AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF FOOD PROCESS ENGINEERING

Doctoral Thesis

Study of confectionery industry waste valorisation for microbial oil production via fermentation using oleaginous yeasts

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ABSTRACT

This doctoral dissertation focused on the valorisation of renewable resources generated by the food industry for the biotechnological production of microbial oil. Microbial oil has been evaluated as potential feedstock for the production of biodiesel.

The first part of this PhD thesis demonstrated that flour-rich waste (FRW) and by-product streams generated by bakery, confectionery and wheat milling plants could be employed as the sole raw materials for generic fermentation media production, suitable for microbial oil synthesis. Wheat milling by-products were used in solid state fermentations (SSF) of *Aspergillus awamori* for the production of crude enzymes, mainly glucoamylase and protease. Enzyme-rich SSF solids were subsequently employed for hydrolysis of FRW streams into nutrient-rich fermentation media. Batch hydrolytic experiments using FRW concentrations up to 205 g/L resulted in higher than 90% (w/w) starch to glucose conversion yields and 40% (w/w) total Kjeldahl nitrogen to free amino nitrogen conversion yields. Starch to glucose conversion yields of 98.2, 86.1 and 73.4% (w/w) were achieved when initial FRW concentrations of 235, 300 and 350 g/L were employed in fed-batch hydrolytic experiments, respectively. Crude hydrolysates were used as fermentation media in shake flask cultures with the oleaginous yeast *Lipomyces starkeyi* DSM 70296 reaching a total dry weight of 30.5 g/L with a microbial oil content of 40.4% (w/w), higher than that achieved in synthetic media. Fed-batch bioreactor cultures led to a total dry weight of 109.8 g/L with a microbial oil content of 57.8% (w/w) and productivity of 0.4 g/(L·h).

This PhD thesis subsequently dealt with the production of crude enzymes via SSF using wheat milling by-products for both fermentation media production using FRW streams and lysis of *Rhodosporidium toruloides* yeast cells. Filter sterilization of crude hydrolysates was more beneficial than heat sterilization regarding yeast growth and microbial oil production. The initial carbon to free amino nitrogen ratio of crude hydrolysates was optimized (80.2 g/g) in fed-batch cultures of *R. toruloides* leading to a total dry weight of 61.2 g/L with microbial oil content of 61.8 % (w/w). A feeding strategy where the glucose concentration was maintained in the range of 12.2 – 17.6 g/L led to the highest productivity (0.32 g/L·h). The crude enzymes produced by SSF were utilised for yeast cell treatment leading to simultaneous release of around 80% of total lipids in the broth and production of a hydrolysate suitable as yeast extract replacement.

The next part of the thesis was related to the utilization of diversified mixed confectionery waste streams in a two-stage bioprocess to formulate nutrient-rich fermentation media for microbial oil production. Solid state fermentation was conducted for the production of crude enzyme consortia to be subsequently applied in hydrolytic reactions to break down starch, disaccharides and proteins into monosaccharides, amino acids and peptides. Crude hydrolysates were evaluated in bioconversion processes using *R. toruloides* DSM 4444 in both batch and fed-batch mode. Under nitrogen-limiting conditions, during fed-batch cultures, the concentration of microbial lipids reached 16.6 – 17.0 g/L with the intracellular content being more than 40% (w/w) in both hydrolysates applied. *R. toruloides* was able to metabolize mixed carbon sources without catabolite repression. The fatty acid profile of the produced lipids was altered based on the substrate employed in the bioconversion process. Microbial lipids were rich in unsaturated fatty acids, with oleic acid being the major fatty acid (61.7%, w/w).

This PhD thesis finally focused on the evaluation of biodiesel production using microbial oil derived from FRW hydrolysates. Crude hydrolyzates were used in fed-batch bioreactor fermentations at a C/FAN ratio of 80.2 g/g with the oleaginous yeast *R. toruloides*. Microbial oil was transesterified into biodiesel with satisfactory performance considering the European standard

EN 14214. This work demonstrated that valorization of food waste for biodiesel production could be feasible.

Scientific area: Design of Food and Biorefinery Processes

Keywords: Food waste valorisation, Bioprocess development, *Rhodosporidium toruloides*, *Lipomyces starkeyi*, Microbial oil, Enzymatic cell lysis, Solid state fermentation, Fed-batch fermentation, Enzymatic hydrolysis, Circular economy, Oleic acid, biodiesel properties

Μελέτη της αξιοποίησης βιομηχανικών αποβλήτων προϊόντων ζαχαροπλαστικής για τη βιοτεχνολογική παραγωγή μικροβιακών ελαίων με χρήση ελαιογόνων ζυμών

Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου
Εργαστήριο Μηχανικής και Επεξεργασίας Τροφίμων

ΠΕΡΙΛΗΨΗ

Η παρούσα διδακτορική διατριβή επικεντρώθηκε στην αξιοποίηση ανανεώσιμων πρώτων υλών, που προέρχονται από υποπροϊόντα βιομηχανιών τροφίμων για τη βιοτεχνολογική παραγωγή μικροβιακού λίπους. Εξετάστηκε η πιθανή χρήση του παραγόμενου μικροβιακού λίπους ως πρώτη ύλη για την παραγωγή βιοντίζελ.

Το πρώτο μέρος της διατριβής έδειξε ότι τα αλευρούχα υπολείμματα (flour-rich-waste-FRW) και τα ρεύματα υποπροϊόντων που προέρχονται από μονάδες αρτοποιίας, ζαχαροπλαστικής και αλέσεως σίτου μπορούν να χρησιμοποιηθούν ως πλήρεις πρώτες ύλες για την παραγωγή θρεπτικών μέσων ζύμωσης, κατάλληλες για τη σύνθεση μικροβιακών λιπιδίων. Τα παραπροϊόντα άλεσης σίτου χρησιμοποιήθηκαν σε ζυμώσεις στερεάς κατάστασης (solid state fermentation-SSF) με το στέλεχος *Aspergillus awamori* για την παραγωγή ακατέργαστων ενζύμων, κυρίως γλυκοαμυλάση και πρωτεάση. Τα πλούσια σε ένζυμα στερεά χρησιμοποιήθηκαν στη συνέχεια για την υδρόλυση των FRW προς παραγωγή πλούσιων σε θρεπτικά μέσων ζύμωσης. Οι κλειστού τύπου υδρολύσεις, με την χρήση αρχικών συγκεντρώσεων FRW έως και 205 g/L είχαν ως αποτέλεσμα αποδόσεις μετατροπής αμύλου σε γλυκόζη υψηλότερες από 90% (w/w) και σε 40% (w/w) απόδοση μετατροπής ολικού αζώτου (Kjeldahl) προς ελεύθερες αμινομάδες αμινοξέων και πεπτιδίων (free amino nitrogen-FAN). Αποδόσεις μετατροπής αμύλου σε γλυκόζη ίσες με 98,2%, 86,1% και 73,4% (w/w), επιτεύχθηκαν σε αντίστοιχες αρχικές συγκεντρώσεις FRW 235 g/L, 300 g/L και 350 g/L μέσω ενζυμικών υδρολύσεων ημισυνεχούς λειτουργίας. Εν συνεχεία, τα υδρολύματα που προέκυψαν από τις ενζυμικές υδρολύσεις των FRW χρησιμοποιήθηκαν ως θρεπτικό υπόστρωμα ζύμωσης προς παραγωγή μικροβιακού λίπους με την ζύμη *Lipomyces starkeyi* DSM 70296. Η παραγωγή μικροβιακής βιομάζας (total dry weight-TDW) που επιτεύχθηκε ήταν 30,5 g/L με λιποπεριεκτικότητα 40,4% (w/w), τιμές υψηλότερες από τις αντίστοιχες που προέκυψαν με την χρήση συνθετικών θρεπτικών μέσων. Οι ζυμώσεις ημισυνεχούς καλλιέργειας σε βιοαντιδραστήρα οδήγησαν σε TDW 109,8 g/L με λιποπεριεκτικότητα 57,8% (w/w) και παραγωγικότητα 0,4 g/(L·h).

Το επόμενο μέρος της διδακτορικής διατριβής αφορούσε στην παραγωγή ακατέργαστων ενζύμων μέσω SSF χρησιμοποιώντας παραπροϊόντα άλεσης σίτου τόσο για την παραγωγή θρεπτικών μέσων ζύμωσης αξιοποιώντας FRW, όσο και για τη λύση των κυττάρων ζύμης του στελέχους *Rhodosporidium toruloides*. Η αποστείρωση με φίλτρο των ακατέργαστων παραγόμενων υδρολυμάτων ήταν ευεργετικότερη σε σύγκριση με την θερμική αποστείρωση όσον αφορά στην ανάπτυξη ζύμης και στην παραγωγή μικροβιακών λιπιδίων. Η αρχική αναλογία άνθρακα προς FAN των ακατέργαστων υδρολυμάτων βελτιστοποιήθηκε (80,2 g/g) σε ζυμώσεις ημισυνεχούς καλλιέργειας του *R. toruloides*, οδηγώντας σε TDW 61,2 g/L με λιποπεριεκτικότητα 61,8% (w/w). Με την εφαρμογή μιας στρατηγική τροφοδοσίας κατά την οποία η συγκέντρωση γλυκόζης διατηρήθηκε σε εύρος 12,2 - 17,6 g/L επιτεύχθηκε η υψηλότερη τιμή παραγωγικότητας (0,32 g/(L·h)). Τα ακατέργαστα ένζυμα που παράχθηκαν μέσω SSF χρησιμοποιήθηκαν για την επεξεργασία των κυττάρων της ζύμης οδηγώντας σε ταυτόχρονη απελευθέρωση περίπου 80% των συνολικών λιπιδίων στο υγρό της ζύμωσης και σε παραγωγή ενός υδρολύματος ως υποκατάστατο εκχυλίσματος ζύμης.

Το επόμενο στάδιο της εργασίας αφορούσε την αξιοποίηση μικτών παραπροϊόντων ζαχαροπλαστικής σε βιοδιεργασία δύο σταδίων προς παραγωγή ενός πλούσιου σε θρεπτικά συστατικά μέσου ζύμωσης κατάλληλου για την παραγωγή μικροβιακού λίπους. Αρχικά διεξήχθη ζύμωση στερεής κατάστασης για την παραγωγή ακατέργαστων ενζύμων που στην συνέχεια χρησιμοποιήθηκαν σε υδρολυτικές αντιδράσεις για την διάσπαση του αμύλου, δισακχαριτών και πρωτεϊνών σε μονοσακχαρίτες, αμινοξέα και πεπτίδια. Τα παραγόμενα υδρολύματα

αξιολογήθηκαν σε διεργασίες βιομετατροπής με την χρήση της ζύμης *R. toruloides* DSM 4444 σε ζυμώσεις κλειστής καλλιέργειας και ημισυνεχούς καλλιέργειας. Υπό συνθήκες περιορισμού του αζώτου, κατά τη διάρκεια ημισυνεχών ζυμώσεων, η συγκέντρωση των μικροβιακών λιπιδίων κυμάνθηκε από 16,6 έως 17,0 g/L με το ενδοκυτταρικό περιεχόμενο να είναι μεγαλύτερο του 40% (w/w). Το στέλεχος της ζύμης ήταν ικανό να μεταβολίζει τις μικτές πηγές άνθρακα χωρίς καταστολή του καταβολισμού. Το προφίλ λιπαρών οξέων των παραγόμενων λιπιδίων μεταβλήθηκε με βάση το υπόστρωμα που χρησιμοποιήθηκε στη διαδικασία βιομετατροπής. Τα μικροβιακά λιπίδια ήταν πλούσια σε ακόρεστα λιπαρά οξέα, με το ελαϊκό οξύ να είναι το κύριο λιπαρό οξύ (61,7%, w/w).
Το τελευταίο μέρος αυτής της διπλωματικής εργασίας περιελάμβανε την αξιολόγηση της παραγωγής βιοντίζελ χρησιμοποιώντας μικροβιακό λίπος που προέρχεται από υδρολύματα FRW. Χρησιμοποιήθηκαν ακατέργαστα υδρολύματα σε ζυμώσεις βιοαντιδραστήρων ημισυνεχούς λειτουργίας σε αναλογία C/FAN 80,2 g/g με το στέλεχος ζύμης *R. toruloides*. Το μικροβιακό λίπος μετεστεροποιήθηκε σε βιοντίζελ με ικανοποιητικές επιδόσεις λαμβάνοντας υπόψη το ευρωπαϊκό πρότυπο EN 14214. Αυτή η εργασία έδειξε ότι η αξιοποίηση των απορριμμάτων τροφίμων για την παραγωγή βιοντίζελ μπορεί να είναι εφικτή.

Επιστημονική περιοχή: Σχεδιασμός Διεργασιών Τροφίμων και Βιοδιυλιστηρίων

Λέξεις κλειδιά: Αξιοποίηση απορριμμάτων τροφίμων, ανάπτυξη βιοδιεγασιών, *Rhodosporidium toruloides, Lipomyces starkeyi*, Μικροβιακό λίπος, ενζυμική λύση κυττάρων, Ζύμωση στερεάς κατάστασης, Ζύμωση ημισυνεχούς λειτουργίας, Ενζυμική υδρόλυση, Κυκλική οικονομία, Ελαϊκό οξύ, Παραγωγή βιοντήζελ, Χαρακτηρισμός ιδιοτήτων

ΔΗΛΩΣΗ ΕΡΓΟΥ

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Μελέτη της αξιοποίησης βιομηχανικών αποβλήτων προϊόντων ζαχαροπλαστικής για τη βιοτεχνολογική παραγωγή μικροβιακών ελαίων με χρήση ελαιογόνων ζυμών

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, 2και 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

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Dissemination activities

A) List of publications - related to this thesis:

1. **Tsakona, S.,** Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Kookos, I. K., Koutinas, A. A. (2014). Formulation of fermentation media from flour-rich waste streams for microbial lipid production by *Lipomyces starkeyi*. *Journal of Biotechnology*, 189, 36–45.
2. Leiva-Candia, D.E., **Tsakona, S.,** Kopsahelis, N., García, I.L., Papanikolaou, S., Dorado, M.P., Koutinas, A.A. (2015). Biorefining of by-product streams from sunflower-based biodiesel production plants for integrated synthesis of microbial oil and value-added co-products. *Bioresource Technology*, 190, 57-65.
3. **Tsakona, S**., Skiadaresis, A.G., Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Kookos, I.K., Koutinas, A.A. (2016). Valorisation of side streams from wheat milling and confectionery industries for consolidated production and extraction of microbial lipids. *Food Chemistry*, 198, 85-92.
4. **Tsakona, S.,** Papadaki, A., Kopsahelis, N., Kachrimanidou, V., Papanikolaou, S., Koutinas, A. (2019). Development of a circular oriented bioprocess for microbial oil production using diversified mixed confectionery side-streams. *Foods*,8(8),300.

B) List of publications – not related to this thesis

1. Galanakis, C.M., Goulas, V., **Tsakona, S**., Manganaris, G.A., Gekas, V. (2013). A knowledge base for the recovery of natural phenols with different solvents. *International Journal of Food Properties*, 16(2), 382-396.
2. **Tsakona, S.,** Galanakis, C.M., Gekas, V. (2012). Hydro-Ethanolic Mixtures for the Recovery of Phenols from Mediterranean Plant Materials. *Food and Bioprocess Technology*, 5(4), 1384-1393.

C) Conference presentations - related to this thesis

1. **Tsakona, S**., Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Koutinas, A. (oral presentation) (2012). Production of generic fermentation feedstock from flour-based industrial waste streams. 8th International Conference on Renewable Resources and Biorefineries (RRB8), 4-6 June, Toulouse, France.
2. **Tsakona, S.,** Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Koutinas, A.A. (poster presentation) (2013). Developing confectionery industry biorefineries. Nanotechnology 2013: Bio Sensors, Instruments, Medical, Environment and Energy - 2013 NSTI

Nanotechnology Conference and Expo, NSTI-Nanotech 2013; Washington, DC; United States; 12 - 16 May.

3. **Tsakona, S.,** Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Koutinas, A.A. (oral presentation) (2013). Valorization of flour-based industrial waste streams for microbial oil production. FaBE 2013 - International Conference on Food and Biosystems Engineering, 30 May-02 June, Skiathos Island, Greece.
4. **Tsakona, S**., Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Koutinas, A.A. (2013). Production of microbial oil from flour-based industrial waste streams. 5ο Πανελλήνιο Συνέδριο «Σύγχρονες Τάσεις στον τομέα των Λιπιδίων», 29 Μαρτίου, Αθήνα, Ελλάδα.
5. Leiva-Candia, D., **Tsakona, S**., Kopsahelis, N., Garcia, I.L., Papanikolaou, S., Dorado, M.P., Koutinas, A. (oral presentation) (2014). Biorefinery development based on sunflower-based biodiesel industry by-products for the production of microbial oil and value-added products. 247th American Chemical Society National Meeting & Exposition: Chemistry and Materials for Energy, 16-20 March, Dallas, Texas, USA.
6. Vlysidis, A., **Tsakona, S.,** Kopsahelis, N., Kookos, I., Koutinas, A. (2017). (oral presentation) Sustainability assessment for the production of microbial oil from food Wastes. 10th World Congress of Chemical Engineering, 1-5 October, Barcelona, Spain.

