of esterification reactions of isopropanol (▲) and 2-ethylhexanol (Δ) with hydrolyzed microbial oil, catalyzed by 10% (w/w) of Novozyme 435 at 60 °C for 24 h.

8.5.2. Enzyme reuse and stability

Immobilized biocatalysts could lead to cost effective enzymatic processes as they are easily recovered from reaction mixtures and they can be reused in several sequential batch reactions. Free fatty acids were utilized for the implementation of 6 repeated batch reactions. The TCY and any losses of lipase activity were monitored after specific batch cycles (Figure 8.7). TCY of isopropyl esters was higher than 90% for the first three sequential cycles. A decrease to 83.8 - 88.2% in the next two batches was observed, which finally reduced to 70.2% after the sixth cycle. TCY yield for 2-ethylhexyl esters was 80.3% after the first cycle, and remained at around 75% in the next three cycles. The TCY was reduced to 63.4% at the fifth batch cycle and finally it was sharply decreased to 22.1% in the last cycle.

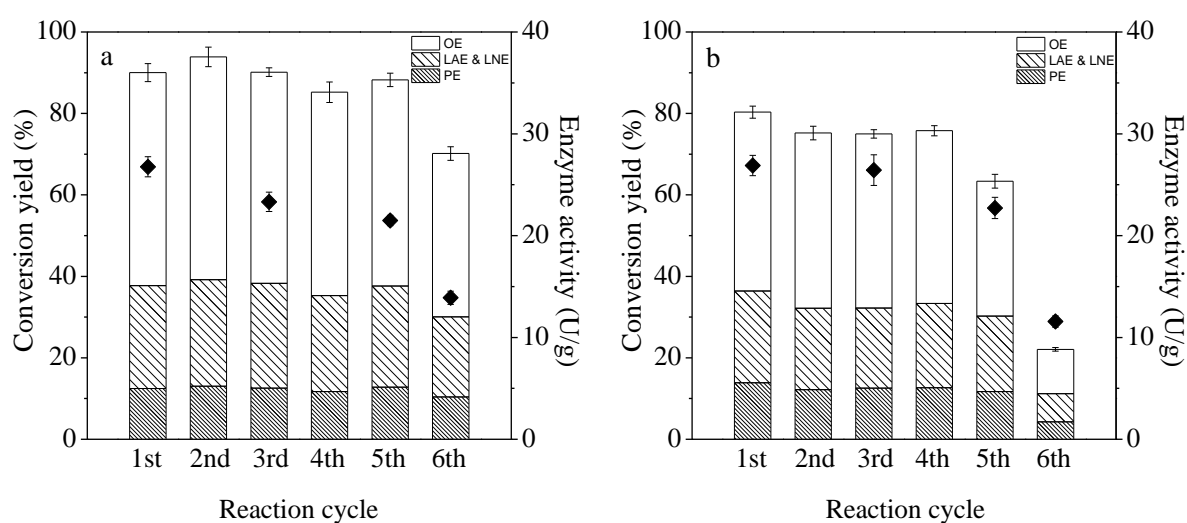


Figure 8.7 Effect of biocatalyst (10% w/w of Novozyme 435) reuse on total conversion yield and individual yields of palmitate (PE), oleate (OE), and sum of linoleate and linolenate esters (LAE & LNE), during esterifications of (a) isopropyl and (b) 2-ethylhexyl alcohol with hydrolyzed microbial oil using 1:1 lipids to alcohol molar ratio at 60 °C for 4 h. Stability of biocatalyst (◆) Novozyme 435 (initial enzyme activity: 27.0±3.0 U/g of immobilized enzyme) via the determination of lipases activity, after 3, 5, and 6 reaction cycles.

The decrease on bioesters yield could be attributed to the enzyme partial deactivation, naturally occurring after several reaction cycles. Also, dissociation and dissolution of the enzyme due to solvent washing after each cycle and the applied solvent free system, have a direct impact on enzyme activity and thus on bioesters formation (Papadaki et al., 2018). The effect of enzyme

reuse on its stability was demonstrated by a subsequent study on lipase activity determination after particular batch cycles.

Lipase activity after the 1st reaction, remained almost stable for both esters (26.8 - 26.9 U/g of immobilized enzyme) in comparison to the initial activity of the enzyme (27.0 U/g of immobilized enzyme). Regarding isopropyl esters, enzyme activity was slightly reduced after the 3rd (23.3 U/g of immobilized enzyme) and 5th cycle (21.5 U/g of immobilized enzyme). Thereafter, lipases activity was decreased to 13.9 U/g of immobilized enzyme after the 6th reaction cycle. In the case of EHA, enzyme activity remained almost stable at 26.4 U/g of immobilized enzyme until the 3rd cycle, then slightly dropped (22.7 U/g of immobilized enzyme) after the 5th batch and finally decreased to 11.6 U/g of immobilized enzyme after the 6th batch.

8.5.3. Characterisation of bio-esters

Density of isopropyl esters was calculated equal to 0.961 g/cm³ and for 2-ethylhexyl esters 0.938 g/cm³. Densities of commercial esters, such as isopropyl and 2-ethylhexyl palmitate and oleate, vary between 0.852 - 0.871 g/cm³.

Iodine values of isopropyl esters (50.9 g I₂/100 g) and 2-ethylhexyl esters (59.8 g I₂/100 g) obtained in this study were quite comparable with palm oil esters (69.7 g I₂/100 g), palm stearin esters (62.1 g I₂/100 g), palm kernel oil esters (58.8 g I₂/100 g) and palm kernel olein esters (61.3 g I₂/100 g) derived from transesterifications of palm oil fractions with oleyl alcohol catalyzed by Lipozyme RM IM (Keng et al., 2009). Lower iodine values varying between 23.4 - 27.5 g I₂ /100 g have been reported by Papadaki et al. (2018) referring to behenyl esters synthesized from various yeast lipids (i.e. *Cryptococcus curvatus*, *Lipomyces starkeyi* and *Rhodospiridium toruloides*) in transesterification reactions catalyzed by Novozyme 435. The high iodine values of the ISA and EHA microbial oil derived esters is attributed to the high unsaturation fraction (73.2%) of the microbial oil used. The high iodine values of these esters are desirable in order to increase their permeability into the upper skin layer (stratum corneum) enhancing their efficiency as moisturizers and softening agents (Keng et al., 2009).

Isopropyl and 2-ethylhexyl esters exhibited respective acid values of 10.6±0.4 mg KOH/g and 9.6±0.4 mg KOH/g. These values are higher than those obtained in enzymatic synthesis of other fatty acid esters (< 1.8) (Keng et al., 2009; Papadaki et al., 2018). Acidity reflects the free fatty

acids present in a compound and low values promote oxidative stability of compounds (Frega et al., 1999).

Saponification value is a molecular weight or chain length indicator. Long chain fatty acids of fats have low saponification values due a low number of carboxylic groups in comparison to short chain fatty acids (Nielsen 1998). Saponification value of 2-ethylhexyl esters (134.2 ± 7.5 mg KOH/g) was found lower than that of isopropyl esters (167.7 ± 4.8 mg KOH/g), because EHA has longer carbon chain length than ISA.

Isopropyl esters and 2-ethylhexyl esters produced in this study had a refractive index of 1.456 and 1.458, respectively. The refractive index is correlated to the saturation degree and it decreases linearly as iodine value decreases. The saturated oils such as coconut oil show values of refractive index in the range of 1.448 - 1.450, while refractive index of unsaturated lipids, such as menhaden oil, is equal to 1.47 (Nielsen 1998).

8.7. Concluding remarks

The present study demonstrated the efficient enzymatic conversion of a GLA-rich microbial oil into esters using the commercial lipase Novozyme 435. Enzymatic synthesis was highly affected by temperature, substrate molar ratio and less by the enzyme quantity. Results showed that the different lipid fractions have a key role in the enzymatic activity and the final conversion yield. Specifically, conversion yield was higher when microbial oil was free of PL and GL+SL fractions. The highest conversion yield achieved using only NL fraction, being 80.1% for isopropyl esters and 73.8% for 2-ethylhexyl esters. Moreover, Novozyme 435 was more effective in terms of conversion yields (90.8% for isopropyl esters and 80.0% for 2-ethylhexyl esters) when hydrolysed microbial oil was utilized as substrate. The successful lipase utilisation for up to 5 repeated reactions without significant decrease of conversion yield, indicates that enzymatic processes may be a cost-effective route for the production of bio-based esters. The production of bioesters from microbial oil rich in GLA creates innovative perspectives for the development of value-added nutraceutical and pharmaceutical products.