D) Conference presentations – not related to this thesis

1. Dimou, C., **Tsakona, S**., Kachrimanidou, V., Kopsahelis, N., Papanikolaou, S., Koutinas, A. (2012). (poster presentation) Evaluation of PHA production from industrial waste streams. 8th International Conference on Renewable Resources and Biorefineries (RRB8), 4-6 June, Toulouse, France.

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CHAPTER 1

Introduction

Over the last decades, climate change has become one of the most relevant environmental challenge. Fossil fuel combustion accounts for 90% of total world`s CO_2 emissions (Oliver et al., 2012). Renewable energy sources are rapidly growing, and their use will increase from 10% of total energy in 2008 to 14% by 2035, mending both energy security and climate change issues (Balsalobre-Lorente et al., 2018). Renewable energy sources could provide 50% of the world's energy needs by 2050 in a target-oriented scenario to prevent dangerous anthropogenic interference with the climate system (Krewitt et al., 2007).

Concern about climate change, population growth, pollution, and the rising prices of essential inputs, such as fuels, has led to increased emphasis on the development of technologies that are perceived to be renewable and sustainable. These urgent necessities for energy and materials are forcing society to shift from fossil-based linear economy to sustainable circular bioeconomy. Key drivers for the bioeconomy include: a) the demand for sustainable renewable biological resources and bioprocesses b) neccesity to manage renewable resources, d) neccesity for energy and food security and e) opportunity to dissociate agricultural growth from environmental degradation through sustainable production employing biotechnology (Rosegrant et al., 2018). Bioeconomy demands renewable feedstocks with perpsectives to generate a spectrum of bio-based products employing multidisciplinary areas of science, management and engineering. Waste deriving from biogenic fractions could serve as an ideal feedstock for the development of a sustainable waste-based bioeconomy incorporated into integrated biorefinery approaches. The critical point is the integration of diverse bioprocesses into industrial production leading waste conversion to viable bio-based products (Dahiya et al., 2018).

The increased awareness and demand for sustainability in the modern society has made terms such as ‘environment-friendly’, ‘green’, ‘bio-based’ and ‘renewable’ quite popular for marketing of many products. The biorefinery concept can provide numerous bio-based products like biofuels, platform chemicals, polymers and fertilizers with enhanced waste treatment efficiency. Recent developments on bio-based production have targeted mainly to biofuels (biodiesel and bioethanol). Thus, the combined production of biofuels, chemicals and materials could lead to economic viable plants simulating the petrochemical refinery.

1.1 Generation of by-products and wastes from food supply chains

Food supply chain waste and by-product streams are generated along any supply chain of food industrial sectors and sub-sectors, from agricultural production up to consumption of food products including food loss before, during or after meal preparation in the household and food discarded in the process of manufacturing, distribution, retail and food service activities (European Commission, 2010). As it is depicted in Figure 1.1, around 90 million t of food waste is generated annually in the EU-27 through food manufacturing (39%, 70 kg per capita), households (42%, 76 kg per capita), food catering services (14%, 25 kg per capita) and retail/wholesale of food products (5%, 8 kg per capita). Food that ends up as being discarded by households represents 25% of food purchased. For the UK, food waste represents an annual loss per household of around 565 euros (European Commission, 2010, Ravindran and Jaiswal 2016). Reports published by FAO, estimate more than 1.3 billion t of annual food supply chain waste corresponding to 50% of food lost or wasted along the supply chain from the agricultural field to the consumer (Parfitt et al., 2010). To illustrate the approximate quantities of food waste produced in Europe, it is mentioned that the losses and wastes generated along with wheat and rye supply chains in Europe are: a) 1.45×10^6 t at agricultural production, b) 2.56×10^6 t at post-harvest handling and storage, c) 7.45×10^6 t during industrial bread baking, d) 1.34×10^6 t during distribution, and e) 16.43×10^6 t at the consumption stage (Gustavsson et al., 2013).

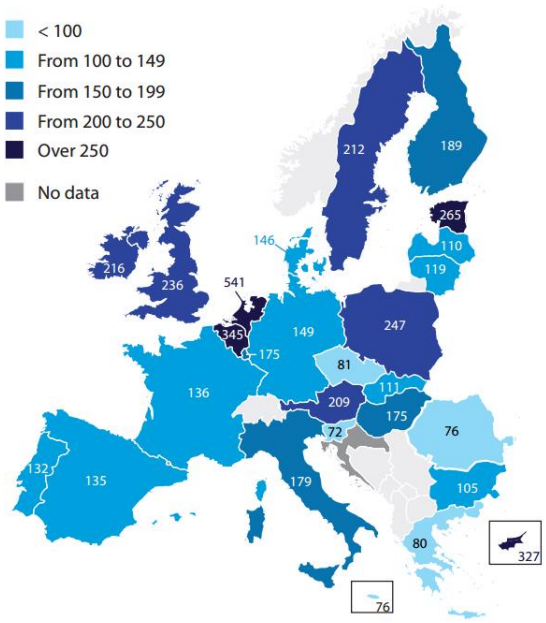

Figure 0-1 Generation of total food waste in the EU (kg per capita)

Food waste is forcasted to increase to around 126 million t in 2020 from about 89 million t in 2006 leading to additional 40% of CO_2 emissions (Monier et al., 2010). Increasingly tighter regulations regarding organic waste, and the demand for renewable chemicals and fuels, are driving the manufacturing industry toward higher sustainability to improve cost-effectiveness and meet customers' demand. The Waste Framework Directive 2008/98/EC designates as top priority the prevention of waste generation, followed by processing for reuse and recycling, with disposal as the least favoured stage of waste management (Figure 1.2). Member states should implement national waste prevention strategies for collection and treatment of biowaste ensuring optimal environmental protection, and the development of innovative technologies for the production of bio-based and biodegradable products from biowaste (Recital 8).

Figure 0-2 Waste hierarchy (Lin et al., 2013)

1.2 Biorefinery development: from food wastes to bioeconomy products

The food sector generates significant quantities of bio-wastes worldwide. Food waste could be used as a feedstock in bioprocesses for the production of a wide spectrum of bio-based products along with its remediation employing innovative sustainable technologies. The viability of bioprocesses could be achieved with the implementation of biorefinery strategies for the utilization of residual organic waste towards diverse product recovery and formulation, ranging from biofuels to commodity chemicals and biofertilizers (Figure 1.3).

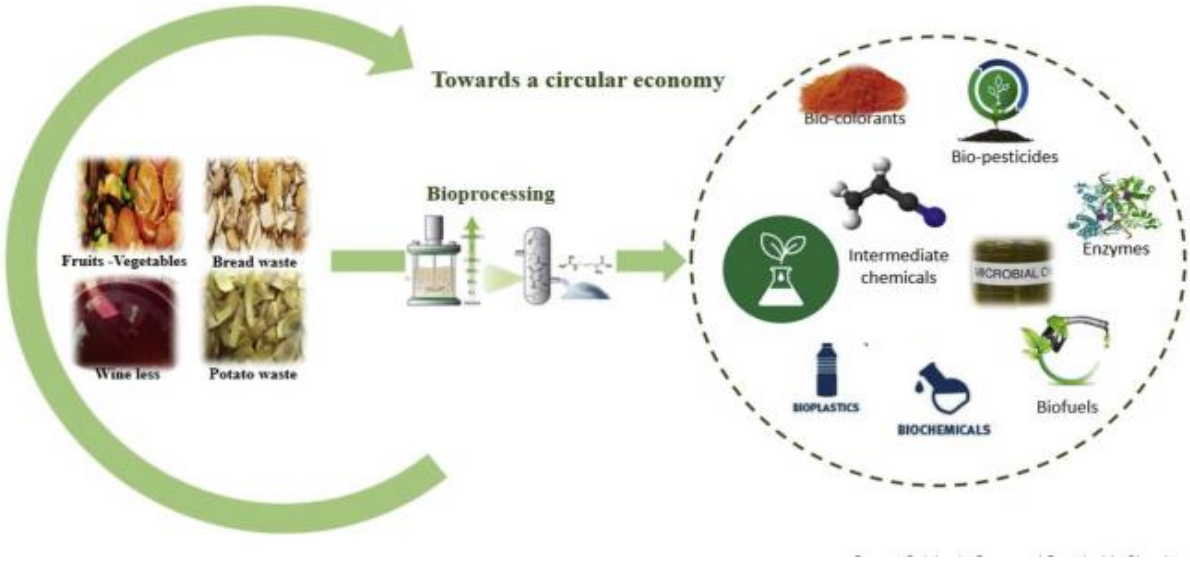

Figure 0-3 Circular valorization of industrial wastes and by-products towards high added-value products (Maina et al., 2017)

Pei et al. (2017) demonstrated an effective strategy for algal oil production using effluent from anaerobic digestion of kitchen waste as fermentative substrate. Biomass production and lipids accumulation of *Chlorella* SDEC-11 and *Scenedesmus* SDEC-13 were enhanced when algal inoculum was pretreated with agricultural phytohormones. Based on the obtained results, the authors claimed that a feasible and economic plant can be built incorporating algae-based biofuel production and anaerobic digestion. Amulya et al. (2015) reported a sustainable multistage process for enhanced productivity of polyhydroxyalkanoates (PHA) from food waste after remediation. After the production of biohydrogen via acidogenic fermentation, the generated, rich in volatile fatty acid effluent was used for PHA production (39.6%). The authors indicated that this integrated multistage process shows great perspectives for further large scale PHA production with simultaneous waste remediation in the framework of biorefinery. Kwan et al. (2015) carried out a techno-economic analysis of food waste valorization via fungal hydrolysis, microalgae cultivation and production of plasticizer, lactic acid and animal feed. A pilot-scale plant was designed with a capacity of 1 metric ton/day of food waste and 20 years lifetime. The first scenario included the co-production of plasticizer and lactic acid while the second one proposed production of plasticizer and animal feed. The viabily of the first scenario was demonstrated by annual net profits, net present value, payback period and internal rate of return of $422,699, $3,028,000, 7.56 years and 18.98%, respectively. Du et al. (2008) proposed a wheat biorefining approach for the fermentative production of succinic acid. Wheat was fractionated into bran, gluten and gluten-free flour. The waste bran was used as a substrate for fungal solid state fermentation to produce crude enzyme consortia which was subsequently utilized for the

hydrolysis of gluten-free flour and gluten to glucose (140 g/L) and free amino nitrogen (3.5 g/L). The addition of $MgCO_3$ in the fermentation media resulted in a succinic acid production higher than 64 g/L demonstrating the solid state based strategy as a successful biorefinery.

Food waste show high complexity due to their diversity and variability. Thus, they can comprise important renewable resources through well designed valorization approaches. Maximum exploitation of the potential of food waste requires synergies from legislation and socio-economics to scientific sector paving the way towards a more sustainable society based on the bio-economy pillars.

The recovery of carbon, nitrogen and phosphorous from food waste by chemical and biological methods enables the recycling of valuable nutrients for the production of chemicals, materials and energy. Valorisation of food waste is not only an environmentally benign waste treatment, but it also benefits the bio-based economy. The concept of the circular economy is emerging as a worldwide strategy to transit from the current linear economy model of production and consumption to efficient resource exploitation (Ghisellini et al., 2016). Within this framework, bio-economy encompasses the holistic valorization of renewable resources towards the development of biorefinery concepts and bioprocessing schemes to produce high value-added products.

CHAPTER 2

State of the art

2.1. Bakery and confectionery waste streams

Flour–rich waste (FRW) and by-product streams are generated by many industrial food sectors belonging mainly to the following categories, as have been classified by the PRODCOM List 2013 (Anonymous 2014a):

- ✓ manufacture of **grain mill products** (PRODCOM code 10.61), constituting the 4th most important food sector in terms of production capacity
- ✓ manufacture of **bread, fresh pastry goods and cakes** (PRODCOM code 10.71), constituting the 7th most important food sector in terms of production capacity
- ✓ manufacture of **rusks, biscuits and preserved pastry goods and cakes** (PRODCOM code 10.72)
- ✓ various types of **confectionery products** and **food for infants** (PRODCOM 10.86.10.60 and 10.86.10.70)

FRW are generated primarily from the manufacturing process, also disposed by consumers and catering services, or are returned from the market as end-of-date products. Specifically, wheat milling by-products (WMB), generated by wheat flour millers, are produced via milling and sifting of wheat and contains mainly bran and varying quantities of endosperm depending on the type of wheat flour produced. All these FRW are rich sources of nutrients, consisting of 86.1% carbohydrates, 7.3% protein and 1.1% fat (Koutinas et al., 2014a) and are mainly used as animal feed, but surplus quantities are treated as wastes.

Considering the production capacity of such waste streams, which is around 800,000 t of bakery waste per year, as recorded in the UK (WRAP 2011), and their significant starch and protein content, a biorefinery strategy could add value to these wastes. Previous studies have presented the perspective of FRW utilization towards the production of platform chemicals, such as succinic acid, and enzymes through microbial fermentations and enzymatic bioconversions (Lin et al., 2013; Koutinas et al., 2014a) (Figure 2.1). This and other circular economy schemes targeting to FRW and other bakery waste streams are described in the next section.

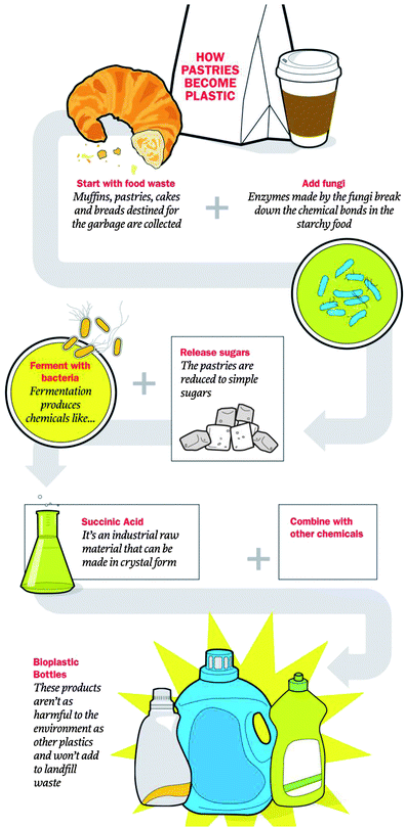

Figure 0-1 Scheme of a proposed bakery waste valorization through microbial and enzymatic processes (Lin et al., 2013)

2.2. Prospects for utilization of flour and starch-based waste streams

Waste valorisation is the most attractive alternative for dealing with residues other than disposal or landfilling. Through conventional valorisation practices such as composting, recycling and burning for energy recovery, less than 50% of the waste can be managed, leaving great margins for extraction of useful components, sustainable bioconversion for the production of bio-based products and cascade recovery of valuable chemicals (Luque and Clark, 2013). A wide variety of case studies have been developed for the conversion of food waste-derived feedstocks into bio-based added value products i.e chemicals, biomaterials and biofuels illustrating the high potential of advanced waste valorization strategies (Luque and Clark 2013; Mirabella et al., 2014; Ravindran and Jaiswal 2016).

The confectionery industry is highly diverse and complex. There are over 12,700 confectionery manufacturers across Europe producing speciality and mass produced products. The increasing demand for different confectionery products due to the fast-moving nature of consumption and consumer preference is exerting huge pressure on confectionery supply chains across the world setting limits to production (Miah et al., 2018). According to an environmental life cycle analysis for a range of different confectionery products, it was

shown that by focusing on raw materials with lower environmental impacts, product reformulations, and reducing waste generation, an aggregated environmental reduction of 46% could by achieved (Miah et al., 2018).

Waste deriving from bakery, confectionery and wheat milling industry are highly promising for the synthesis of bio-based products via a biotechnology route. Particularly, food processing wastes, deriving from confectionery and bakery industry, consist of mono-, di- or polysaccharides (especially starch), protein and other macromolecules that could be converted into assimilable carbon, nitrogen and nutrient sources through enzymatic hydrolysis. The essential enzymes for the hydrolysis processes can be produced via solid state fermentations utilizing solid food waste streams (Koutinas et al., 2014a).