CHAPTER 9

**Microbial oil production by *Lipomyces starkeyi*,
Cunninghamella echinulata and *Mortierella isabellina* via
fermentation using hemicellulose hydrolysate as feedstock**

9.1. Introduction

Pretreatment of spent sulphite liquor (SSL) via nano-filtration or ultrafiltration, is a vital step for the reduction of its high organic load (Jönsson, 2016) prior to utilization as carbon sources in bioprocesses. Also, the efficient valorisation of SSL through fermentation is considered challenging due to large proportion of pentoses and the presence of inhibitory compounds, such as lignosulphonates (LS), phenolics and organic acids. Oleaginous fungi of the phylum *Zygomycota* (i.e *Mortierella isabellina*) and yeasts (i.e *Lipomyces starkeyi*) have been previously reported to produce lipids, when cultivated on lignocellulosic-derived hydrolysates (Kam, 2015). Genome mapping of *L. starkeyi* has revealed the presence of an acetyl-CoA synthase gene which is strongly indicative of the natural capacity of this yeast to metabolize inhibitors derived from hydrolysis of hemicellulosic biomass (Xavier et al., 2017). *Zygomycetes* have been suggested as effective microbes for phenolic compounds removal (Bellou et al., 2014) and potent GLA producers in scaling up processes (Sayegh et al., 2016). Fed-batch or continuous fermentation practice could lead to high cell densities undoubtedly beneficial for the industrialization of microbial oil production (Huang et al. 2013).

Production of high value added metabolites e.g succinic acid (Alexandri et al., 2016; Ladakis et al., 2018), poly-hydroxybutyrate (Weissgram et al., 2015) and ethanol (Helle et al., 2004), could enhance economics of pulp and paper industry in addition to revenues coming from LS market as dispersants, precipitates, binders, and adhesives (Jönsson, 2016). In this perspective, the use of SSL for the production of microbial oil rich in polyunsaturated fatty acids (PUFA) e.g γ -linolenic acid (GLA), offers great potential considering that similar studies have not been reported in the literature.

GLA is an essential fatty acid with numerous applications in food industry as dietary supplement, nutraceuticals as encapsulation preparations, cosmetics for the formulation of natural ingredient-based cosmetics and in the medical sector (Sergeant et al., 2016; Bellou et al., 2014). Currently, the main source for GLA extraction is vegetables, such as borage and black currant seeds with a GLA-content up to 21% (Sergeant et al., 2016). Many attempts have been also performed for the production of GLA-rich microbial oil from fungus achieving high GLA concentrations up to 29.4% (Saad et. al., 2014).

The objective of this study was the valorisation of spent sulphite liquor as fermentation feedstock for microbial oil production using oleaginous yeast and fungal strains. The aim was to maximize microbial lipids production through the evaluation of different fermentation

conditions, such as carbon to nitrogen ratio and LS concentration in batch and fed-batch fermentations. The fatty acid composition was also assessed during all fermentations. In the case of fungal fermentations, the optimum conditions were identified for the production of a GLA-rich microbial oil.

9.2. Shake flasks fermentations using *L. starkeyi*

9.2.1. Effect of C/FAN ratio

The yeast strain *L. starkeyi* was cultivated on nanofiltrated SSL and the effect of C/FAN ratio on the fermentation efficiency was evaluated. Maintaining a desirable C/N ratio and a constant carbon source concentration could enhance lipid biosynthesis and productivity. The presence of acetic acid (1.3 g/L) and phenolic compounds (0.1 g/L) in SSL could lead to a synergistic inhibition, suppressing yeast growth and lipid accumulation (Zhao et al., 2012; Xavier et al., 2017). Nevertheless, the cell growth (8 - 11 g/L) in all the examined C/N ratios (Figure 9.1a) indicated the high tolerance of the strain to these inhibitors. Acetic acid was consumed after 48 h and the hemicellulosic sugars were efficiently assimilated by *L. starkeyi* with a final consumption varying between 90.3 - 94.7% (Figure 9.1a). FAN was depleted after 48 h except for the C/FAN ratio of 104, at which FAN was slowly consumed until 120 h without total depletion. This can explain the low lipid concentration achieved in this case (1.2 g/L). The most effective C/N ratio was 173 regarding biomass production (11.2 g/L), lipid content (25.2%), yield (0.09 g/g) and productivity (0.04 g/L/h). The assimilation pattern of individual consumption of glucose, xylose, galactose, mannose and arabinose (Figure 9.1b) was similar in all the examined C/FAN ratios. During the first 24 h of fermentation, the yeast strain metabolized only glucose and thereafter, glucose was simultaneously consumed (94.2%) with xylose (95.2%) and mannose (68.3%), followed by galactose (73.2%) and arabinose (59.6%).

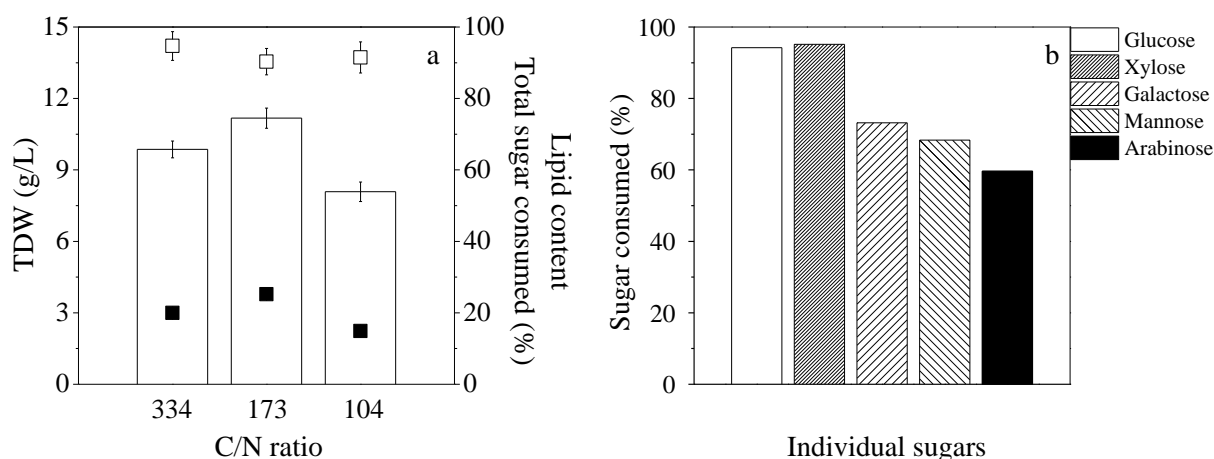


Figure 9.1 (a) Effect of C/FAN ratio on (a) total dry weight (TDW), lipid content (■) and total sugar consumption (□), during shake flask fermentations of *L. starkeyi* using nano-filtrated spent sulphite liquor. (b) Individual sugars consumption at C/FAN ratio of 173.

Analysis of fatty acid composition of microbial oil obtained at different C/FAN ratios revealed no significant variations. Oleic acid (C18:1) (42-46%) and palmitic acid (C16:0) (32-36%) were the predominant fatty acids, followed by palmitoleic (C16:1), stearic (C18:0) and linoleic acid (C18:2).

Table 9.1. Effect of C/FAN ratio on fatty acid profile of microbial oil produced by *L. starkeyi* in shake flask fermentations using nano-filtrated spent sulphite liquor.

C/N ratio	Time (h)	Fatty acid methyl esters (%)							
		C14:0	Δ^9 C14:1	C16:0	Δ^7 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	Others
334	48	0.7	1.8	35.3	5.9	6.2	41.9	5.2	3.0
	96	0.8	1.5	35.9	5.1	6.0	44.0	5.8	0.9
173	48	0.4	1.4	34.8	7.2	4.4	46.1	3.7	2.0
	96	0.5	1.6	32.1	6.2	5.0	45.8	5.2	3.6
104	48	0.6	3.9	31.6	5.9	6.7	42.1	5.9	3.3
	96	0.6	1.4	33.0	6.1	4.4	44.8	5.9	3.8

9.2.2. Effect of lignosulphonates concentration

Several studies have demonstrated improvement of fermentation efficiency for succinic acid production when pretreated SSL was used as nutrient source, implying the inhibitory effect of LS (Alexandri et al., 2016; Pateraki et al., 2016). Figure 9.2 shows the effect of different LS

concentrations on *L. starkeyi* metabolism. Biomass production was slightly enhanced with increasing LS concentration. Total sugar consumption was higher than 90% in all cases, with glucose and xylose being almost entirely depleted. However, total consumption of arabinose ranged within 22 - 45%. Lipid content varied from 16% to 20% at LS concentrations of 10 - 70 g/L, and it was sharply decreased (11%) at 90 g/L LS. Yield and productivity were inversely related to LS concentrations varying between 0.06-0.08 g/g and 0.01-0.03 g/L/h, respectively. Moreover, at LS concentrations of 70 g/L and 90 g/L, a prolonged lag phase was observed (data not shown).

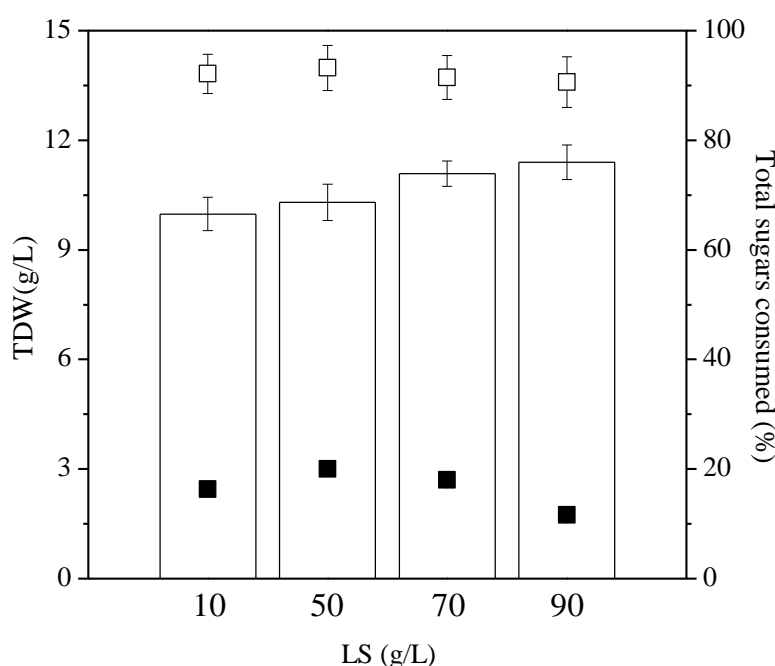


Figure 9.2 Effect of lignosulphonates (LS) concentrations on total dry weight (TDW), lipid content (■) and total sugar consumption (□), during shake flask fermentations of *L. starkeyi* using sugar-simulated spent sulphite liquor at C/FAN ratio of 173.

Results concerning fatty acid profile of lipids showed similar patterns at different LS concentrations (Table 9.2). C18:1 and C16:0 were produced in similar proportions. It was observed that C16:0 content was higher (~40%) than shake flask fermentations using nanofiltrated SSL containing 5 g/L LS (32 - 36%) (Table 1). The composition of the fermentation feedstock could alter the fatty acid composition in yeast lipids. Studies for lipids production on molasses (Vieira et al., 2014), potato starch (Wild et al., 2010) and willow wood sawdust hydrolysate (Wang et al., 2014) by *L. starkeyi* have respectively reported enhanced

C18:2 (18%) production against C16:0 (21%), increased C18:1 (55%) and increased C16:1 (10%).

Table 9.2. Effect of lignosulphonates (LS) concentration on fatty acid profile of microbial oil produced by *L. starkeyi* in shake flask fermentations using sugar-simulated spent sulphite liquor at C/FAN ratio of 173.

LS (g/L)	Time (h)	Fatty acid methyl esters (%)					
		C16:0	Δ^7 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	Others
10	48	39.7	6.6	3.2	46.7	3.0	0.8
	96	41.1	4.8	3.7	45.8	2.1	2.5
50	48	39.2	5.5	3.6	45.4	3.2	3.1
	96	40.2	4.7	4.6	46.3	3.1	1.1
70	48	37.0	5.2	6.8	35.6	9.3	6.1
	96	39.5	4.7	3.6	47.7	2.0	2.5
90	48	39.6	4.7	3.9	46.4	1.8	3.6
	120	39.6	4.4	3.6	47.5	1.8	3.1

9.3. Fed-batch in bioreactor using *L. starkeyi*

Fed-batch cultures could promote cell growth leading to high cell densities and simultaneously deteriorate the inhibitory effect caused by high nutrient concentration by regulating the flow rate of the feeding medium. The cultivation of *L. starkeyi* was carried out in fed-batch mode applying a continuous feeding strategy and different C/FAN ratios. Continuous feeding has been reported to keep the cellular metabolism active, enhancing biomass and lipids yield (Karamerou et al., 2017). In all the applied treatments concerning C/FAN ratios, FAN was consumed within 24 - 48 h, triggering thereafter the secondary metabolism for lipids production.

9.3.1. Effect of C/FAN ratios

Initially, a fed-batch experiment was implemented at the best C/FAN ratio (173), as indicated by shake flask fermentations. TDW and lipid content were gradually increased (Figure 9.3) reaching their maximum values of 27.1 g/L and 49.1%, respectively, after 261 h. Biomass and lipids yield on total consumed sugars were 0.17 g/g and 0.12 g/g, respectively. Sugars

consumption rate was slow during the first stage of the fermentation, while after FAN depletion it remained almost stable (0.3 – 0.5 g/L/h). Feeding supply was stopped at 261 h. Regarding assimilation pattern of individual sugars consumption, glucose, xylose, galactose and mannose were consumed together with glucose and mannose reaching higher consumption rates, followed by xylose and galactose. Arabinose was the less assimilated carbon source and its accumulation was observed at the end of fermentation.

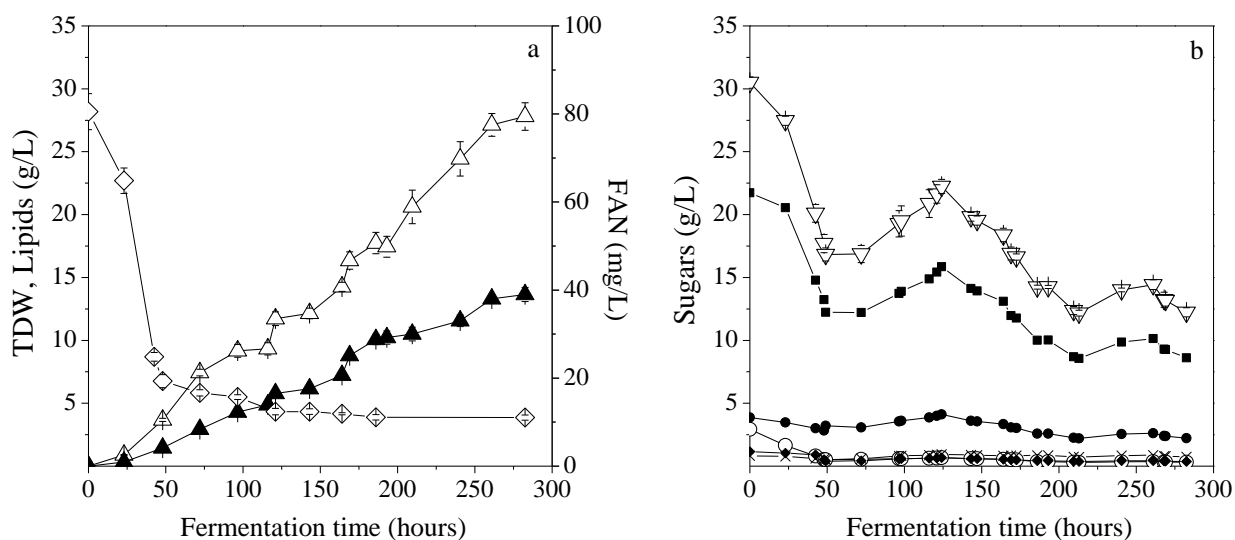


Figure 9.3 Fed-batch fermentation of *L. starkeyi* in bioreactor using sugar-simulated spent sulphite liquor at C/FAN ratio of 173. (a) Time course of total dry weight (TDW) (Δ), lipid accumulation (\blacktriangle) and free amino nitrogen (FAN) (\diamond) consumption. (b) Time course of total sugars (∇), glucose (\circ), xylose (\blacksquare), galactose (\bullet), mannose (\blacklozenge) and arabinose (\times) consumption.

Subsequent fermentations were conducted at a C/FAN ratio of 51 (Figure 9.4). Productivity increased until 72 h and it was decreased thereafter. The consumption rate followed the same trend reaching maximum values (1.2 - 1.7 g/L/h) from 39 h to 68 h, while it was gradually decreased until the end of the fermentation (237.5 h). At 164 h, TDW production was equal to 49 g/L with a lipid content of 30.8%, while respective values were maximized to 53.2 g/L and 35.6% at 212 h.