Bakery waste including pastry and cakes have been previously investigated for the production of value-added products through novel approaches targeting to sustainability and biorefinery development. Pastries are characterized of higher starch (44.6%) and lipid content (35.2%) to those of cakes, whereas cakes have higher sugar (11.9% fructose and 22.7% sucrose) and protein (17.0%) content (Arancon et al., 2013). Case studies dealing with the bioconversion of bakery hydrolysate into bio-plastics i.e poly(3-hydroxybutyrate) (PHB) and platform chemicals i.e succinic acid, highlight the significant potential of advanced waste valorization strategies including simultaneous hydrolysis and fungal autolysis, as well as solid state fermentation and submerged fermentation for the formulation of a rich fermentative generic feedstock (Arancon et al., 2013; Zhang et al., 2013). Direct production of lactic acid based on simultaneous saccharification and fermentation of mixed restaurant food waste in combimation with specific downstream processing resulted in a pure L(+)-lactic acid formulation of 702 g/L (Pleissner et al., 2017). Biocolorants and enzymes have also been efficiently produced utilizing bakery waste as a nutrient source (Haque et al., 2016). In another study, waste bread was used as the sole nutrient source for the production succinic acid production by *Actinobacillus succinogenes.* Hydrolysates of waste bread were transformed to fermentative substrate rich in glucose and free amino nitrogen via fungal solid state fermentation and sequential enzymatic hydrolysis. Succinic acid production of 47.3 g/L with an overall yield of 0.55 g succinic acid per g bread was reported (Leund et al., 2012).

Wastewaters from confectionery industries showed good performance for the microbial production of xanthan gum (24 g/L). A cost model for a production facility using a simulation software revealed that the bioprocess was in the range of being an investment of

interest (Bajić et al., 2017). The utilization of fermentation media derived from waste and by-product streams of confectionery industries could lead to highly efficient production of bacterial cellulose. Flour-rich hydrolysates were used as the sole fermentation media resulting in bacterial cellulose production of 13 g/L with the bacterial strain *Komagataeibacter sucrofermentans* (Tsouko et al., 2015).

All these bakery and confectionery waste streams could be further exploited as fermentative feedstock for many other added-value products, such as microbial oil, as it is proposed in this PhD thesis.

2.3. Oleaginous yeasts and microbial oil production

Microbial lipid production represents a significant field of research during the last decades. The increasing interest towards alternative pathways for cost-effective production of biofuels from renewable resources has set the ground for intensive search on heterotrophic (yeast and fungi) or phototrophic (algae) organisms, capable of accumulating high oil contents, accompanied by high bioconversion yields (Papanikolaou and Aggelis, 2010).

Microbial oil is a secondary metabolite, refering to intracellular storage lipids mainly composed of triacylglycerols (TAGs). Other components present in microbial oil are free fatty acids, neutral lipids i.e monoacylglycerols, diacylglycerols, and steryl-esters, sterols and polar fractions including phospholipids, sphingolipids and glycolipids (Papanikolaou and Aggelis, 2011a). 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). Oleaginous microorganisms can accumulate intracellular lipids to more than 20% of their total mass dry weight (Ratledge, 1991).

Microorgansims, such as microalgae, yeasts (e.g. *Candida*, *Cryptococcus, Lipomyces*, *Rhodotorula, Rhodosporidium, Trichosporon*), fungi (e.g. *Mortierella*, *Cunninghamella*) and bacteria (e.g. *Rhodococcus*, *Mycobacterium*) can accumulate intracellularly high amounts of microbial lipids. Among oleaginous yeasts, specific focus was given in this thesis for *Lipomyces strakeyi* and *Rhodosporidium toruloides*, as they have shown high potential in microbial oil production, accumulating up to 76% (w/w, db) lipids under nitrogen-limited conditions (Angerbauer et al., 2008; Meng et al., 2009; Li et al., 2006; Park et al., 2018).

Depending on the oleaginous strain and the applied cultivation method, microbial lipids may present a diversified fatty acid composition attributing varying properties to the produced lipid bodies. In many cases, the produced microbial lipids present similar fatty acid composition to vegetable oils (Meng et al., 2009; Papanikolaou and Aggelis, 2011). For instance, palm oil mainly consists of palmitic acid (44.5%) and oleic acid (39.6%), whereas the predominant fatty acids in soybean oil, rapeseed oil and sunflower oil are oleic and linoleic acids with respective values 25.9% and 52.8%, 65.9% and 17.1%, 28.2% and 60.3% (Leiva-Candia et al., 2015). Microbial oil mainly consists of TAGs with a fatty acid composition rich in C18 and C16 fatty acids (Meng et al., 2009). The microbial oil produced by *Rhodotorula toruloides* contains primarily oleic (around 48%) and palmitic (around 33%) acids simulating palm oil composition. The fatty acid profile of lipids produced by *Lipomyces starkeyi* contains around 30% palmitic and 55% oleic acid, a composition quite similar to palm oil (Li et al. 2007).

Research has focused on the improvement of microbial oil production efficiency (Leiva-Candia et al., 2014, Papanikolaou et al., 2013, Shen et al., 2013) and evaluation of its cost-competitiveness (Koutinas et al., 2014b). The production cost of purified microbial oil at an annual production capacity of 10,000 t, a bioreactor productivity of 0.54 g/(L·h), a TDW of 106.5 g/L and microbial oil content of 67.5% (w/w) has been estimated at $3.4 per kg when the cost of glucose is negligible (Koutinas et al., 2014b). The unitary cost of purified microbial oil production at negligible expenditure for the carbon source could be closer to that of vegetable oils at bioreactor productivities higher than 2.5 g/(L·h) (Koutinas et al., 2014b). Increasing the price of glucose to $400 per kg corresponds to a microbial oil production cost of $5.5 per kg and therefore higher bioreactor productivities should be achieved in order to create a cost-competitive process.

The great majority of microorganisms including prokaryotes and eukaryotes can produce lipids for regulal cellular metabolism and structural purposes. Origin of microbial oil dates back to 1985 when first microbial oil was produced from *Mucor circinelloides (*Yellapu et al., 2018). Since then, many microbes including microalgae, yeasts (i.e. *Candida, Cryptococcus, Lipomyces, Rhodotorula, Rhodosporidium, Trichosporon*), fungi (i.e. *Mortierella, Cunninghamella*) and bacteria (i.e. *Rhodococcus, Mycobacterium*) have been discovered and investigated for their ability to accumulate high amounts of microbial oil (Tsouko et al., 2016). The strain *Lipomyces starkeyi* has demonstrated the potential to satisfy the required criteria to achieve industrial implementation. Lin et al. (2011) reported the production of a TDW of 104.6 g/L with a microbial oil content of 64.9% (w/w) at a

productivity of 1.2 g/(L·h) via fed-batch bioreactor cultures of *L. starkeyi* AS 2.1560 using glucose-based mineral medium. Tapia et al. (2012) carried out fed-batch bioreactor cultures of a mutant strain of *L. starkeyi* DSM 70296 cultivated on mixtures of glucose and xylose to achieve a TDW of 88.7 g/L with a microbial oil content of 55.2% (w/w) at a productivity of 0.29 g/(L·h). Among the various oleaginous microorganisms, *Rhodosporidium* species have been widely investigated for microbial oil production via the valorization of an ample range of carbon sources (Xu and Liu, 2017). Specifically, *R. toruloides* exhibits advantages over other oleaginous yeasts, including rapid proliferation and high lipid accumulation on low-cost resources (Park et al., 2018). Leiva-Candia et al. (2015) reported a microbial oil production ranging between 18.1 - 19.2 g/L using different sunflower meal hydrolysates, deriving from the fractionation of sunflower meal, along with crude glycerol as fermentation supplements.

2.4. Agroindustrial substrates for microbial oil production

Microbial oil could be applied in many sectors, as a substiture of plant oils. For instance, it can be used as alternative raw materials for the production of biofuels and oleochemicals (Leiva-Candia et al., 2015; Paulino et al., 2017; Soccol et al., 2017). Nevertheless, the main problem is the higher production cost of microbial oil compared to vegetable oils. The solution seems to be the cost reduction through the use of waste streams as fermentation media, especially in the case that integrated biorefineries are developed for the production of various end-products. It should be highlighted that the development of biorefineries based on the utilisation of low-cost substrates for lipid production, aiming to reduce production cost and eliminate the disposal of agro-industrial wastes in the environment, has become a necessity towards the implementation of a circular economy (Athenaki et al., 2017).

Various agro-industrial substrates have been utilised by oleaginous microorganisms for microbial oil production as it is presented in Table 2.1. Tsouko et al. (2016) has reviewed various fermentation schemes for microbial oil production. Besides the utilization of pure substrates, indicative examples of the used renewable materials are the sugar-based or sugar-enriched wastes, molasses, vegetable oils, crude-waste industrial hydrophobic materials (e.g. industrial free-fatty acids, waste fats, crude fish oils, soap-stocks etc) or even biodiesel by-products.

The conversion of waste streams into fermentation media require the development of advanced upstream processing strategies that exploit the full potential of complex biological

entities. The renewable resources shall contain all the necessary nutrients for microbial growth and oil accumulation. 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 (e.g. protein-rich rapeseed or sunflower cakes), meat-and-bone meal, sewage sludge, 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 (e.g. waste bread, whey). 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 (Evans and Ratledge, 1984).

Table 0-1 Literature-cited results on microbial oil production using pure sugars and renewable resources.

Microorganism	Cultivation mode	Carbon source	Total dry weight (g/L)	MO content (%, w/w)	Productivity (g L^{-1} h^{-1})	Reference
Yeast species						
Yarrowia lipolytica	single-stage continuous	Glucose	9.2	25	0.08	Aggelis and Komaitis, 1999
Yarrowia lipolytica		Crude glycerol	8.1	43	0.11	Papanikolaou and Aggelis, 2002
Yarrowia lipolytica	shake flask	Stearin	15.2	52	N.M.	Papanikolaou et al., 2007
Candida sp. 107	single-stage continuous	Glucose	18.1	37.1	0.4	Gill et al., 1977
Candida curvata	single-stage continuous	Glucose	13.5	29	0.16	Evans and Ratledge, 1983
		Sucrose	16	28	0.18	
		Lactose	18	31	0.22	
		Xylose	15	37	0.27	
		Ethanol	11.5	35	0.2	
Apiotrichum curvatum	batch	Glucose	14.5	45.6	N.M.	Hassan et al., 1993
Apiotrichum curvatum	batch	Whey	21.6	36	0.119	Ykema et al., 1988
	recycling		85	35	0.372	
	continuous		20	36	0.382	
	partial recycling		91.4	33	0.995	

Cryptococcus curvatus	fed-batch	Glycerol	118	25	0.59	Meesters et al., 1996
Lipomyces starkeyi	shake flask	Glucose & Xylose	20.5	61.5	N.M.	Zhao et al., 2008
Lipomyces starkeyi		Glucose & Sewage sludge	9.4	68	N.M.	Angerbauer et al., 2008
Lipomyces starkeyi	fed-batch	Glucose	153	54	0.59	Yamauchi et al., 1983
Trichosporon fermentans	shake flask	Glucose	24.1	56.6	N.M.	Zhu et al., 2008
		Sucrose	19.5	62.6	N.M.	
		Xylose	17.1	57.8	N.M.	
		Lactose	16.9	49.6	N.M.	
		Fructose	21.5	40.7	N.M.	
		Molasses	36.4	35.3	N.M.	
Trichosporon fermentans	shake flask	Mannose	22.7	50.4	N.M.	Huang et al., 2009
		Galactose	23.6	59	N.M.	
		Cellobiose	15.8	65.6	N.M.	
Rhodosporidium toruloides	fed-batch	Glucose	106.5	67.5	0.54	Li et al., 2007
Rhodotorula gracilis	continuous	Glucose	9.60	49.8	0.096	Choi et al., 1982
Rhodotorula glutinis	shake flask	Monosodium glutamate wastewater	25	20	N.M.	Xue et al., 2008

Rhodotorula glutinis	fed-batch	Glucose	185	40	0.88	Pan et al., 1986
Rhodotorula glutinis	shake flask	Crude glycerol	8.17	52.91	0.058	Saenge et al., 2011a
	fed-batch		13.77	60.70	0.116	
Rhodosporidium toruloides	batch in fermenter	Pure glycerol	18.8	58.7	N.M.	Xu et al., 2012
	5 L fermentor	Crude glycerol	26.7	69	N.M.	
Pichia kudriavzevii	26 L fed-batch bioreactor	Crude glycerol	32.1	23	0.05	Sankh et al., 2013
Rhodosporidium toruloides	batch fermenter	Pure glycerol	43	45.8	0.164	Kiran et al., 2013
	fed-batch	Crude glycerol	31.1	41.7	0.108	
Rhodosporidium toruloides	fed-batch bioreactor	Crude glycerol	37.4	51.3	0.17	Leiva-Candia et al. 2015
Cryptococcus curvatus	fed-batch bioreactor	Crude glycerol	34.6	52.9	0.11	Leiva-Candia et al. 2015
Candida freyschussii	fed-batch bioreactor	Crude glycerol	82	34.15	0.28	Raimondi et al., 2014
Rhodosporidium toruloides	fed-batch bioreactor	Crude glycerol	24.9	45	N.M.	Yang et al., 2014

Yarrowia lipolytica	7 L fed-batch bioreactor	Glucose & VFAs	41.02	40.22	0.33	Fontanille et al., 2012
	7 L bioreactor-fed batch	Glycerol & acetic acid	40.93	38.43	0.33	
Lipomyces starkeyi	shake flask	Glucose & monosodium glutamate wastewater	4.6	24.7	0.01	Liu et al., 2012
Lipomyces starkeyi	shake flask	Glucose & fishmeal wastewater	17.6	15.3	0.01	Huang et al., 2011a
R. glutinis	5-L fermenter	Starch wastewater	60	30	0.3	Xue et al., 2010
	300-L fermenter		40	35	0.35-0.47	
Fungal species						
Cunninghamella echinulata	shake flask	Glucose	15	46	N.M.	Fakas et al., 2009
Cunninghamella echinulata	shake flask	Starch	13.5	28	N.M.	Papanikolaou et al., 2007
		Pectin	4.1	10	N.M.	
Mortierella isabellina	shake flask	Glucose	27	44.6	N.M.	Fakas et al., 2009
Mortierella isabellina	shake flask	Starch	10.4	36	N.M.	Papanikolaou et al., 2007
		Pectin	8.4	24	N.M.	

Mucor sp. RRL001	shake flask	Tarioca starch	28	17.8	N.M.	Ahmed et al., 2006
Mortierella ramanniana	commercial-scale batch bioreactor	Glucose	62	46.1	N.M.	Hiruta et al., 1997

The table was adapted from Tsouko et al., (2016).
N.M.: not mentioned

2.4.1. Dairy industry wastes

Many other waste and by-product streams from the food industry could be used for the production of microbial oil, such as sewage sludge and wastewaters. Whey constitutes a significant waste stream from the dairy industry and its valorisation is an important environmental target. The yeast *Cryptococcus curvatus* fermented cheese whey resulting in microbial oil content of around 60% (w/w) (Ratledge 1991). Other studies have evaluated pretreated cheese whey or deproteinized whey permeate treated with galactosidase utilizing *Cryptococcus curvatus* and *Mortierella isabellina* respectively. The results showed high microbial oil accumulation reaching a content of 65% (w/w) and a lipid productivity of 4.68 g/L/d was reported in the case *Cryptococcus curvatus*, whereas a lipid concentration of 17.1 g/L was achieved from Mortierella isabellina (Seo et al., 2014; Demir et al., 2013).

2.4.2. Sugar refining industry wastes

The sugarcane process generates significant quantities of side streams, predominantly molasses and bagasse. About 3 t of bagasse are produced per 10 t of sugarcane processed, whereas it is estimated that from 100 t of sugarcane around 3 - 7 t of molasses are generated (Gharib-Bibalan, 2018). Molasses is considered one of the most economically important by-products of sugar industries. It is a viscous and dark sugar-rich syrup, which remains after the final crystallization stage and separation of raw sugar as a result of a circular path of washing and heating the cane sugar with hot water. The sugar-rich composition of molasses along with its significant content in proteins, minerals, B group vitamins and antioxidants, among others (Gharib-Bibalan, 2018; Veana et al., 2014), suggests it as a good substrate for microbial fermentations. More specifically, fermentation of molasses by *Trichosporon fermentans* resulted in a total dry weight of 36.4 g/L with a microbial oil content of 35.3% (w/w) (Zhu et al., 2008). In the case of *Candida tropicalis*, *Candida lipolytica* and *Rhodotorula mucilaginosa* yeasts trains the respective lipid contents of 59.9% (w/w), 46.8% (w/w) and 69.5% (w/w) were achieved (Karatay and Dönmez, 2010). In addition, the cultivation of *Trichosporon capitatum* on cane molasses using a 10% total sugar concentration led to a total dry biomass of 17.3 g/L and a lipid content of 37.6% w/w (Wu et al., 2011).