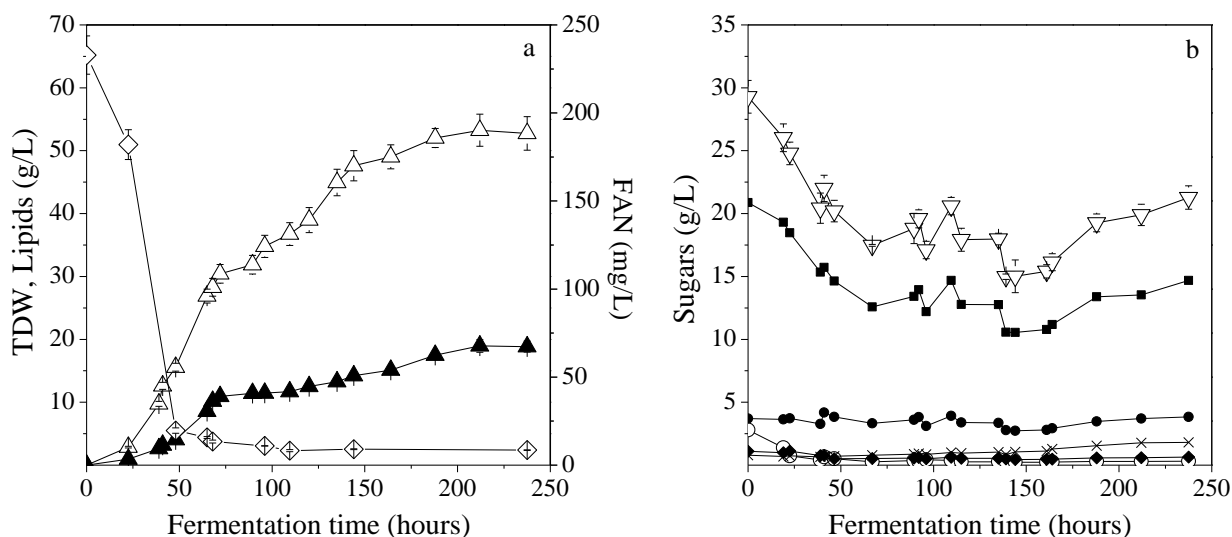


Figure 9.4 Fed-batch fermentation of *L. starkeyi* in bioreactor using sugar-simulated spent sulphite liquor at C/FAN ratio of 51. (a) Time course of total dry weight (TDW) (Δ), lipids accumulation (\blacktriangle) and free amino nitrogen (FAN) consumption (\diamond). (b) Time course of total sugars (∇), glucose (\circ), xylose (\blacksquare), galactose (\bullet), mannose (\blacklozenge) and arabinose (\times) consumption.

Figure 9.5 presents the results concerning fed-batch fermentation of *L. starkeyi* at a C/FAN ratio of 33. The efficiency of this fermentation was demonstrated by the high TDW production which was 81.1 g/L with an intracellular lipid content of 50.4% (158 h). Biomass yield was increased to 0.25 g/g, in comparison to the previous experiments, whereas lipids yield was similar (0.10 g/g) (Table 9.4). FAN was almost entirely depleted at 46 h, accompanied by a slow consumption rate. Consumption rate varied within 3.2 - 5.6 g/L/h from 48 h to 112 h of fermentation while it was gradually decreased thereafter.

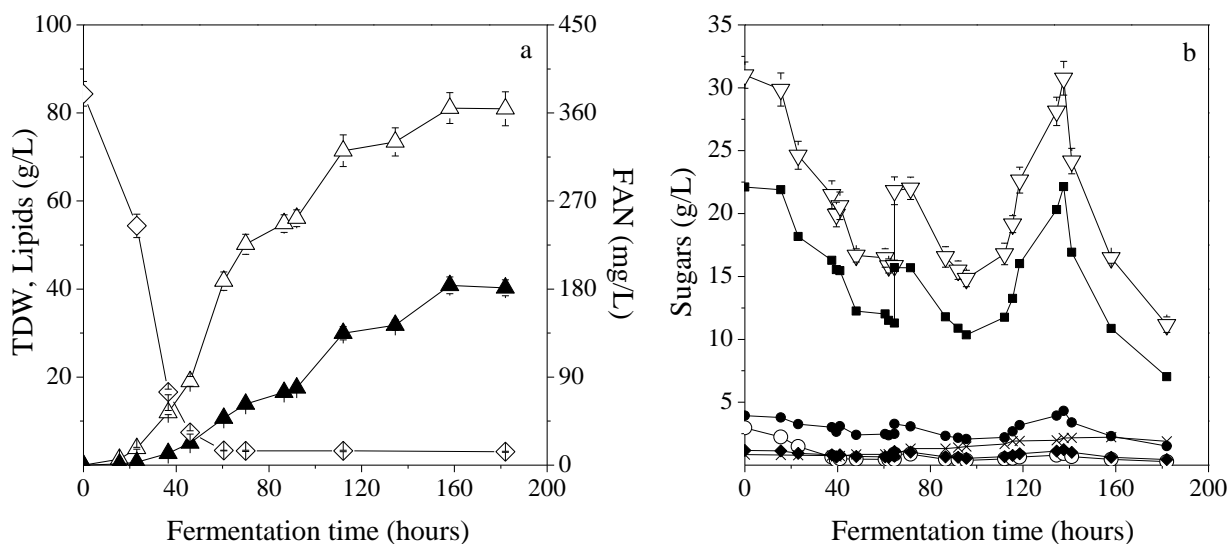


Figure 9.5 Fed-batch fermentation of *L. starkeyi* in bioreactor using sugar-simulated spent sulphite liquor at C/FAN ratio of 33. (a) Time course of total dry weight (TDW) (Δ), lipids accumulation (\blacktriangle) and free amino nitrogen (FAN) (\diamond) consumption. (b) Time course of total sugars (∇), glucose (\circ), xylose (\blacksquare), galactose (\bullet), mannose (\diamond) and arabinose (\times) consumption.

A lower C/FAN ratio of 26 was also evaluated in fed-batch fermentation, which led to the highest TDW production of 90.4 g/L with an intracellular oil content of 40.2%. The biomass and oil yields based on sugar consumption (158.5 h) slightly decreased in comparison to the previous C/FAN ratio (Table 9.4). The applied nitrogen concentration in this part could be considered as an indicator for establishing thresholds considering fermentation efficiency (Table 9.4). The maximum consumption rate (3 - 5.7 g/L/h) was monitored from 37.5 h to 94 h.

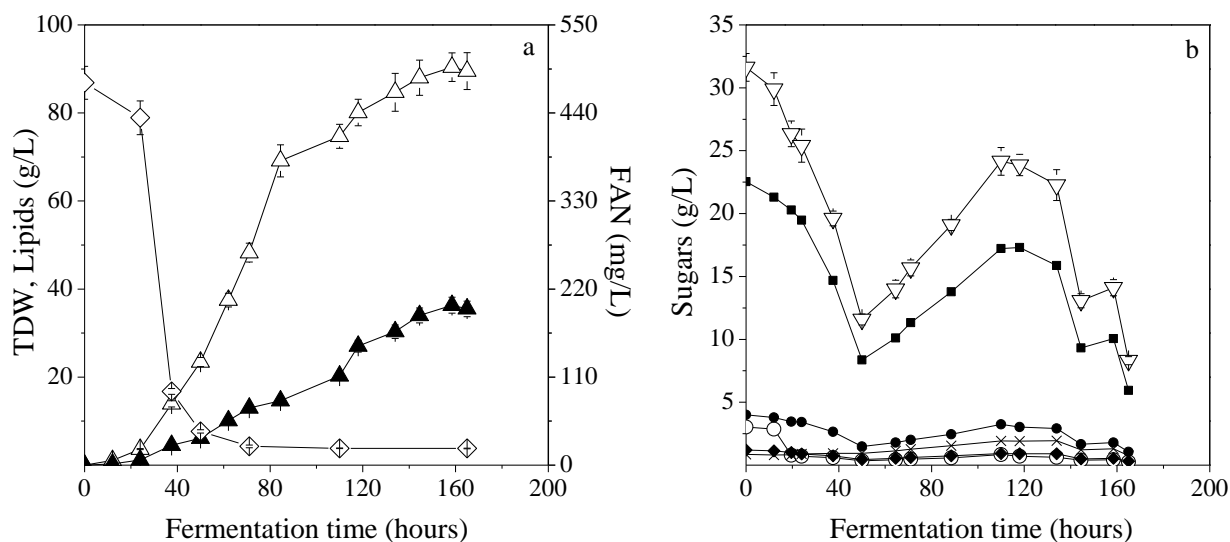


Figure 9.6 Fed-batch fermentation of *L. starkeyi* in bioreactor using sugar-simulated spent sulphite liquor at C/FAN ratio of 26. (a) Time course of total dry weight (TDW) (Δ), lipids accumulation (\blacktriangle) and free amino nitrogen (FAN) consumption (\diamond). (b) Time course of total sugars (∇), glucose (\circ), xylose (\blacksquare), galactose (\bullet), mannose (\blacklozenge) and arabinose (\times) consumption.

Table 9.3 presents the fatty acid profile of microbial oil produced by *L. starkeyi* in fed-batch fermentations at different C/FAN ratios. Although fatty acid methyl esters were determined for a wide range of time periods during fermentations, no worth noting variations were observed (data not shown). Microbial oil of *L. starkeyi* showed a typical fatty acid pattern (Sutanto et al., 2018), mainly consisted of oleic acid (around 50%) and palmitic acid (37 - 41%).

Table 9.3. Effect of C/FAN ratio on fatty acid profile of microbial oil produced by *L. starkeyi* in fed-batch fermentations in bioreactor using nano-filtrated spent sulphite liquor (5 g/L lignosulphonates).

C/N ratio	Time (h)	Fatty acid methyl esters					
		C16:0	Δ^7 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	Others
173	261	41.1	4.5	5.2	48.8	0.0	0.4
51	212	40.1	4.0	5.1	49.8	0.7	0.4
33	158	36.7	1.1	9.3	50.4	2.4	0.2
26	158.5	37.8	1.3	8.5	49.6	2.6	0.0

Table 9.4 depicts a synopsis of fed-batch fermentations efficiency evaluating the aforementioned C/FAN ratios. It was demonstrated that productivity was enhanced with decreasing C/FAN ratios while lipids yield showed a slightly downward trend.

Table 9.4 Fed-batch fermentation efficiency cultivating *L. starkeyi* on nano-filtrated spent sulphite liquor under various C/FAN ratios. Data represent maximum valued obtained at each individual fermentation.

C/FAN ratio	TDW (g/L)	Lipids (g/L)	Biomass yield (g/g)	Lipids yield (g/g)	Productivity (g/L/h)
173	27.1	13.3	0.17	0.12	0.05
51	53.2	18.9	0.20	0.09	0.09
33	81.1	40.9	0.25	0.10	0.26
26	90.4	36.3	0.24	0.07	0.23

9.4. Shake flasks fermentations by *C. echinulata* and *M. isabellina*

In this section, the potential of *C. echinulata* and *M. isabellina* to convert SSL into high added value lipids rich in polyunsaturated fatty acids was investigated. Various C/FAN ratios and LS concentrations were assessed for both fungal strains.

9.4.1. Effect of C/FAN ratios

Several C/FAN ratios (Figure 9.7) were evaluated during shake flask fermentations by *C. echinulata* (248, 164, 101, 87) and *M. isabellina* (388, 213, 119). The best C/FAN ratios were 101 and 213 for *C. echinulata* and *M. isabellina*, respectively, resulting to high microbial oil content, around 60%. At these C/FAN ratios, TDW was 6.5 g/L for *C. echinulata* and 9.4 g/L for *M. isabellina*. Ratios lower than those, resulted in reduced microbial oil concentrations (Figure 9.7a). In the case of *M. isabellina*, yield achieved in the fermentation with 5 g/L of LS (0.19 g/g) was reduced by 54% when 50 g/L of LS were applied. *C. echinulata* was highly tolerant at increasing LS concentration since yield attained at 5 g/L of LS (0.14 g/g) was diminished just by 28% in the extremely high LS concentration of 120 g/L.

Total sugars consumption for *M. isabellina* was higher than 90% in all cases (Figure 9.7b). *C. echinulata* consumed only 60% of total sugars when the C/FAN ratio of 248 was applied, while in the other ratios, a sufficient sugars consumption was achieved (82 - 92%) (Figure 9.7b).

Individual sugars consumption analysis, at specific ratios (Figure 9.7c) showed that arabinose was poorly assimilated (7-38%) by both fungal strains while glucose and xylose were almost totally consumed (96-100%). Galactose and mannose were equally consumed (57%) by *C. echinulata* while *M. isabellina* utilized slightly higher amounts (74-87%). Biomass yield was increased with increasing C/FAN ratios achieving maximum values of 0.23 g/g and 0.31 g/g for *C. echinulata* (ratio of 101) and *M. isabellina* (ratio of 213) respectively.

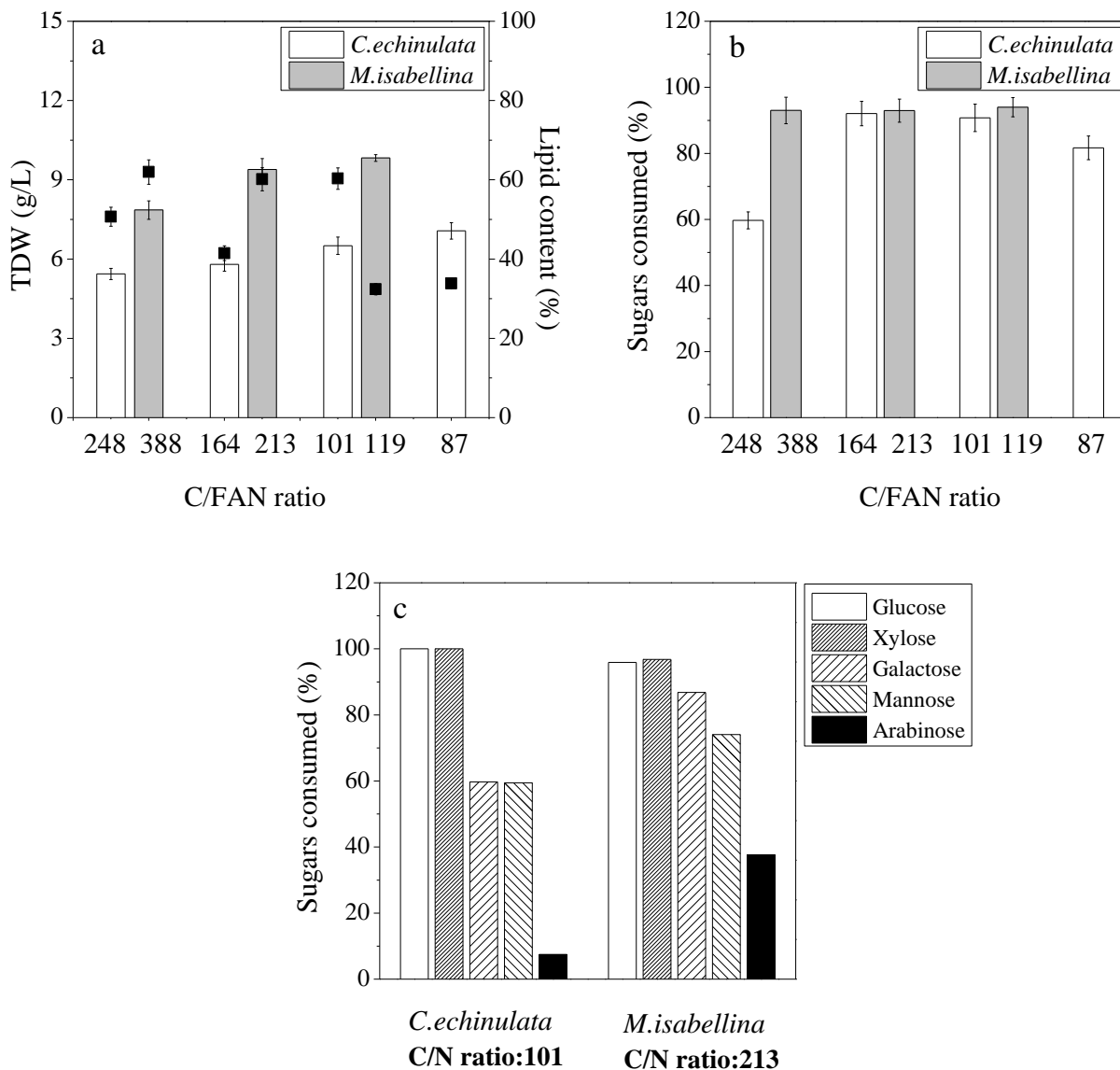


Figure 9.7 Shake flask fermentations of *C. echinulata* and *M. isabellina* using nano-filtrated spent sulphite liquor. Effect of C/FAN ratio on (a) total dry weight (TDW), lipid content (■) and (b) total sugar consumption. (c) Consumption of individual sugars at the best C/FAN ratio.