2.4.3. Other industrial wastes

Shrimp processing wastes (e.g. heads, shells) are rich in chitin, a natural polysaccharide consisting of repeated units of N-acetyl-glucosamine (NAG). Chemical or enzymatic processes may be applied to obtain this amino sugar (NAG) to be further utilised as carbon source for the production of microbial oil, as it has been proposed by Zhang et al. (2011). *Cryptococcus curvatus* produced 18.78 g/L biomass concentration, during the growth phase, but the accumulation of oil was induced at the death phase in the absence of NAG, presenting a maximum lipid content of 28.4% (w/w).

Olive oil mill wastewaters (OMWs) have been evaluated also as feedstocks for fermentation (Yousuf et al., 2010). *Lipomyces starkeyi* achieved 28.6% (w/w) lipid content using 50% diluted OMWs. Several other microorganisms have been reported to produce lipids from OMWs, such as *Yarrowia lipolytica* and *Zygomycetes*, with a simultaneous removal of color and phenolic compounds (Sarris et al., 2011; Bellou et al., 2014). Interestingly, the addition of OMWs stimulated lipid accumulation in the microorganisms tested (Sarris et al., 2011). Palm oil mill effluent (POME), the waste stream produced by palm oil production processes, has also been evaluated for the production of oil using the oleaginous red yeast *Rhodotorula glutinis* (Saenge et al., 2011b).

Rhodotorula glutinis has produced significant amounts of lipids in starch-derived wastewater. In particular, a 5 L bioreactor fermentation resulted in 60 g/L of biomass with a 30% (w/w) lipid content, whereas cultivation in a 300 L bioreactor led to the production of 40 g/L of biomass and 35% lipid content (Xue et al., 2010). Municipal wastewater has also been evaluated for the production of lipids by *Cryptococcus curvatus*, *Yarrowia lipolytica*, and *Rhodotorula glutinis*, but the lack of micronutrients resulted in low biomass and lipid yields (Chi et al., 2011). Similarly, *Lipomyces starkeyi* was cultivated in fishmeal wastewater reaching the highest biomass concentration of 17.6 g/L and 2.7 g/L of lipid yield (Huang et al., 2011a).

2.5. Adding value to microbial oil

The need to replace petrochemically derived products with bio-based ones has paved the way for research and development towards vegetable oil utilisation for the production of oleochemicals due to their non-toxic and readily biodegradable nature (Böttcher et al., 2008, Buchholz and Bornscheuer, 2005, Metzger and Bornshcheuer, 2006). Moreover, worldwide

demand for natural fatty acids and glycerol, nowadays available as a crude by-product stream from biodiesel production processes using natural fats and oils, is expected to grow about 9.8% annually, from the current manufacturing value of $7.7 billion in 2011 to $13.5 billion through 2017, based on the expected continuous increase of prices of key vegetable oils and animal fats (Anonymous, 2014a, Anonymous, 2014b). The composition of microbial lipids is comprised of long chain saturated and unsaturated fatty acids, a fact that justifies their general acceptance as suitable starting material for biodiesel production (Vincente et al., 2009). Therefore, the utilisation of microbial lipids as precursors for oleochemical synthesis can be considered as a promising path, especially within a biorefinery concept.

Under the viewpoint of bio-economy transition, emphasis should be given to novel applications of microbial lipids for the production of lipid-based products with improved quality and specific applications (Papadaki et al., 2017). The production of food products with a limited amount of saturated fat has emerged to be of paramount importance for food industries during the last years. For instance, high unsaturated vegetable oils (i.e. soybean oil) are preferred for the production of oleogels, which are considered a healthier substitute for trans and saturated fats in food products (Mert and Demirkesen 2017). Bharathiraja et al. (2017) reviewed the application of microbial lipids in food formulations, stating how they could replace plant-derived lipids (e.g., cocoa butter, palm oil) and further utilized as stabilizing and thickening agents, emulsifiers, and water-binding compounds. Equally, given the high content of oleic acid in the produced lipids, it could be also used as a substitute for cocoa butter, based on the high content of unsaturated fatty acids.

Apart from texture, fatty acids can also regulate the aroma and flavor of specific types of food. Likewise, fatty acids can be implemented in pharmaceutical applications, and more specifically, to formulate fortified foods and/or beverages with polyunsaturated fatty acids, entailing possible health effects (Bharathiraja et al., 2017). Concerning microbial lipids from *R. toruloides*, Papadaki et al. (2019) conducted a study on the valorization of yeast lipid derivatives to formulate bio-based wax esters through a two-stage biocatalysis reaction. Implementation of molasses as fermentation supplement led to the production of 8.1 g/L microbial oil, containing mainly oleic acid (51%), palmitic acid, and stearic acid. The generated oleogels, using olive oil as the base oil, successfully simulated commercial margarine, thus demonstrating their potential for future applications in spreadable fat-products.

Furthermore, microbial oil has attracted increasing attention as a feedstock for biodiesel production due to characteristics, such as short growth cycle, low environmental footprint, high lipid content, and no requirement on agricultural land (Subramaniam et al., 2010). *R. toruloides* ranks among the most promising with biomass concentration and lipid content reaching respective values of 100 g/L and 70% (Li et al., 2007). It is also highly resistant to inhibitory compounds. Microbial oil-based biodiesel properties are inseparably dependent on microbial oil chemical composition and structure and more specifically unsaturation degree of the methyl-esters, number of double bonds, length and branching of the carbon chain (Tsouko et al., 2016). So far, numerous studies have demonstrated that microbial oil could be regarded as a potential raw material for biodiesel production. Soccol et al. (2017) reported a pilot-scale fed-batch strategy for biodiesel production from microbial oil produced by *Rhodosporidium toruloides*. A lipid productivity of 0.44 g/(L·h) was obtained using low-cost sugarcane juice and urea. Biodiesel showed very good performance with all properties being classified within ASTM standards and the blend B20 microbial oil, when operating at 2,500 rpm, presented lower pollutant emissions. In another study, *Trichoderma reesei* was cultivated in glucose and resulted in 30 g/L biomass production with a lipid yield of 32.4% and a saturation of 49.7%. Lipase-catalyzed transesterification showed a biodiesel production efficiency of 96.1%. The biodiesel properties satisfied ASTM and EN specifications (Bharathiraja et al., 2017). Li et al. (2008) claimed that microbial oil produced during fed-batch fermentations of *R. toruloides* could be converted into biodiesel with a cetane number (CN) higher than 51, which meets the minimal CN standards set by ASTMD 6751, DIN 51606 and EN 14214. Thiru et al. (2011) developed a low-cost substrate based on crude glycerol resulting in high cell density (69 g/L) fed-batch cultivation of *Cryptococcus curvatus* for the production of microbial oil (48%). Biodiesel was produced with a yield of 90% while properties were in good accordance with the international established standards. Leiva-Candia et al. (2015) estimated biodiesel properties produced from microbial oil derived from *R. toruloides*, *L. starkeyi* and *C. curvatus* cultivated on biodiesel by-product streams. The properties of FAMEs derived from the produced lipids were similar to biodiesel produced from palm oil. Large-scale production of biodiesel faces limitations and challenges. Some solutions should be urgently exploited and designed for future industrialization, including development of cheap carbon sources, screening and modification of oleaginous microorganisms, exploitation of high-efficiency cell wall breaking and oil extraction technology and development of novel catalysts and regeneration techniques.

Another field of considerable interest is biolubricants production from microbial oil derived from several yeast strains. Up to 55% of lubricants end up into the environment through evaporation, spillage and total-loss lubrication (Willing 2001). Conventional lubricants are petroleum-based (70-90%) and consequently toxic and non-biodegradable, causing huge damages to the ecosystem. The biodegradable and non-toxic nature of microbial oil in combination with good friction properties and low shearing stress makes it a potent candidate for bio-based lubricant production (Bandhu et al., 2018). Papadaki et al. (2018) evaluated the production of neopentyl glycol esters using microbial oil. Neopentyl glycol esters derived from lipids of *Rhodosporidium toruloides* and *Cryptococcus* curvatus showed respective conversion yields of 88% and 82.7% demonstrating their potential to substitute conventional lubricants. In recent years, research has focussed on the production of microbial oil rich in oleic acid. The global oleic acid market was 218.9 thousand t in 2013, with a revenue of $344.6 million. High-oleic acid oils present excellent thermal and oxidative stability and these characteristics made them suitable for the production of biolubricants and hydraulic fluids (Park et al., 2017). It is noteworthy, that only high-oleic vegetable oils are currently used for these purposes (Park et al., 2017). Hence, the production of high-oleic microbial oil offers the opportunity to produce biolubricants from renewable resources that do not compete food.

2.6. Microbial oil biosynthetic pathway

Microbial oil production by oleaginous microorganisms on sugar based and nitrogen limited media is a process consisted of two stages. During the growth phase, all nutrients are available for microbial cells formation and carbon sources are highly assimilated by the microorganisms. The produced lipids contain mainly polar fractions i.e sphingolipids, phospholipids as an indispensable part of their membrane. During the lipid accumulation phase, microbial oil mainly consists of neutral lipids (TAGs) while the uptake rate of carbon sources is slower compared to the previous phase (Fakas et al., 2006).

2.6.1. *De novo* lipid synthesis

De novo accumulation of intracellular lipids occurs in the case that microbial growth is carried out on sugar-based substrates. It is a secondary anabolic activity consisted of the intermediate cellular metabolism and the biosynthesis of TAGs (Figure 2.2) and it is

enhanced after the nitrogen depletion from the culture medium due to metabolic overflow at carbon excess conditions. The glycolysis pathway that microorganisms undergo for sugars degradation, generates pyruvic acid, which enters the mitochondrion matrix through the mitochondrial membrane. Acetyl-CoA either enters the Krebs cycle, or it is transported to the cytoplasm to give fatty acids (Papanikolaou and Aggelis, 2011).

In the oleaginous microorganisms, acetyl-CoA which is the precursor of fatty acids biosynthesis, derives from breakdown of citric acid that has been previously accumulated inside the mitochondria followed by its transportation into the cytosol (Fakas et al., 2008). Changes in the intracellular concentration of various metabolites after depletion of nitrogen into the fermentation substrate trigger lipid synthesis (Ratledge 1994). Nitrogen depletion causes an immediate decrease of intracellular adenosine monophosphate (AMP) AMP since, the microorganism cleaves it in inosine monophosphate and NH_4^+. The latter serves as an additional nitrogen source for synthesis of cell material after the extracellular nitrogen limitation (Evans and Ratledge 1985). Krebs cycle undergoes changes due to this tremendous reduction of intracellular AMP. Iso-citric acid is accumulated in the mitochondrion due to deactivation of NAD^+ isocitrate dehydrogenase, being in equilibrium with citrate. When citric acid reaches a critical value, citrate enters the cytoplasm in exchange with malate (Evans et al., 1983). Citric acid is cleaved by the ATP-citrate lyase (ATP-CL, key enzyme of lipid biosynthesis in oleaginous microorganisms) in acetyl-CoA and oxaloacetate. Fatty acids are generated by quasi-inverted b-oxidation reactions of acetyl-CoA (Evans and Ratledge 1985). In case that ATP-CL is absent, nitrogen exhaustion leads to accumulation of citric acid in the cytoplasm and its eventual excretion into the culture medium i.e citric acid production by *Aspergillus niger* or will inhibit 6-phosphoro-fructokinase resulting in the intracellular accumulation of polysaccharides (Papanikolaou and Aggelis, 2009).

2.6.2. Triacylglycerol synthesis

After the biosynthesis of fatty-CoA esters, reserve lipids are incorporated into TAGs via esterification with glycerol following the pathway of a-glycerol phosphate acylation. Initially glycerol-3-phosphate (G-3-P) is acylated to lysophospatidic acid (LPA) by G-3-P acyltranferase at the sn-1 position. LPA is further acylated to phosphatidic acid (PA) by LPA acyltransferase in the sn-2 position. PA is dephosphorylated by phosphatidic acid phosphohydrolase (PAP) to release diacylglycerol (DAG). DAG is acylated either by

diacylglycerol acyltransferase or phospholipid diacylglycerol acyltransferase to produce TAGs. Considering the structure of TAGs, in the case of the oleaginous microorganisms, the glycerol sn-2 position is mostly occupied by unsaturated fatty acids (Ratledge and Wilkinson 1988; Papanikolaou and Aggelis 2011a).

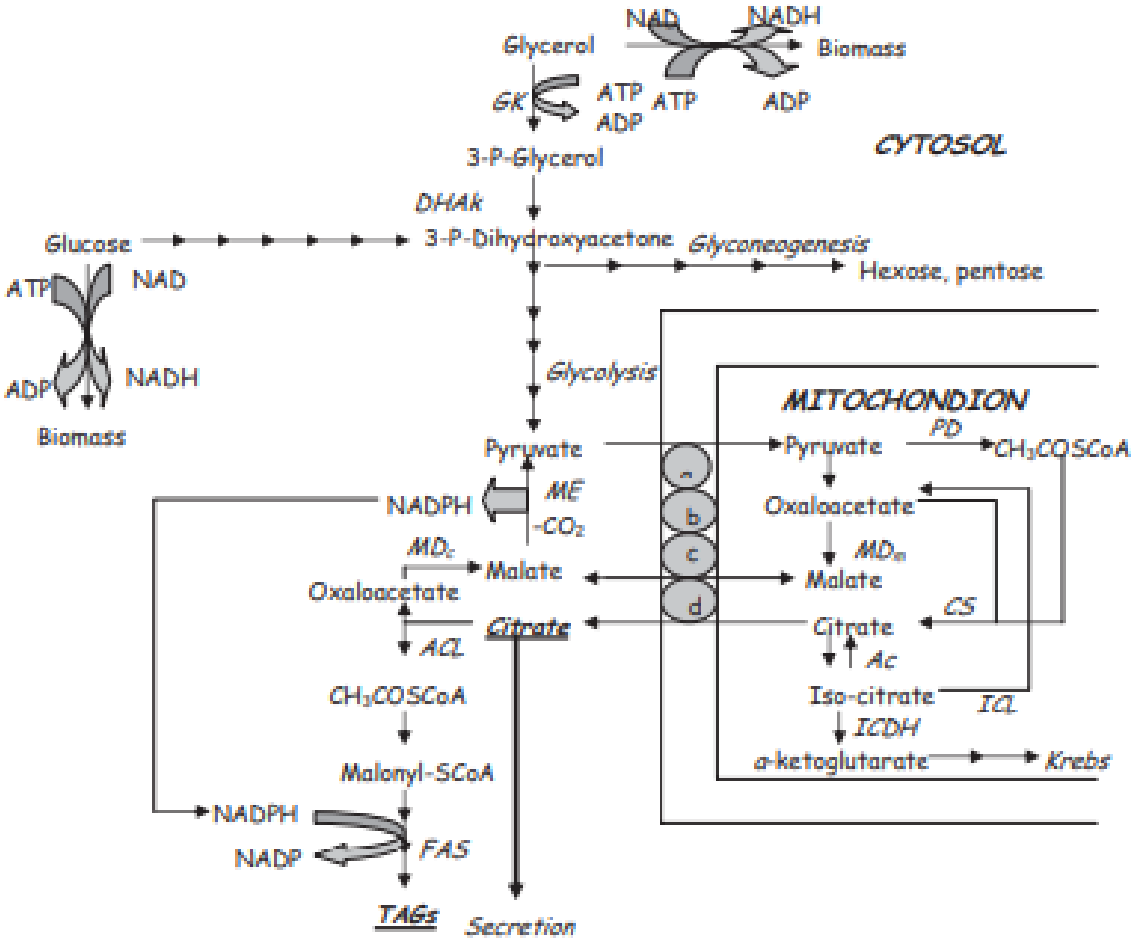

Figure 0-2 Pattern of intermediate metabolism and *de novo* fatty acid biosynthesis (TAGs: triacylglycerol; a, b, c: systems transporting pyruvic acid from cytosol to mitochondrion and inversely; d: system transporting citric and malic acid from cytosol to mitochondrion and inversely; ACL: ATP-citrate lyase; FAS: fatty acid synthetase; ICDH: iso-citrate dehydrogenase; MDc: malate dehydrogenase (cytoplasmic); MDm: malate dehydrogenase (mitochondrial); PD: pyruvate dehydrogenase; CS: citrate synthase; ICL: iso-citrate lyase) (Papanikolaou and Aggelis, 2011)

2.6.3. Degradation of microbial oil in oleaginous microorganisms

Oleaginous microorganisms tend to consume the produced intrecellular oil in cases of exhaustion or decrease of the carbon sources present in the fermentation environment. In fact, reserve lipid turnover generally occurs when the extra-cellular flow rate of aliphatic chains is considerably decreased. TAG lipases and STE hydrolases are activated for the utilization of TAGs and steryl-esters (STEs) from lipids via the cleavage of the esters and the generation of fatty acids that will be subsequently catabolized by β-oxidation (Wältermann et al., 2000). Reserve lipid turnover is accompanied by formation of lipid-free material suggesting that untracellular lipids act as precursor in the biosynthesis of cell materials (Papanikolaou and Aggelis 2011a). In the case that *de novo* lipid accumulation has been proceded, lipid turnover is principally performed through the glyoxylic acid by-pass pathway, while cellular nitrogen is responsible for the biosynthesis of new lipid-free material. Degradation of lipids when *ex novo* accumulation had been previously performed is conducted exclusively through the glyoxylic acid anaplerotic by-pass pathway (Papanikolaou and Aggelis 2011a).

2.6.4. *Ex novo* lipid synthesis

There are limited yeast strains that can grow on hydrophobic materials i.e fats, vegetable oil, soap stocks, fatty esters and simultaneously accumulate considerable intacellular lipids. *Ex novo* lipid production occurs when fats or other hydrophobic compounds are utilized as sole carbon and energy sources by microorganisms (Papanikolaou and Aggelis 2011b).

Ex novo lipid production presents greta alterations in a biochemical level as compared to *de novo* synthesis. Fat-based materials, after their enzymatic hydrolysis to free fatty acids, enter the microbial cell via active transportation and they are utilized either for microbial growth (energy for cell growth and maintenance) or for the synthesis of intracellular fatty acids. They are degraded, by virtue b-oxidation reactions, to smaller acyl-CoAs and acetyl-CoA chains providing intermediate metabolites necessary for the formation of cellular products (Papanikolaou and Aggelis 2003). Accumulation of reserve lipid occurs simultaneously with cell growth, in the presence of assimilable nitrogen in the fermentative environment. Fatty acid synthetase and ATP-citrate lyase (ACL) are inhibited by exogenous aliphatic chains and consequently there is no *de novo* lipid synthesis from the acetyl-CoA (Papanikolaou et al., 2007).

2.7. Downstream separation and purification process of microbial oil

The so far commercial microbial oil production includes the production of high added-value oils rich in polyunsaturated fatty acids for nutritional purposes. The production of microbial oil resembling plant oils is not efficient from the economic point of view to reach commercialization (Leong et al., 2018). The obstacles are mostly related to the extraction and purification of microbial oil, high energy requirements and labor cost (Gorte et al., 2020). Consequently, efficient cell disruption methods need to be developed for successful lipids recovery.

The downstream separation and purification (DSP) process for microbial oil recovery starts with biomass separation from the fermentation broth. Numerous separation methods have been investigated including centrifugation, coagulation, filtration, and flotation, thus the majority of them presents high cost. More particularly, centrifugation of the culture media to separate microbial biomass from liquid phase accounts for 20 - 30% of the total production cost (Dickinson et al., 2017).

The extraction of lipids from microbial cells constitutes a two-step procedure: the disintegration of cells by pretreatment methods applying physical, chemical or enzymatic techniques followed by lipid recovery with organic solvents from the biomass i.e a non-polar solvent (chloroform) and a polar solvent (methanol) to extract the lipids from dry biomass according to protocols proposed by Folch et al. (1957) and Bligh & Dyer (1959) (Figure 2.3). These DSP techniques can be applicable only at a lab scale.

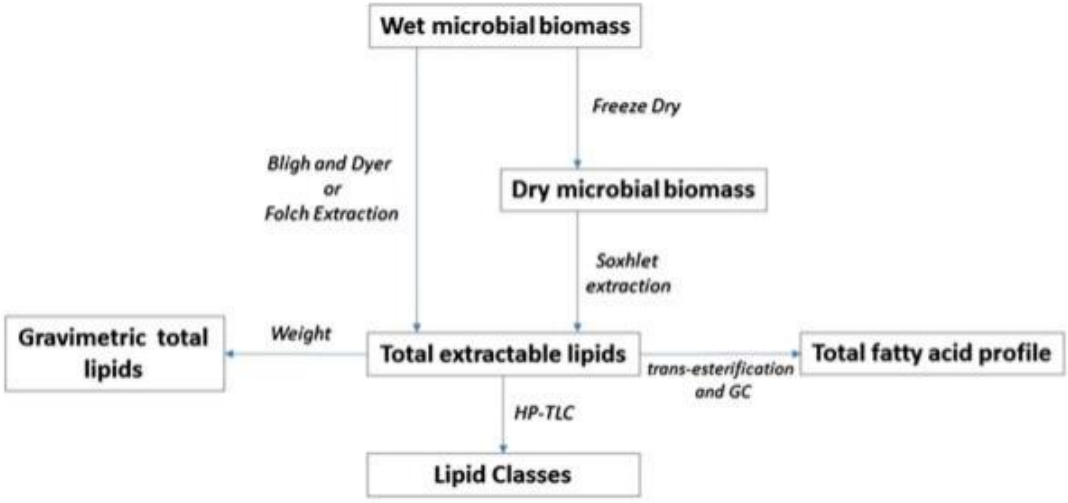

Figure 0-3 Schematic presentation of a typical analytical DSP protocol for the recovery and characterization of microbial oil (Meullemiestre et al., 2015).

In Figure 2.4, the available cell disruption techniques are presented. The first classification is the mechanical or non-mechanical method. Mechanical methods i.e bead milling and ultrasonication show good performance at an industrial level. They have been effectively applied in several microbial strains including algae (Lee et al., 2010), yeasts (Channi et al., 2016; Zhang et al., 2014) and fungi (Klimek-Ochab et al., 2011).

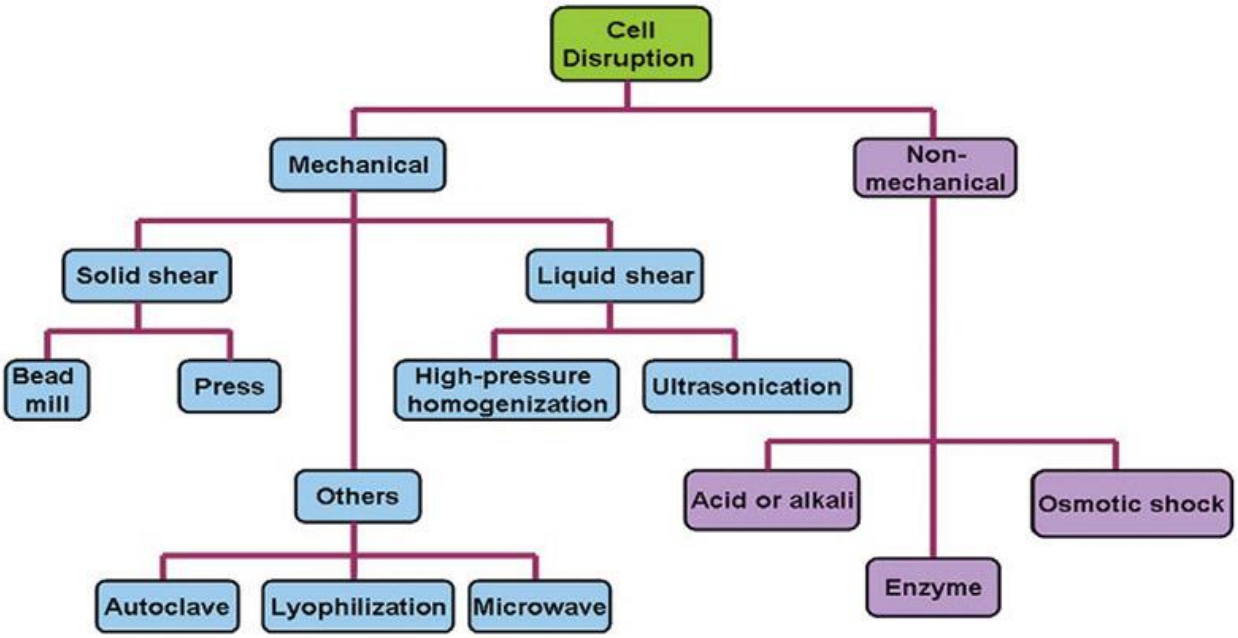

Figure 0-4 The classification of the cell disruption techniques.

Non-mechanical cell disruption is divided into physical (microwave-assisted lipid extraction and elecrophoration) and chemical techniques. Microwave-assisted lipid extraction could be effective in terms of yield and cost thus applicability in this field is hampered due to heat and free radical creation, damaging eventually PUFAs (Günerken et al., 2015). Elecrophoration is characterized by low energy requirements rendering it applicable in large-scale practice. It has been implemented for lipid extraction from cyanobacteria (Liu et al., 2011) and microalgae (Flisar et al., 2014) with microbial oil recovery up to 50%. Surfactant-assisted lipid extraction is a highly efficient method normally applied in wet biomass and it employs eco-friendly and biodegradable chemicals. Efficiencies varying between 82 - 100% have been reported when a variety of surfactants were used for the extraction of lipids from several algal species (Yellapu et al., 2018; Wu et al., 2017). Supercritical fluid extraction mostly with CO_2 is a promising technique due to the fact that CO_2 is a gas of low viscosity, high diffusivity and proper critical temperature and pressure. Notwithstanding CO_2

extraction is energy intensive, high extraction efficiency up to 99% has been reported in microbial oil obtained by the yeast strain *Rhodotorula glutinis* (Duarte et al., 2017).

2.7.1. Enzyme-assisted cell lysis

The biological pretreatment of microbial cells is a greener approach for cell disruption and microbial oil extraction than the chemical techniques. The use of enzymes as the biological catalysts differs from chemical catalysts, for instance the enzymatic reactions require mild-reaction conditions in terms of pH, temperature or pressure combined with short duration, eco friendly nature and relatively low energy requirements (Puri et al., 2012). Enzymes present also higher specificity enabling the regulation of catalysis by changing reaction parameters, such as product and substrate concentration, pH and temperature (Meullemiestre et al., 2015).

As can be observed in the schematic presentation of enzyme-assisted extraction (Figure 2.5), the enzymes modify the solid surface of cell and the intracellular oil is being released. Actually, enzymes hydrolyze the structural polysaccharides of the cell wall as well as the proteins associated with lipid bodies. In practice, the use of consortia of enzymes results in better cell wall lysis than a single enzyme. The enzymatic pretreatment of microbial biomass is highly dependent on the components of the cell wall of each individual oleaginous microorganism (Patel et al., 2018). Various degrading enzymes are required including xylanase, cellulase, amylase, pectinase, and hemicellulase (Sowbhagya et al., 2010). Meullemiestre et al. (2015) also reported that the enzymatic extraction of lipid from microorganisms, enzymes like cellulase, snailase (a complex of more than 30 enzymes, including cellulase, hemicellulase, galactase, proteolytic enzyme, pectinase, β-glucuronidase etc.), neutral protease, alkaline protease and trypsin are required.

Up to date there are few attemps employed for microbial oil recovery using the enzyme-assisted technique. *R. toruloides* biomass was treated with the recombinant β-1,3-glucomannanase plMAN5C, resulting in around 96.6% lipids recovery using ethyl acetate at room temperature and atmospheric pressure without dewatering (Jin et al., 2012). The microalga *C. vulgaris* was treated with cellulases for 72 h achieving a hydrolysis efficiency of the cell wall carbohydrates of 85.3% combined with high microbial oil recovery (Cho et al., 2013). In another study, enzymatic pretreatment of *L. starkeyi* cells was reported as non-effective for subsequent microbial oil recovery, due to the the presence of sulfide bonds in

the cell wall enhancing strength and rigidity of organelles (Bonturi et al., 2015). Zhang et al. (2018) investigated cell disruption of the microalga *Scenedesmus* sp. applying various enzymes, i.e cellulase, xylanase and pectinase. After optimization of enzymatic concentration, temperature, pH and incubation time, it was demonstrated that the combination of cellulase, xylanase, and pectinase for 190 min, enhanced the lipid extraction yield by 96.4% compared to the untreated biomass. Another oleaginous marine microalga, *Nannochloropsis* sp., was treated with cellulase and mannanase resulting in the improvement of lipid extraction yields from 40.8% to over 73% (Maffei et al., 2018).

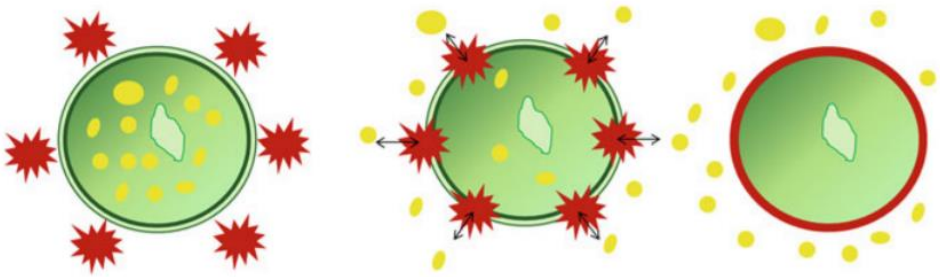

Figure 0-5 Schematic presentation of enzymatic-assisted extraction (Meullemiestre et al., 2015).

All extraction methods considered so far either employing a green extraction process or using organic solvents are dependent on the lipid composition, types of lipid fraction and their interaction with membrane protein. An efficient extraction practice should combine high recovery yields, limited contamination, and simplification of the downstream processing. Further researches should focus on the scalability and energy demands as well as the techno-economic analysis of the whole extraction process.

2.8. Biodiesel

The demand for energy independence, sustainable and clean energy supply is expected to propel the demand for biofuels worlwide. On account of higher mandates for biofuel blending in automotive fuels and increasing government support for eco-friendly alternatives, the global consumption of biofuel is expected to further grow at a significant level. Biofuels (ethanol and biodiesel) represent the majority share of renewables in global energy demand.

The current EU policy for renewable energy is established in the EU Energy and Climate Change Package (CCP) and the Fuel Quality Directive. One of the mandatory goals of the

Package is a 20% binding target for renewable energy in the overall energy mix of the EU. In the Renewable Energy Directive, specific sustainability requirements are defined for conventional (food based) liquid biofuels. On June 14, 2018, parties forged a political agreement committing member states to a new overall renewable energy target of 32% by 2030 (Anonymous 2018).

Biodiesel includes traditional biodiesel, fatty acid methyl ester (FAME) and hydrogenated vegetable oil. Biodiesel refers to a vegetable oil-, animal fat-, waste cooking oil-, and algae lipids-based diesel fuel consisting of long-chain alkyl (methyl, ethyl, or propyl) esters. Specifications for FAMEs based on EN 14214 standard are given in Table 2.2. Biodiesel is biodegadable and it is considered as an ideal source of renewable and clean energy as its carbon content is originally fixed from the atmosphere (Talebian-Kiakalaieh et al., 2013). The major obstacle of biodiesel commercialization is the price of feedstock, which accounts for around 70% of production cost, and production technology (Yan et al., 2014).

Table 0-2 Requirements for FAMEs specified by EN 14214 standard (www.biofuelsystems.com/biodiesel/specification.htm)

Property	Units	Limits
Density at 15°C	kg/m³	860-900
Viscosity at 40°C	mm²/s	3.5-5
Flash point	°C	>101
Cetane number	-	>51.0
Cu band corrosion (3h, 50 °C)	rating	Class 1
Oxidation stability (110 °C)	hours	>8
Acid value	mg KOH/g	<0.5
Iodine value	-	<120
Linolenic acid methylester	% (m/m)	<12
Polyunsaturated methylester		<1
Methanol		<0.2
Ester		>96.5
Sulfated ash content		<0.02
Monoglyceride		<0.7
Diglyceride		<0.2
Triglyceride		<0.2
Free Glycerine		<0.02
Total Glycerine		<0.25

Group I metals (Na+K)		<5
Group II metals (Ca+Mg)		<5
Water content	mg/kg	<500
Sulfur		<10
Total contamination		<24
Phosphorus		<4

The EU is the world's largest biodiesel producer representing about 75% of the total transport biofuels market. The structure of the EU biodiesel sector is very diverse and plant sizes range from an annual capacity of 2.3 million L owned by farmers to 680 million L owned by large multi-national companies (Figure 2.6) (Anonymous 2018). Rapeseed oil is the dominant biodiesel feedstock in the EU accounting for 45% of total production in 2017 followed by used cooking oil (21%) and palm oil (18%). The use of soybean and palm oil in biodiesel is limited by the EU biodiesel standard DIN EN 14214. Soybean-based biodiesel does not comply with the iodine value prescribed by the standard while palm oil-based biodiesel does not provide enough winter stability in northern Europe. Blend of both feedstocks could overcome these deficiencies and meet the standard requirements. Sunflower oil only comprised 1% of the total biodiesel feedstock (Anonymous 2018).

Figure 0-6 EU biodiesel producers (Anonymous 2017)

2.8.1. Biodiesel production processes

Utilization of edible oil for biodiesel production has been widely investigated in the wide literature. Nonetheless, the cultivation of arable land for the production of edible oil as biodiesel feedstock at the same time that food crisis becomes of high severity sets great limitations to this alternative. Another obstacle is the fact that large-scale plantation of vegetable oil leads to biodiversity causing problems to the compliance of the biodiesel with the specifications of Standards (Yan et al., 2014). Research has foced on the formulation of non-edible oil as biodiesel feedstock that do not compete with agro-land for crops, including microbial oil and waste oil.