Microbial oil produced by *C. echinulata* in all studied C/FAN ratios, mainly consisted of C18:1 (35.4 - 45.4%) and C16:0 (15.9 - 19.9%) followed by C18:2 (10.7 - 14.7%), GLA (10.0 -

18.8%), C18:0 (6.0 - 10.2%) and C16:1 (1.3 - 2.0%) acids. Decrease of linoleic acid with fermentation time was rationally accompanied with an increase in GLA content. The highest GLA content of 18.8% was achieved at C/FAN ratio of 101. Increase in essential and polyunsaturated fatty acids with increasing concentrations of nitrate- and ammonium-based nitrogen sources has been previously reported for lipids produced by marine microalga (Xu et al., 2001).

Lipids deriving from *M. isabellina* consisted mainly of C18:1 (47.5 - 52.1%), C16:0 (20.1 - 27.8%) and C18:2 (12.2 - 19.7%), followed by lower amounts of C18:0 and C16:1. Distribution of fatty acids at various C/FAN ratios was irregular with small fluctuations.

Data illustrating microbial oil concentration and GLA alterations when various C/FAN ratios were employed, are shown in Figure 9.8. These data correspond to maximum values obtained at the point that was considered as the end of each fermentation. In the case of *C. echinulata*, ratios and GLA seemed to be positively related. The maximum GLA percentage of 18.8% was achieved at a C/FAN ratio of 101. Regarding *M. isabellina*, GLA alterations were irrelevant to the applied ratios varying between 1.9 - 3.8%.

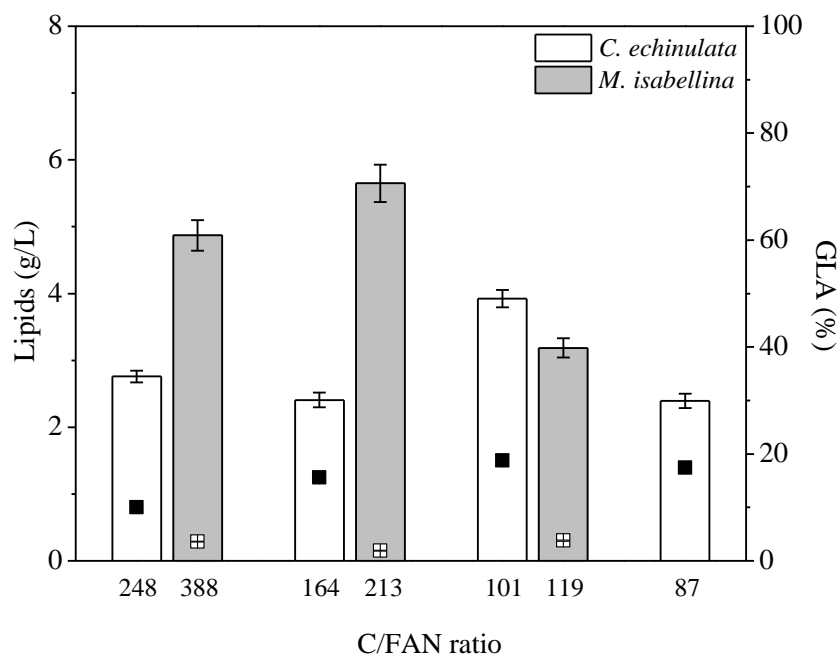


Figure 9.8 Maximum microbial oil production and γ -linolenic (GLA) content in shake flask

fermentations of *C. echinulata* (■) and *M. isabellina* (□), using nano-filtrated spent sulphite liquor at various C/FAN ratios.

9.4.2. Effect of lignosulphonates concentration

The impact of various LS concentrations on microbial growth and lipid production was assessed in shake flask fermentations using the best C/FAN ratios for each fungal strain (Figure 9.9). Lipids content of *C. echinulata* was gradually decreased with increasing LS concentration while biomass growth seemed to be induced yielding to 0.33 ± 0.05 g/g, when 50 - 120 g/L of LS were applied. At LS concentrations of 50 - 120 g/L, TDW of 5.5 - 7 g/L was produced after 48 h of fermentation while at 5 g/L LS the equivalent value was 0.7 g/L. *M. isabellina* was strongly affected by increasing LS concentration (>5 g/L) (Figure 3.7a, 3.9a). Lipids content was reduced to 40% and biomass yield was decreased to 0.22 ± 0.01 g/g. Lipid productivity was dropped 4 folds and 5 folds respectively when 10 g/L and 50 g/L of LS were added in the fermentation broth.

As depicted in Figure 9.9b, total sugars consumption by *C. echinulata* was in the range of 91 - 97% for all LS concentrations except for 120 g/L (71%). *M. isabellina* consumed respectively 97% and 75% total sugars in fermentations with 10 g/L and 50 g/L LS.

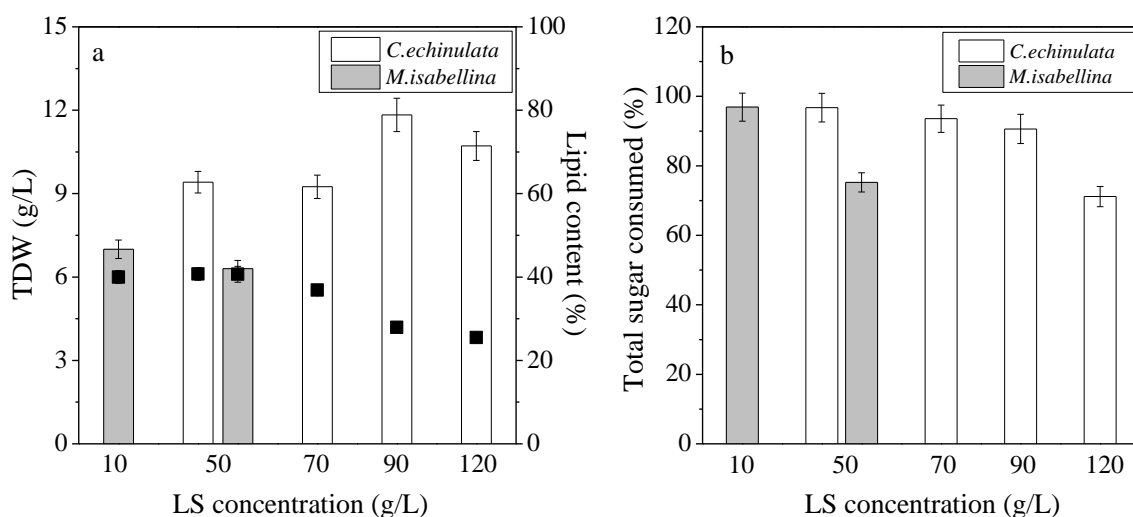


Figure 9.9 Effect of lignosulphonates (LS) concentration on (a) total dry weight (TDW), lipid content (■) and (b) total sugar consumption, during shake flask fermentations of *C. echinulata* and *M. isabellina*, using sugar-simulated spent sulphite liquor at C/FAN ratio of 101 and 213, respectively.

Concerning fatty acid composition of *C. echinulata*, C16:0 was increased from 13.5% to 18.7% and C18:1 from 40.6% to 55.7% at LS concentrations from 50 g/L to 120 g/L. Results showed that LS concentration higher than 50 g/L, negatively affected polyunsaturated fatty acids biosynthesis. Linoleic acid was reduced from 18.1% to 9.3% with increasing LS concentration (50 - 120 g/L). Also, GLA was decreased from 20.7% (50 g/L LS) to 8.9% (120 g/L LS) (Figure 9.10).

Considering fatty acid composition of *M. isabellina* microbial oil, C18:1 increased with fermentation time, up to 55% at 10 and 50 g/L LS, whereas C16:0 decreased to around 23% at the same LS concentrations. C18:2 decreased with increased LS concentrations, while GLA was gradually enhanced up to 5.5% at 50 g/L LS (Figure 9.10).