Biodiesel can be produced via direct use of vegetable oil and blends, micro-emulsification, thermal cracking, and transesterification (Ma et al., 1998). Vegetable oil has high viscosity so its direct use as biodiesel could cause serious problem to the engine due to inability of complete combustion. Micro-emulsification and pyrolysis could enhance the properties of vegetable oil thus these practices are not effective for the formulation of a proper fuel. Transesterification (Figure 2.7) is the most commonly used method for large-scale production of biodiesel having advantages, such as gentle reaction conditions and wide oil feedstock feasibility (Yan et al., 2014). Catalysts for transesterification include bases, acids, and enzymes. Catalysis using bases is highly affected by moisture and free fatty acid contents of oils resulting in the formation of soaps and therefore low production efficiency and complicated separation of biodiesel. Acidic catalysis is applied in feedstocks that present high acid values. This process requires higher amount of alcohol, reaction temperature and pressure and slower reaction rate in comparison to the previous method (Sinha et al., 2008). Industrial practice for biodiesel production employs mostly base catalysts (Shahid and Jamal 2011).

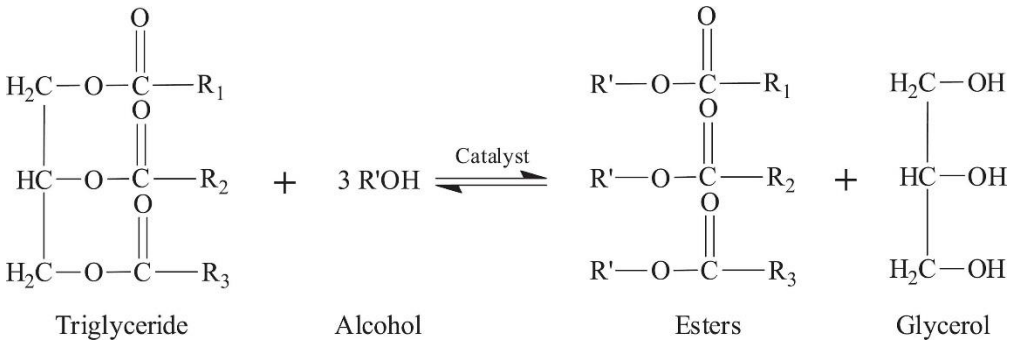

Figure 0-7 Transesterification reaction (Patel and Shah 2015)

By the aforementioned, it can be concluded that chemical catalysis is energy-intensive and faces problems with the recovery of glycerol and catalyst from the product, management of alkaline wastewater, and interference of free fatty acids and water in the reaction (Shahid and Jamal 2011). A highly promising alternative constitutes enzyme-catalyzed processes. Biocatalytic reactions involving mainly lipases (Figure 2.8), occur under mild conditions, are not energy intensive, offer simple dowstream separation of the product and show high selectivity. Large-scale application of enzymatic processes is so far limited due to the high cost of lipases. Thus, as lipases production is becoming viable with their reuse in immobilized form, these processes show great potential for biodiesel production (Yan et al., 2014).

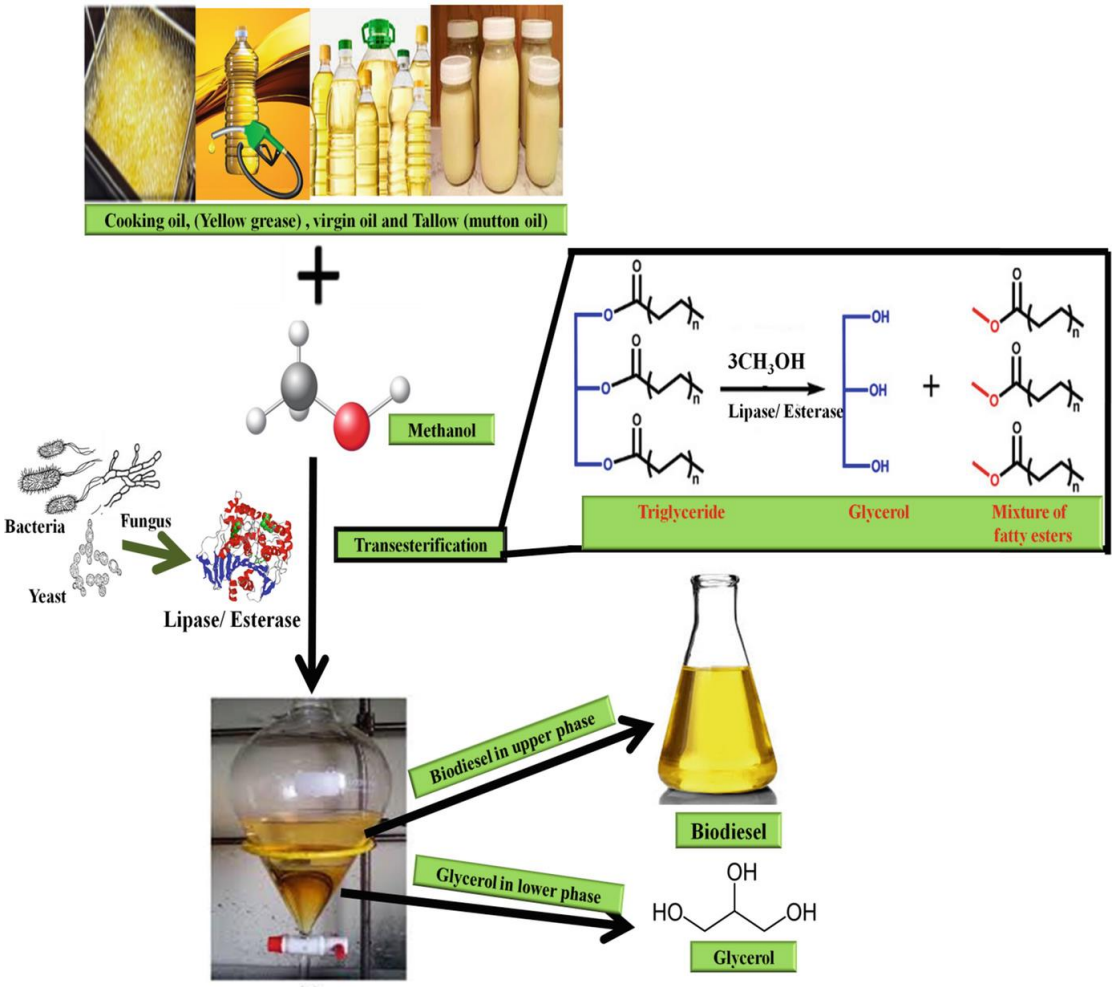

Figure 0-8 Biodiesel production through enzymatic catalysis (Sharma et al., 2019)

2.8.2. Biodiesel production from microbial oil: Properties and characteristics

Microbial oil produced by various microorganisms i.e. bacteria, yeast, fungi and microalgae have attracted increasing attention as a feedstock for biodiesel production due to characteristics such as short growth cycle, low environmental footprint, high lipid content, and no requirement on agricultural land cultivation (Subramaniam et al., 2010). Bacteria are less capable of producing lipids as they only can synthesize specific lipids, while microalgae, yeasts and fungi have been considered as key lipid producers (Ma et al., 2018). As it is depicted in Figure 2.9, biodiesel production can be produced following the conventional production route; collection of biomass, lipid extraction and transesterification of the extracted lipids, or by the direct method. In the later method, transesterification involves using directly the wet oleaginous biomass (Yousou et al., 2016).

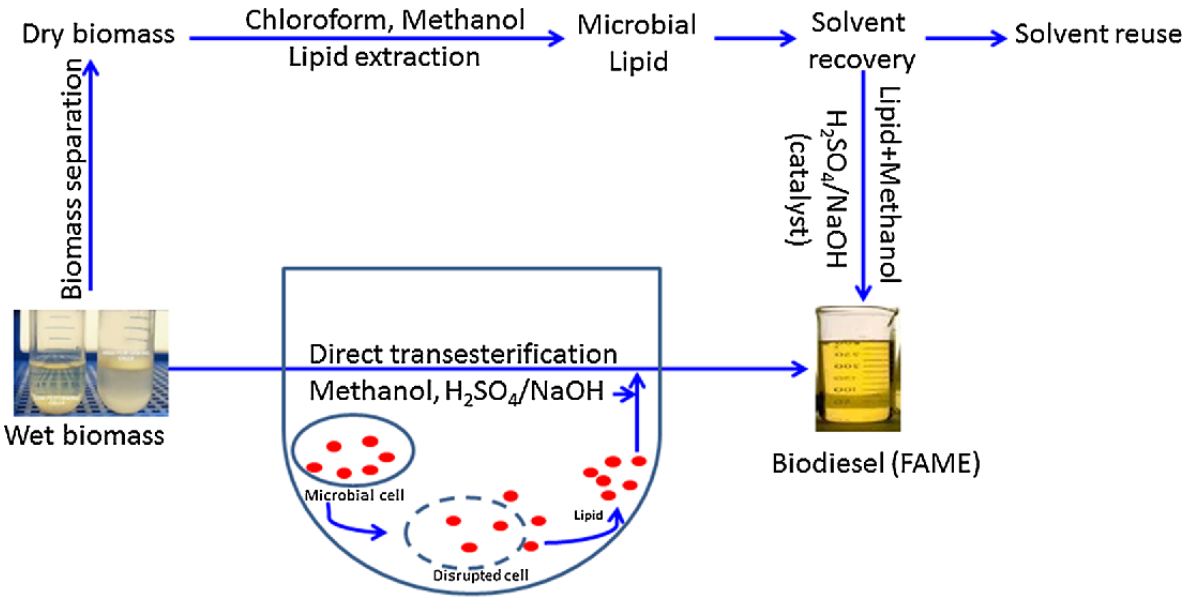

Figure 0-9 Conventional and direct transesterification methods for biodiesel production from microbial lipids (Yousouf et al., 2016).

Many oleaginous yeasts with high yields of intracellular lipids have been reported as favorable for biodiesel production including *Rhodotorula mucilaginosa* (Yen et al., 2016), *Rhodosporidium toruloides* (Gao et al., 2016), *Yarrowia lipolytica* (Mathiazhakan et al., 2016), *Lipomyces starkeyi* (Calvey et al., 2016), *Trichosporon fermentans* (Shen et al., 2013), *Trichosporon cutaneum* (Wang et al., 2016) and *Trichosporon oleaginosus* (Meo et al., 2017). The lack of polyunsaturated fatty acids in the lipids produced by these yeasts

render them suitable for biodiesel production. *R. toruloides* is one of the most promising yeast strains for biodiesel production (Li et al., 2007).

Microbial oil produced by yeast and fungal strains should be compared with vegetable oils in order to show the possibility of substitution of current raw materials used for biodiesel production. Microbial oil-based biodiesel should conform with the requirements of quality biodiesel standards ASTMD 6751 (USA), DIN 51606 (Germany) and EN 14214 (Table 2.1) (European Organization). Biodiesel properties are inseparably dependent on microbial oil chemical composition and structure and more specifically unsaturation degree of the methyl-esters, number of double bonds, length and branching of the carbon chain (Tsouko et al., 2016). Generally, viscosity increases with increasing length of the fatty acid chain while a decrease in chain length and an increase in unsaturation degree leads to higher densities. Cetane number increases with longer fatty acid carbon chains and more saturated molecules. Highly saturated fatty acids result in increased flash point and decreased iodine values (Jahirul et al., 2015). The characterization of biodiesel for its fuel performance is considered an expensive and time consuming process which lacks in reproducibility and requires high quantities of sample (Khot et al., 2018). Consequently, prediction and mathematical models as well as software packages have been developed to predict biodiesel properties from FAMEs composition (Tsouko et al., 2016).

Microbial oil is a renewable feedstock for biodiesel production without compromising food and water supply. It can be produced from various carbon sources by microorganisms, which also can be efficiently cultivated and harvested via assorted technologies and pathways. However, large-scale production faces limitations and challenges. Some solutions should be urgently developed and designed for future industrialization, including development of cheap carbon sources, screening and modification of oleaginous microorganisms, exploitation of high-efficiency cell wall breaking and oil extraction technology and development of novel catalysts and regeneration technique.

CHAPTER 3

Objectives

This PhD thesis focuses on the development of a biorefinery strategy towards the production of microbial oil, upon the valorization of flour-rich, wheat milling, bakery and confectionery wastes and side-streams (Figure 3.1).

Initially, an enzymatic bioprocess was developed based on wheat milling by-products, namely wheat bran, aiming at the production of a crude enzyme consortia. The fungal strain *Aspergillus awamori* was employed for solid state fermenttaion and the resulted crude proteases and amylases were further utilized for the hydrolysis of confectionery and bakery wastes. The hydrolytic process led to the formulation of nitrogen-rich and sugar-rich hydrolysates, which were evaluated as fermentation media for microbial oil synthesis by the oleaginous yeasts *Lipomyces strakeyi* and *Rhodosporidium toruloides.*

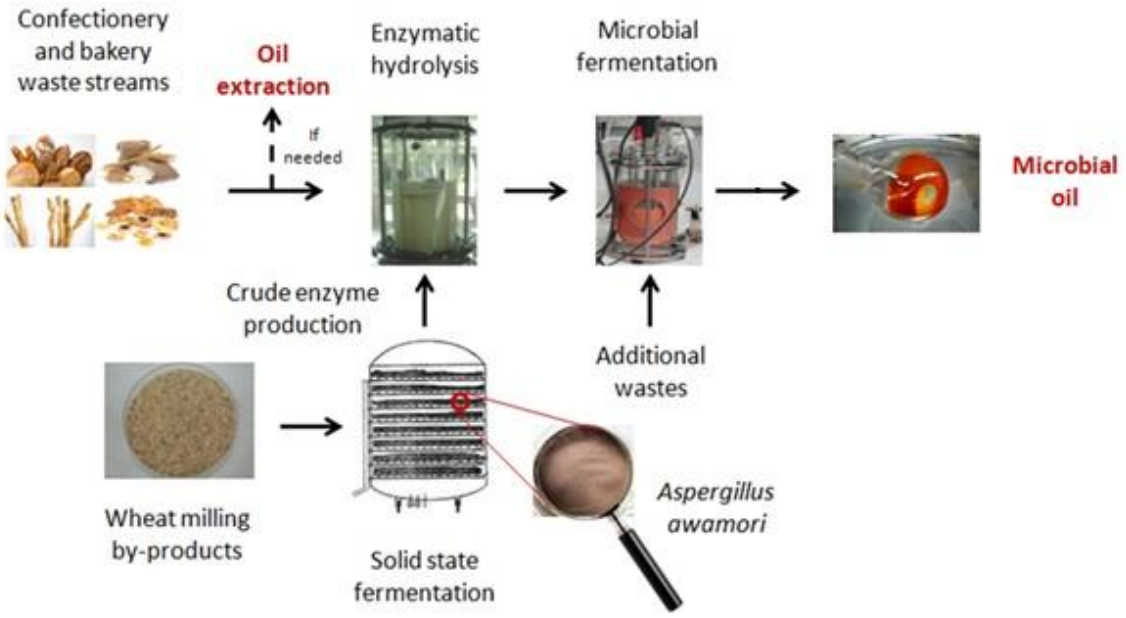

Figure 0-1 General biorefinery scheme for the production of microbial oil through the valorization of confectionery and bakery waste streams.

More specifically the main objectives of the experimental work are presented as follows:

- Identification of the most suitable valorization process of each wheat milling, confectionery and bakery side-stream based on the chemical composition of each one
- Development of a bioprocess for the production of crude fungal enzymes by *A. awamori* through solid-state fermentation. In this case, wheat bran was utilized as the fermentation medium, whereas the effect of initial moisture content and the solid substrate on enzyme production were evaluated.

- Development of an enzymatic hydrolysis process towards the formulation of nutrient-rich fermentation media containing both carbon and nitrogen sources. The hydrolytic process was based on the valorization of a) flour-rich wastes, b) confectionary side-streams and c) mixed confectionery side-streams.
- Optimization of the hydrolytic process of the selected food wastes
- Evaluation of fermentation conditions for microbial oil production using the oleaginous yeasts *L. starkeyi* and *R. toruloides.*
- Development of an enzymatic cell disruption methodology, applying the crude enzyme consortia produced by *A. awamori,* as an alternative and eco-friendly method to recover microbial lipids.
- Assess the production of biodiesel using the micobial lipids produced by *R. toruloides*.

CHAPTER 4

Materials and methods

4.1. Renewable resources

Wheat milling by-products (WMB) were used as substrate for solid-state fermentation of *A. awamori*. The starch, protein, phosphorus and moisture content of WMB used in this study were 12 %, 20 %, 1.1 % and 9.7 % (w/w), respectively.

Waste streams including mixed food for infants (MFI), mixed confectionery waste streams (MCWS), and flour-rich waste (FRW) were supplied by Jotis S.A., a Greek confectionery industry that produces a wide range of confectionery products and food for infants. The starch, protein and moisture content of FRW was (w/w) 84.8%, 7.3% and 5% respectively. FRW were tested as additional solid substrate in SSF to evaluate the potential to enhance the production of amylolytic and proteolytic enzymes. All waste streams were involved in enzymatic hydrolytic experiments aiming to produce a generic fermentation medium. MFI contained (w/w) 33% starch, 17% sucrose, and 27% lactose while a similar composition was determined for MCWS with (w/w) 32.3% starch, 16% sucrose, 27% lactose and 7% lipids. Lipids were extracted with n-hexane prior to utilization. Mixed waste streams (MWS) were formulated by mixing MFI:MCWS:FRW at a ratio of 1:1:1 and they presented a final composition of (w/w) 50% starch, 11% sucrose, and 18% lactose.

4.2. Microbial strains, pre-culture conditions and preservation

The fungal strains *Aspergillus awamori* 2B.361 U 2/1 was kindly provided by Professor Colin Webb (University of Manchester, UK), and originally obtained from ABM Chemicals, Ltd. (Woodley, UK). *A. awamori* was used for the production of crude enzyme consortia (mainly amylolytic) through solid-state fermentation (SSF), which were subsequently used in hydrolytic experiments. Spores were preserved in silica particles and their reconditioning was carried out using PBS solution containing 8 g/L NaCl, 0.2 g/L KCl, 1.4 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4. *A. awamori* was stored at 4 °C in agar slopes containing 5% (w/v) wheat bran (WB) and 2% (w/v) 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 5 days. Deionized water supplemented with Tween 80 was added in flasks with the grown fungus and after

vigorous shaking using glass beads of 4 mm diameter, a spore suspension of 2×10^6 spores/mL was obtained which was used as inoculum for SSF.

The oleaginous yeast strains *Lipomyces starkeyi* DSM and *Rhodosporidium toruloides* DSM 4444 (purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH) were employed in fermentations for microbial oil production and they were purchased from DSMZ culture collection. The strains were maintained at 4 °C on agar slopes, containing glucose (10 g/L), yeast extract (10 g/L), peptone (10 g/L) and agar (2%, w/v). A liquid medium of the same composition in glucose, yeast extract and peptone was used for the preparation of fermentation inocula and it was incubated at 28°C (*Rhodosporidium toruloides)* or 30°C (*Lipomyces starkeyi*) in an orbital shaker, at agitation rate of 180 rpm for 24 h.

In the case of *L.* starkeyi, it was observed that efficient microbial oil production in bioreactor cultures is highly dependent on the production of a dense inoculum. To achieve this in fed-batch bioreactor fermentations, pre-cultures were produced in FRW hydrolysates that led to improved fermentation efficiency.

4.3. Crude enzyme production via solid state fermentation

The crude enzyme consortia production was conducted through SSF in 250 mL Erlenmeyer flasks, which contained 5 g (dry basis, db) of substrate. SSF was carried out at 30 °C using various ratios of WMB to FRW (100:0, 90:10, 80:20, 70:30 and 60:40, on a weight basis), moisture content (50, 55, 60, 65, 70 and 75%, w/w) and fermentation duration to evaluate the production of glucoamylases and proteases. The moisture content of the substrate was adjusted by inoculating with the fungal spore suspension (2×10^6 spores/mL). The solid substrates were sterilized at 121°C for 20 min and after inoculation all cultures were incubated at 30°C. At regular intervals, the fermented solids were suspended in sterile water and macerated using a blender under aseptic conditions, to evaluate glycoamylase and protease activity during fermentation. The data represent the mean values of duplicate experiments.

4.4. Enzymatic hydrolysis process

4.4.1. Production of flour rich waste (FRW) hydrolysate

Optimum conditions found for SSF were applied to produce the crude enzyme consortia and to further utilize them to promote hydrolysis in FRW wastes. In particular, SSF were

macerated using a kitchen blender after suspension in 500 mL sterilised tap water. FRW hydrolysates were produced by mixing varying quantities of FRW streams with the enzyme-rich suspension in 1 L Duran bottles. Mixing of the suspension was achieved using magnetic stirrers. The effect of pH (3.5, 4, 4.5, 5, 5.5, 6, 6.5 and uncontrolled pH) and temperature (30, 40, 45, 50, 55, 60 and 65°C) was evaluated in two different sets of experiments. All subsequent hydrolytic experiments were carried out at the optimum temperature of 55 °C and uncontrolled pH conditions. FRW hydrolysis was optimized using different initial enzyme activities and five initial FRW concentrations (98, 140, 175, 205 and 235 g/L, on a wet basis). Four initial glucoamylase (0.24, 0.49, 0.73 and 0.97 U/mL) and protease (4.03, 8.06, 12.9 and 16.13 U/mL) activities were used. Hydrolytic experiments were carried out in both batch and fed-batch mode. In the latter case, FRW hydrolysis started by adding only 80% of the enzyme-rich suspension, while the remaining 20% was added at approximately 6 h. This approach enhanced the final starch to glucose conversion yield at FRW concentrations higher than 205 g/L.

Samples were collected at random intervals and the solids were separated via centrifugation (9,000 g for 10 min). The supernatant was filtered (Whatman No1) to remove insoluble materials and was subsequently used for the determination of glucose and free amino nitrogen (FAN) content. FRW hydrolysates used as fermentation media were initially filter-sterilised using a 0.2 μm filter unit (Polycap TM AS, Whatman Ltd.) and the pH of the hydrolysate was adjusted to the optimum pH value of 6.0 for yeast strains growth using 5 M NaOH.

4.4.2. Production of bakery and confectionery based hydrolysates

The fermented solids of five flasks containing solely WMB (after 3 days of incubation at 30°C) were suspended in 500 mL sterilized tap water and subsequently macerated using a kitchen blender. After centrifugation (9000 × g for 10 min), individual hydrolysis of mixed food for infants (MFI), (mixed confectionery waste streams) MCWS and (mixed waste streams) MWS at initial solids concentrations of 50 g/L, 100 g/L and 150 g/L was performed by adding the supernatant in 1 L Duran. The suspension was mixed employing magnetic stirrers, and enzymatic hydrolysis was carried out at 55°C and uncontrolled pH conditions. At the end of enzymatic hydrolysis, the produced hydrolysates were treated like the FRW hydrolysates, as described in the Section 4.4.1.

4.5. Microbial fermentations for lipids production

4.5.1. Batch shake flask fermentations

Shake flask fermentations were conducted in 250 mL Erlenmeyer flasks with a working volume of 50 mL. To ensure the reliability of microbial oil quantification, the content of a whole flask was used for each sample. Each shake flask was inoculated with 1 mL of a 24 h exponential pre-culture (around 1-3×10^7 cells per mL) as mentioned in Section 4.2. Flasks were incubated at 30°C (for *L. starkeyi*) or 27°C (for *R. toruloides*) in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at an agitation rate of 180 rpm. In all experiments it was desirable to maintain the pH in a value greater than 5.2, therefore an appropriate volume of KOH (5M) was periodically and aseptically added into the flasks when needed.

Shake flask cultivations of *L. starkeyi* were carried out using either FRW hydrolysate or a synthetic medium. In the latter case the composition of the medium was (in g/L): glucose, 105.0; yeast extract, 2.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.5; $MgSO_4 7H_2O$, 1.5; $FeCl_3 6H_2O$, 0.15; $ZnSO_4 7H_2O$, 0.02; $MnSO_4 H_2O$, 0.06; $CaCl_2 2H_2O$, 0.15.

Shake flasks of *R. toruloides* were conducted using commercial carbon sources based on the composition of hydrolysates obtained after enzymatic reactions, as indicators of the yeast performance in these hydrolysates. More specifically, commercial glucose, sucrose, fructose, and galactose were individually evaluated for yeast proliferation and microbial oil accumulation. Subsequently, shake flask cultivations were performed using the hydrolysates of the three waste streams, as described in Section 4.10.2.2

Samples were taken at regular intervals to assess sugar and nitrogen consumption along with biomass and lipid concentration.

4.5.2. Bioreactor fermentations

The bioreactor (New Brunswick Scientific Co, USA) employed in batch and fed-batch fermentations had a total volume of 3 L bioreactor and a working volume of 1.5 L. The temperature, aeration and pH value were controlled at 30°C (for *L. starkeyi*) or 27°C (for *R. toruloides*), 1 - 1.5 vvm and 6.0 by automatic addition of 5 M NaOH, respectively. A 10% (v/v) inoculum was employed using a 24 h exponential pre-culture. The agitation rate was controlled in the range of 150 - 500 rpm in order to maintain the dissolved oxygen concentration above 20% of saturation. FRW hydrolysates were used at the beginning of all batch and fed-batch fermentations as the sole source of nutrients.

In the case of *L. starkeyi*, fed-batch fermentation was initiated in batch mode and when the glucose concentration was reduced to less than 20 g/L a concentrated glucose solution (60%, w/v) was added in the bioreactor. Similar additions were performed up to 200 h fermentation when both TDW and lipid production were terminated.

In the case of *R. toruloides*, the first set of fed-batch fermentations focused on the optimisation of the initial carbon to FAN ratio (100.4, 80.2, 58.2, 47.2 and 31.9 g/g). The carbon corresponded to the carbon content in glucose, whereas the FAN corresponded to the nitrogen contained in the free amino groups of amino acids and peptides in the hydrolysate. Different FRW hydrolysates were mixed in order to obtain the appropriate C/FAN ratio in each fermentation. A glucose concentration in the range of 55 - 60 g/L was used at the beginning of all fed-batch fermentations. The initial FAN concentrations employed in the five fed-batch fermentations were 220, 294, 397, 492 and 682 mg/L. A concentrated glucose solution (70%, w/v) was used as feeding medium when the glucose concentration was reduced to less than 20 g/L. In one fed-batch fermentation, the feeding medium contained 70% (w/v) of glucose concentration and 1% (w/v) of yeast extract concentration, equivalent to around 500 mg/L of FAN concentration. The selected feeding mode was either based on consecutive pulses or targeted the maintenance of glucose concentration at approximately the same level. The duration of each fed-batch fermentation was up to 150 h when both TDW and lipid production were terminated. The second set of fed-batch fermentations was carried out using MFI and MCWS hydrolysates at the optimum C/FAN ratio of the first set of experiments. Fed-batch fermentation strategy was achieved by the periodic addition of a concentrated solution derived from each hydrolysate (70%, w/v). The feeding solution was added in the bioreactor under aseptic conditions to sustain microbial proliferation and microbial oil synthesis. Production of total dry weight (TDW) and lipid synthesis indicated the termination of the bioprocess, which lasted up to 120 h.

Samples were taken periodically from the bioreactor throughout fermentation. They were centrifuged (9,000×g for 10 min) to separate yeast cells from the supernatant. The supernatant was used for the analysis of glucose, FAN and inorganic phosphorus (IP), while the yeast biomass was used for the analysis of TDW, endo-polysaccharides and intracellular lipids. Fermentations were carried out in duplicate and the respective analyses in triplicate. Data presented are the mean values of those.

4.5.3. Disruption of the yeast cells

Yeast cell disruption of *Rhodosporidium toruloides* was investigated either via autolysis or enzymatic hydrolysis using the crude enzyme consortia produced via SSF of *A. awamori*. The disruption of yeast cells was identified by quantifying the dry weight at different reaction times and by analysing the FAN concentration in the reaction medium that was increased due to protein hydrolysis. The reduction of TDW and the increasing FAN concentration indicated yeast cell disruption and protein hydrolysis. The lipids were quantified in both the yeast cells and the aqueous suspension.

The yeast cells were harvested after the end of a batch fermentation in FRW hydrolysates. The initial yeast cell concentration in both autolysis and enzymatic hydrolysis reaction was 28.5 g/L with an intracellular lipid content of 32.28% (w/w). Autolysis of yeast cells was carried out at 50°C and uncontrolled pH. In the case of enzymatic hydrolysis of yeast cells, the enzyme suspension was produced following the same protocol presented above using an enzyme-rich aqueous extract that was produced by suspending SSF solids in 250 mL sterilised tap water. The initial protease activity in enzymatic cell lysis was 13.5 U/mL. Prior to initiation of enzymatic lysis of yeast cells, the fermentation broth was boiled at 100°C for 2 min, in order to inactivate endogenous enzymes and prevent autolysis of yeast cells. Then, the aqueous extract rich in crude enzyme consortia produced via SSF of A. awamori were added to the cell suspension and the mixture was incubated at 50°C under agitation at 200 rpm to achieve cell lysis. An additional sample of heat-treated cells without crude enzymes was used as control. After the end of incubation, the mixture was centrifuged (9000×g, 4°C, 10 min), the supernatant was removed and Folch solution was added to the cell debris. After centrifugation of the mixture (9000×g, 4°C, 5 min), the solvent phase was removed for the analysis of lipids and the residual cell debris was dried at 105°C for 24 h for the determination of lipid-free yeast mass. The lipids released in the liquid were analysed after removal of the water via lyophilisation and extracting the remaining lipids using a Folch solution.

4.6. Analytical methods

4.6.1. Determination of sugar content

The concentration of sugar content in fermentation broth and in hydrolysates was determined using a High Performance Liquid Chromatography (HPLC) (Shimadzu) equipped with a Bio-rad Aminex HPX-87H column (300 mm length x 7.8 mm internal diameter) coupled to a differential refractometer. The mobile phase was a solution of 10 mM H2SO4 with 0.6

mL/min flow rate and 65 °C column temperature. Monosaccharides were also determined with a Shodex SP0810 column at 60 °C with 1 mL/min flow rate of pure water as the mobile phase.
Calibration curves of the sugars were as follows:

- Sucrose: y = 6.50143e-006x, (R2= 0.9994)
- Glucose: y=7.2726e-006x, (R2=0.9996)
- Frucrose: y = 6.90429e-006x, (R2 = 0.9999)

4.6.2. Determination of free amino nitrogen and inorganic phosphorus

Free amino nitrogen (FAN) concentration was quantified 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 $Na_2HPO_4.2H_2O$, 5 g/L ninhydrin and 3 g/L fructose were dissolved in deionized water and mixed. Around 60 g/L KH_2PO_4 were gradually added to the mixture until a pH of 6.6-6.8 was achieved.
Dilution reagent: 2 g of KIO_3 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 the calibration curve of Figure 4.1 (y=4.431x-0.0331, R^2=0.9993) was determined by diluting glycine standard solution to obtain different concentrations of FAN (0.5, 1, 1.5 and 2 mg/L).

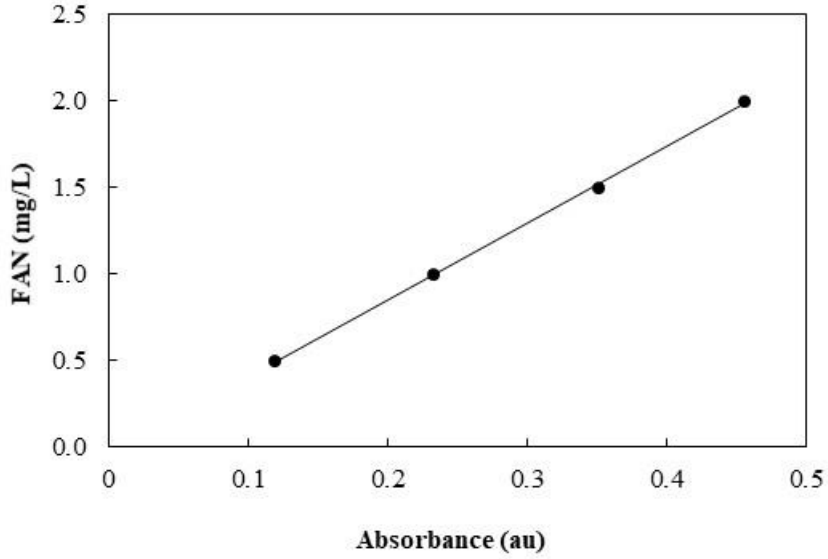

Figure 0-1 Calibration curve of free amino nitrogen colorimetric method.

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 colour 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 of Figure 4.2 ($y=12.156x+0.1666$, $R^2=0.9975$) after dilutions to obtain different IP concentrations (1, 2, 5, and 8 mg/L).