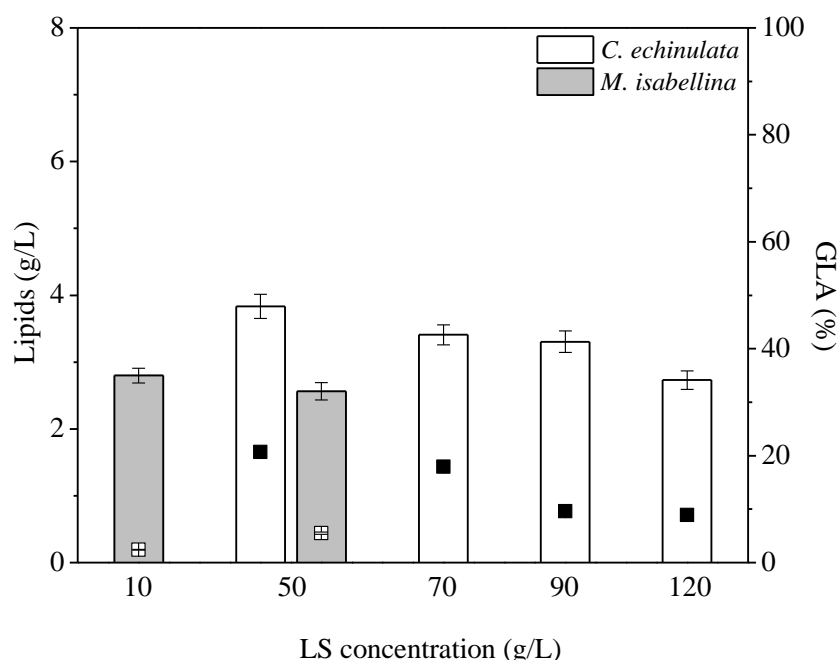


Figure 9.10 Microbial oil production and corresponding γ -linolenic (GLA) percentages by *C. echinulata* (■) and *M. isabellina* (□) cultivated in 250 mL shake flasks containing nano-filtrated and simulated spent sulphite liquor under various lignosulphonates (LS) concentration and specific C/FAN ratios (213 for *M. isabellina*) (101 for *C. echinulata*) (maximum lipids and GLA production).

9.5. Lignosulphonates and phenolic compounds removal

Along with microbial oil production, LS and phenolic compounds removal were determined. Studies focusing on microbial LS degradation are limited and mostly refer to *Basidiomycetous* fungi that possess a lignolytic enzymatic complex including lignin peroxidase, manganese peroxidase and/or laccase (Eugenio et al., 2008; Selin and Sundman, 1972). LS and phenolic compounds assays were determined for all microbial strains, in batch fermentations, at the best C/FAN ratios. According to the results, microbial strains were unable to degrade LS contained in nano-filtrated SSL. This could be explained by the fact that lignolytic enzymes have not been reported to be present in *Zygomycetes* and yeast strains (Eugenio et al., 2008; Bellou, 2014). With respect to phenolic compounds, no reduction was observed throughout the fermentation course of *L. starkeyi*. Total phenolics removal by *M. isabellina* and *C. echinulata* was $56\pm 2.1\%$ and $61\pm 2.8\%$, respectively, after 48 h of fermentation. Despite the lack of lignolytic enzymes in these fungi, the phenolics reduction in the fermentative broth, could be attributed to their absorption by the mycelia other than oxidation (Bellou, 2014).

9.6. Fed-batch fermentations by *C. echinulata* on spent sulphite liquor

In subsequent experiments, *C. echinulata* was cultivated in fed-batch mode utilizing sugar-simulated spent sulphite liquor containing either 5 g/L or 90 g/L of LS (Figure 9.11). At 5 g/L of LS, FAN was depleted after 27 h and TDW reached a maximum value of 12.2 g/L at 100.5 h of fermentation. Biomass yield was determined as 0.36 g/g. At 100.5 h lipid production reached its maximum value of 6.8 g/L (56% intracellular content) with a yield of 0.2 g/g and a productivity of 0.07 g/L/h. Total sugars consumption was slow during the first 27 h, while thereafter it remained at 0.2-0.4 g/L/h (until 113 h). Feeding supply was stopped at 93.5 h of fermentation.

In the case of utilizing media with 90 g/L LS, FAN was depleted later (70 h) than in fermentation with 5 g/L LS (Figure 9.11c). TDW was maximized (9.9 g/L) at 118 h, containing 27% of lipids. Yield of biomass and lipids were 0.41 g/g and 0.11 g/g, respectively, while productivity was 0.02 g/L/h. Total sugars consumption rate was 0.1 g/h during the first 24 h, increased to 0.4-0.5 g/h for the next 50.5 h and decreased to 0.1-0.2 g/h thereafter. Feeding was stopped after 120.5 h. Conclusively, fermentation efficiency in terms of the aforementioned parameters, was highly inhibited in comparison to 5 g/L of LS.

In both fermentations, glucose was firstly metabolized by the fungal strain, followed by partial depletion of mannose and xylose during the first 24 h. Thereafter, glucose, mannose and xylose were consumed simultaneously while arabinose was poorly metabolized resulting in its accumulation at the end of fermentation. Galactose generally followed a slow catabolic rate.

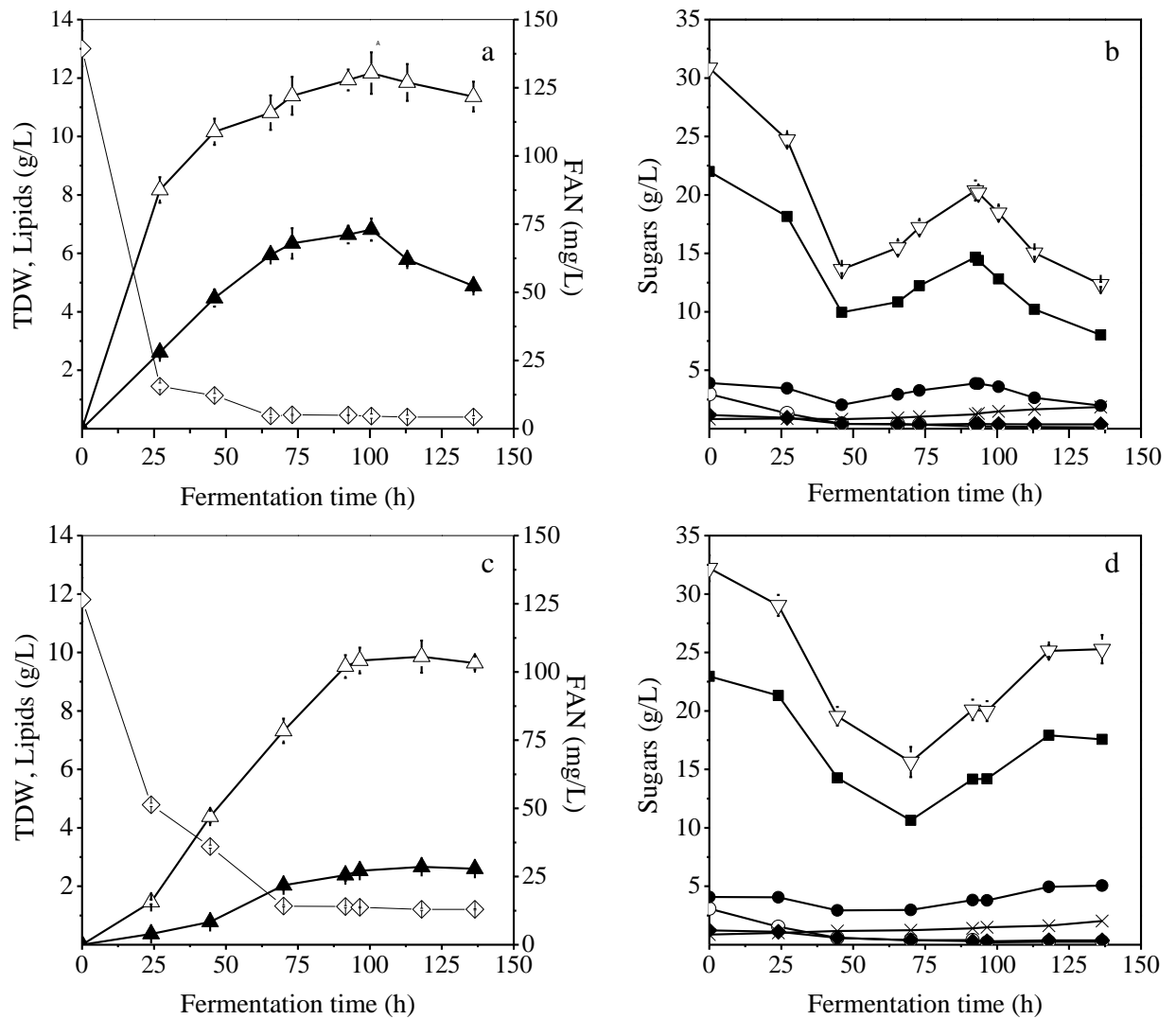


Figure 9.11 Fed-batch fermentation of *C. echinulata* in bioreactor using sugar-simulated spent sulphite liquor (5 g/L lignosulphonates a, b; 90 g/L lignosulphonates c, d) at C/FAN ratio of 101. (a) Time course of total dry weight (TDW) (Δ), lipids accumulation (\blacktriangle) and free amino nitrogen (FAN) consumption (\diamond). (b) Time course of total sugars (∇), glucose (\circ), xylose (\blacksquare), galactose (\bullet), mannose (\blacklozenge) and arabinose (\times) consumption.

Fatty acid variations of microbial oil produced under both LS concentrations is depicted in Table 9.5. Distribution of fatty acids showed small alterations between different fermentations. It is worth noting that in the case of 90 g/L of LS, myristoleic acid percentage was 10.9% in the early phase of fermentation and gradually decreased to 0.1% after 136.5 h. Oleic acid and palmitic acid were the main fatty acids. GLA formation was strongly affected in fed-batch experiments, probably due to the application of aeration and agitation. Respective values of 9.3% after 100.5 h and 5.6% after 118 h were achieved at 5 g/L and 90 g/L of LS. These values were considerably lower compared to the GLA obtained in batch fermentations (18.8%). Saad et al. (2014) demonstrated that *C. bainieri* produced lower amounts of GLA in low aeration rate and agitation. This indicates that further study should be carried out focusing on GLA production through the optimization of aeration and agitation.

Table 9.5. Fatty acid profile of microbial oil produced by *C. echinulata* in fed-batch fermentations in bioreactor using sugar-simulated spent sulphite liquor with 5 g/L or 90 g/L lignosulphonates (LS) at C/FAN ratio of 101.

LS (g/L)	Time (h)	Fatty acid methyl esters (%)							Others
		Δ^9 C14:1	C16:0	Δ^7 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{6,9,12}$ C18:3	
5	46	0.7	20.6	0.7	9.4	48.3	11.5	8.8	-
	65.5	0.6	19.0	1.1	9.2	49.3	11.5	9.3	-
	100.5	0.5	18.6	0.7	7.8	50.9	12.2	9.3	-
	136	1.0	17.8	0.7	5.7	48.2	14.0	11.6	1.0
90	44.5	10.9	21.8	6.6	8.3	44.3	6.5	1.6	-
	72	5.6	29.8	3.0	10.3	41.1	7.6	2.6	-
	118	4.8	22.3	2.7	7.0	45.8	11.8	5.6	-
	136.5	0.1	19.5	1.4	5.2	51.2	13.9	8.7	-

9.7. Concluding remarks

The present study showed that *L. starkeyi* and *C. echinulata* were highly tolerant strains to increased LS concentrations. Fed-batch bioreactor cultures of *L. starkeyi* under the optimum C/FAN ratio, resulted in high cell density fermentation (81.1 g/L) and high lipid content (50%). *Cunninghamella echinulata* was able to produce PUFAs-rich microbial oil (60%) with simultaneous phenolics removal when cultivated in batch fermentation utilizing SSL as

substrate. GLA production was strongly affected in fed-batch experiments of *C. echinulata* probably due to the application of aeration and agitation. Although fungi may offer appropriate cell factories for the production of lipids, they are inferior to yeasts, in terms of growth rates and productivities. This was proven in the current study with productivities achieved, being lower than 0.05 g/L/h. Fermentation yields increased with decreasing C/FAN ratios until a threshold, and thereafter decreased, as substrates rich in nitrogen sources lead to poor microbial oil accumulation by microbes. Increasing LS concentration negatively affected fermentation, particularly in the case of *M. isabellina*.

CHAPTER 10

Conclusions and future perspective

The conversion of waste streams into fermentation media would require the development of advanced upstream processing strategies that exploit the full potential of complex biological entities. This thesis, provides an approach of waste valorisation through the development of microbial and enzymatic bioprocesses contributing to the growth of bioeconomy and the elimination of the severe environmental impact caused by industrial activities.

Among the different chemicals, versatile molecules that act as building blocks for other chemicals and materials are of immense necessity. Microbial oil from yeast stains, microbial oil rich in polyunsaturated fatty acids from fungal stains, cosmetic esters, biolubricants, phenolic compounds and crude proteolytic enzymes were strategic targets within the frame of this thesis. Their global market covers a vast array of sectors including food, feed, pharmaceuticals and oleochemicals.

Carbon and nitrogen sources choice has a great impact on the downstream processing and process economics. The maximum theoretical yield of microbial oil when glucose is utilized as fermentative substrate is 0.32 g/g. the exploitation of xylose increases more the product yield (~ 0.34 g/g), due to the fact that oleaginous microorganisms utilize exclusively the phosphoketolase pathway for xylose assimilation (Papanikolaou and Aggelis, 2011). The narrow gap between the prices of carbon source and products, particularly in case of bulk chemicals ordains the exploration for cheap nutrient sources. The production of microbial oil was implemented from side streams of a palm oil production process (Chapter 5). *Lipomyces starkeyi* was efficiently cultivated on palm kernel cake hydrolysate as the nitrogen-sources mixed with commercial carbon sources. Microbial oil was additionally produced utilising waste streams of the pulp and paper industry (Chapter 8, 9). Spent sulphite liquor, mainly composed of xylose, provided the carbon necessary for microbial proliferation and microbial oil accumulation. Lignocellulosic cakes deriving from the palm oil, cotton oil and castor oil manufacture were finally used as carbon and nitrogen matrices for the SSF of lipids production employing various fungal strains (Chapter 7). Result showed that lipid production (120.8 mg/g fermented solids) from cotton seed cake via packed-bed reactor fermentation is feasible, but better process and reactor design are needed to improve the final lipids yield of this process.

The selection of inhibitor-tolerant microbial strains and high cell density fermentation provide important tools for maximizing productivities and titres of the desired products. In Chapter 9, several shake flasks fermentations with *Lipomyces starkeyi* and *Cunninghamella echinulata*, under different concentrations of inhibitory lignosulphonates compounds, demonstrated their

strong ability to grow in the presence of inhibitory compounds and to consume all C5 and C6 sugars present in the spent sulphite liquor. This could lead to the utilisation of lignocellulosic resources for the production of oleochemicals and fuels. Fed-batch practise is an alternative that could promote natural producer strains and make feasible the industrialization of the applied bioprocess. Fed-batch fermentation with *Lipomyces starkeyi* and *Cunninghamella echinulata* resulted in enhanced biomass (81.1 g/L and 12.2 g/L respectively) and lipids concentrations (40.9 g/L and 6.8 g/L respectively) (Chapter 9).

Two-step consolidated bioprocesses based on the utilisation of the first stage products as the raw material for the formulation of the target product provides a promising alternative in the era of bioeconomy and circular economy. Chapter 5, dealt with the development of a bioprocess based on the production of proteolytic crude enzymes via solid state fermentation (SSF) carried out in tray bioreactors and a fully controlled rotating drum bioreactor. The produced enzyme consortia, was subsequently utilized as the biocatalysts for the production of generic nutrient supplement for microbial oil production. Chapters 7 and 8 focused on the efficient microbial oil production using spent sulphite liquor or various cakes after oil removal and its application as the raw material for cosmetic ester and biolubricants formation via enzymatic synthesis. Results showed that the different lipid fractions have a key role in the enzymatic activity and the final conversion yield of cosmetic esters (Chapter 8). Specifically, conversion yield was higher when microbial oil was free of phospholipids and glycolipids plus sphingolipids fractions. Novozyme 435 was more effective in terms of conversion yields (90.8% for isopropyl esters and 80.0% for 2-ethylhexyl esters) when hydrolysed microbial oil was utilized as substrate. The successful lipase utilisation for up to 5 repeated reactions without significant decrease of conversion yield, indicates that enzymatic processes may be a cost-effective route for the production of bio-based compounds. In the case of biolubricants (Chapter 7), microbial oil extracted from fermented cotton seed cake, was used for enzymatic ester production with Lipomod 34-MDP and neopentyl glycol. The highest conversion yield (80%) was achieved after 2 h reaction. In Chapter 6, phenolic compound extracted from the by-product streams of the palm oil production were used as a source of natural antioxidants with PKC possessing the highest total phenolic content with adequate antioxidant activity index. The incorporation of PKC phenolic extracts resulted in 60 % increase of its induction time. The exploitation of palm oil residues as a source of phenolic compounds could possibly find application in the food industry.

The results presented in this PhD thesis show that lignocellulosic renewable resources constitute promising feedstock for the production of value added metabolites thus the applied bioprocesses deserve further optimisation. Future research should give insight to a genome level of microorganisms. Further metabolic engineering of these microorganisms for enhanced production is possible. Sustainability analysis including techno-economic evaluation and life cycle analysis of the proposed bioprocesses should be also considered.

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