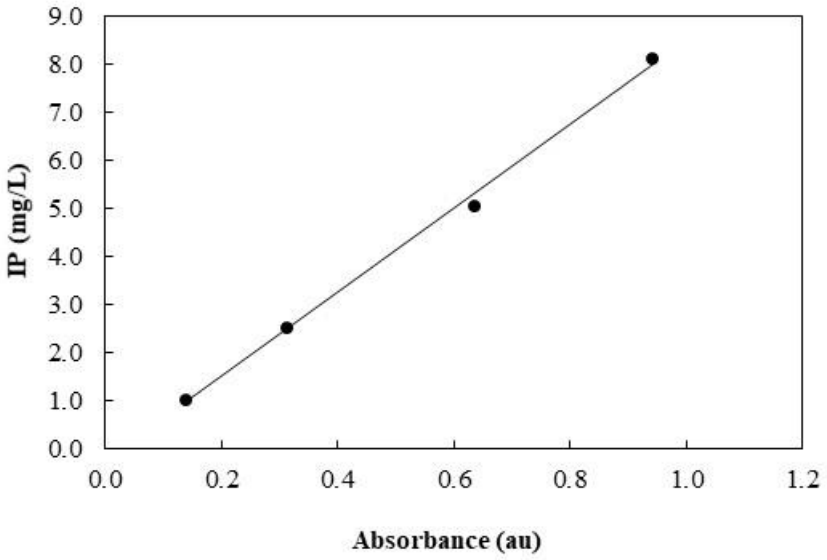

Figure 0-2 Calibration curve of inorganic phosphorus colorimetric method.

4.6.3. Determination of total Kjeldahl nitrogen

Total Kjeldahl nitrogen (TKN) content was measured using a Kjeltek 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).

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

$$\text{TKN}\ (\%) = \frac{\big((\text{Vs} - \text{Vb}) \times \text{N} \times 14.007\big) \times 100}{\text{w}}$$

where, *Vs = volume of 0.1N HCl consumed for the sample in mL,*
Vb = volume of 0.1N HCl consumed for the blank in mL,
N = normality of HCl, and
w = mg of the sample

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

4.6.4. Enzymatic activity assay

Glucoamylase activity was assayed by measuring the glucose concentration produced during hydrolysis of 20 g/L (db) pure starch in 0.2 M phosphate buffer at pH 6.0 and 55°C within 15 min. TCA (5%) was used to stop the reaction (ratio 1:1). Gelatinization of starch suspensions at 75°C for 20 min was used to increase enzyme susceptibility. One unit (U) of glucoamylase activity was defined as the amount of enzyme that releases 1 mg glucose in 1 min under the assay conditions.
Protease activity was measured by the production of FAN during hydrolysis of 15 g/L (db) casein in 0.2 M phosphate buffer at pH 6.0 and 55°C within 30 min. TCA (5%) was used to stop the reaction (ratio 1:1). One unit (U) of proteolytic activity was defined as the amount of enzyme that releases 1 μg FAN in 1 min under the reaction conditions.

4.6.5. Determination of total dry weight, microbial oil and intracellular polysaccharides

TDW was determined by drying the yeast biomass produced in shake flask and bioreactor fermentations at 105°C for 24 h. Microbial oil analysis was carried out by disrupting the dry mass of yeast cells and extraction of oil using either the method proposed by Folch et al. (1957) or heat treatment with HCl (Tapia et al. 2012). In the latter case, hydrochloric acid (6 mL of 4 M HCl) was added for each 500 mg of dry yeast mass and the mixture was heated at 80°C for 1 h. Cellular debris was removed by centrifugation (9000×g, 4°C, 10 min) and the lipids were extracted with a chloroform/methanol mixture at a ratio of 2:1 (v/v). Prior to solvent separation via evaporation, the extracted oil was washed with 0.88% KCl (w/v).
Quantification of total intra-cellular polysaccharides (IPS) was also carried out during fed-batch bioreactor cultures. The dry yeast mass was initially disrupted by heat treatment with HCl as mentioned previously in the case of lipid analysis. Cellular debris was removed by centrifugation and the supernatant was neutralized to pH 7.0 with 2 M NaOH. The final

volume was adjusted to 20 mL. The concentration of total sugars was determined with the assay of 3,5-dinitrosalicylic acid (Miller et al., 1959) and IPS were expressed as glucose equivalents.

4.6.6. Fractionation of microbial oil

A known amount of crude lipid extract was dissolved in 25 ml of Folch solvent (2:1, v/v chloroform - methanol), washed with 5 ml of 0.88% w/v KCl solution, dried over anhydrous Na_2SO_4, and solvent was completely removed at the rotary evaporator. The organic phase was collected and vacuum evaporated to obtain the clarified microbial oil for its subsequent fractionation. Fractionation of the lipid samples was performed using a column (25 × 100 mm) of of silica gel 60 silanized (0.063 - 0.2 mm, Merck), activated by heating 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 weight of each fraction was gravimetrically determined after evaporation of the solvent in a rotary vacuum evaporator and dried under reduced pressure. Each lipid fraction was thereafter re-suspended in chloroform/methanol 1:1 v/v, collected into a vial and stored at –18°C, until further analysis.

4.6.7. Determination of fatty acid composition of microbial oil

The analysis of fatty acid composition of microbial oil was carried out through production of fatty acid methyl esters (FAME) following a two-step reaction with methanol using sodium methoxide (MeONa) and HCl as catalysts. 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 lipase. FAMEs were analysed by a Gas Chromatography Fisons 8060 unit equipped with a chrompack column (60 m × 0.32 mm) and a FID detector. Helium was used 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 CH_2Cl_2, 47885-U, Merck).

4.6.8. Biodiesel properties characterization

According to EN 14103 standard, FAMEs yield was measured using a Perkin Elmer GC model Clarus 500 (Waltham, Massachusetts, US) coupled to a flame ionization detector and comprising a 30 m × 0.25 mm Elite 5-ms (0.25 μm particle diameter) capillary column. Mono- (MG), di- (DG) and triglycerides (TG) and glycerol determination was carried out following EN 14105 standard, using SGE BPX5 capillary column (12 m length, 0.32 inner diameter and 0.25 ml film).

Oxidation stability was analyzed in a professional biodiesel Rancimat, from Metrohm (Herisau, Switzerland) following EN 14112 standard. The calorific value was analyzed using an IKA bomb calorimeter C200 (Staufen, Germany) and following ASTM D240 standard. Density was measured following EN ISO 3675 protocol. Acid value was analyzed following EN14104 standard. Kinematic viscosity was determined using a glass capillary-type viscometer (Cannon-Fenske, size 150) following EN ISO 3104 standard. Water content was measured using a Karl Fischer titrator, model DL32 Mettler Toledo (Columbus, US) following EN ISO 12937 standard. Flash point (FP) was analyzed by Seta Flash series 3 plus, from Instrumentation Analitica S.A. (Madrid, Spain) according to EN ISO 3679.

CHAPTER 5

Formulation of fermentation media from flour-rich waste streams for microbial lipid production by *Lipomyces starkeyi*

5.1. Introduction

Recent research has focused on the improvement of microbial oil production efficiency (Leiva-Candia et al., 2014, Papanikolaou et al., 2013) and evaluation of its cost-competitiveness (Koutinas et al., 2014b). Utilising crude renewable resources rather than commercial and purified carbon sources and nutrient supplements are of paramount importance towards the development of sustainable microbial oil production processes.

In this chapter, the research focused on the production of a generic fermentation feedstock from WMB and FRW streams that could be used for microbial oil production. WMB were used in SSF of *A. awamori* for the production of all the enzymes required to convert starch into glucose, protein into directly assimilable amino acids and peptides, and generate an inorganic source of phosphorus via phytic acid hydrolysis. Enzyme-rich SSF solids were mixed with FRW suspensions for the production of fermentation feedstocks that were subsequently evaluated for microbial oil production using the oleaginous yeast strain *L. starkeyi* DSM 70296. The FRW used in this study were produced by a confectionery industry line producing food for infants and flour-based confectionery products. Therefore, starch was the source of carbohydrate contained in this waste stream.

5.2. Solid state fermentation for crude enzyme production

One of the targets of this study is the development of a two-stage bioprocess for the production of a generic fermentation feedstock relying entirely on FRW and by-product streams generated from wheat flour mills, bakeries and confectionery production processes. Flour contains mainly starch and protein that should be hydrolysed into glucose and directly assimilable nitrogen sources such as amino acids and peptides in order to create highly efficient fermentation media. WMB is a suitable substrate for SSF. It could be also used for the generation of various micronutrients including phosphorus and other minerals. The combination of these waste and by-product streams results in a self-sustained process generating the required hydrolytic enzymes and nutrient supplements for microbial bioconversions. The hydrolytic enzymes were produced via SSF using the fungal strain *A. awamori*, a well-known microorganism for the production of glucoamylase, protease and phytase (Koutinas et al., 2005; Koutinas et al., 2007; Wang et al., 2009).

These enzymes were subsequently employed to hydrolyse FRW and remaining components (e.g. phytic acid) from WMB. Simultaneous lysis of fungal biomass contributed to enhanced

nutrient release in the final hydrolysate (Koutinas et al., 2005). One major advantage of this process as compared to conventional starch or flour hydrolysis is the fact that the majority of FRW streams are already subjected to thermal treatment and for this reason the gelatinisation step could be omitted.

5.2.1. Effect of initial moisture content

Evaluation of SSF by *A. awamori* aimed to maximise primarily glucoamylase and subsequently proteolytic activity. Figure 5.1 (a, b) present the effect of initial moisture content (50, 55, 60, 65, 70 and 75%) and incubation duration on glucoamylase and protease production using WMB as the sole substrate. Maximum glucoamylase (16.6 U/g) and proteolytic (208.6 U/g) activities were achieved at an initial SSF moisture content of 65%. The duration of each experiment presented in Figure 5.1a was 48 h. Enzyme activities at different initial moisture contents were also measured in shorter and longer SSF duration and the results observed showed the same optimum initial moisture content. Further increase in initial moisture content resulted in a significant decrease in both enzyme activities, a fact mainly attributed to the interference of moisture on the physical properties of the solid particles which could cause limited oxygen transfer (Lonsane et al., 1985).

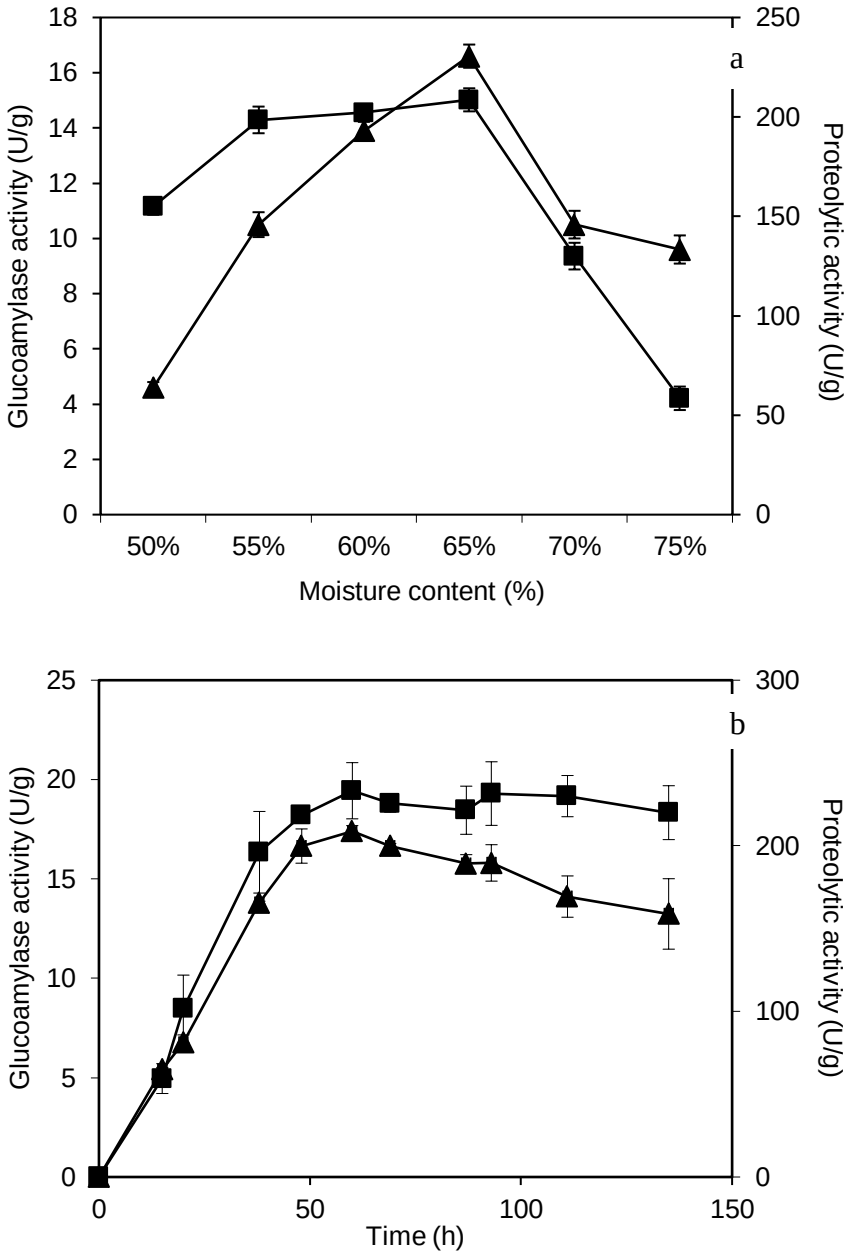

Figure 0-1 Glycoamylase (▲) and proteolytic (■) activity of SSF carried out by *A. awamori* on wheat milling by-products using (a) different initial moisture contents at 48 h and (b) an initial moisture content of 65%.

As it is depicted in Figure 5.1b, maximum glucoamylase (17.4 U/g) and proteolytic (233.6 U/g) activities were achieved at 60 h during an SSF process using only WMB as solid substrate with an initial moisture content of 65%. At prolonged SSF duration, glucoamylase activity was gradually reduced, whereas proteolytic activity remained constant.

5.2.2. Effect of solid substrate

A set of experiments was also carried out using various ratios of WMB to FRW streams generated from a process line producing food for infants. Enzyme production was reduced with increasing content of FRW (data not shown). This could be attributed to the low particle size of this particular FRW stream. Previous literature-cited studies have indicated wheat pieces, pastries and mixed food waste as suitable substrates for multi-enzyme production by *A. awamori* (Wang et al., 2007; Wang et al., 2009; Lam et al., 2013; Pleissner et al., 2014). Wang et al (2009) reported the utilisation of wheat pieces and waste bread for the production of 81.3 U/g and 78.4 U/g of glucoamylase activities, respectively, after more than 130 h of SSF using the same strain of *A. awamori*. It should be stressed that up to approximately 50 h of SSF the production of glucoamylase was lower than 5 U/g (Wang et al., 2009). Contrary to glucoamylase production, the proteolytic activity produced from wheat pieces and waste bread was significantly lower (less than 80 U/g) than this study (Wang et al., 2009). Wang et al. (2007) reported the production of almost 40 U/g of glucoamylase activity and 20 U/g of protease during SSF of *A. awamori* when wheat bran was used as solid substrate which was produced by a simplified wheat milling process using a laboratory mill. However, the high starch content (38.8%) of this bran-rich stream is not common in industrial wheat milling by-products. Lam et al. (2013) reported the production of 253.7 U/g of glucoamylase after 10 days of SSF of *A. awamori* using pastry waste as the sole solid substrate. Pleissner et al. (2014) reported the valorisation of bakery waste from canteens (cakes, pastry & bun) for the production of glucoamylase and proteases using the fungal strains of *A. awamori* and *A. oryzae*.

In subsequent experiments, enzyme production for FRW hydrolysis were produced at 60 h SSF using WMB as the sole substrate with initial moisture content of 65%.

5.3. Flour-rich waste hydrolysis

FRW hydrolysis was carried out by mixing macerated SSF solids with unprocessed FRW. The addition of crude enzymes and macerated fungal mass into the hydrolysis mixture denoted the onset of complex hydrolytic reactions that involved not only the hydrolysis of FRW macromolecules, but also the hydrolysis or even autolysis (caused by oxygen depletion) of macerated fungal cells. The autolysis of the fungus is reported to promote regeneration of valuable nutrient components from fungal biomass (Koutinas et al., 2005).

The production of glucose and FAN during FRW hydrolysis was optimised regarding reaction temperature, pH, initial FRW concentration and initial activities of glucoamylase and protease.

5.3.1. Effect of pH

Figure 5.2 presents the effect of pH (3.5, 4, 4.5, 5, 5.5, 6, 6.5 and uncontrolled pH) on the highest glucose and FAN concentration produced during hydrolysis of 140 g/L (wb) initial FRW concentration. All experiments were carried out at 55°C. One experiment was carried out under uncontrolled pH conditions to evaluate the potential of avoiding pH control during hydrolysis. The maximum glucose production was achieved under uncontrolled pH conditions (Figure 5.2). The FAN concentration achieved under uncontrolled pH conditions was similar to the one produced at pH 5.0. For this reason, subsequent experiments were carried out without pH control as in this way a more cost-competitive process could be developed.

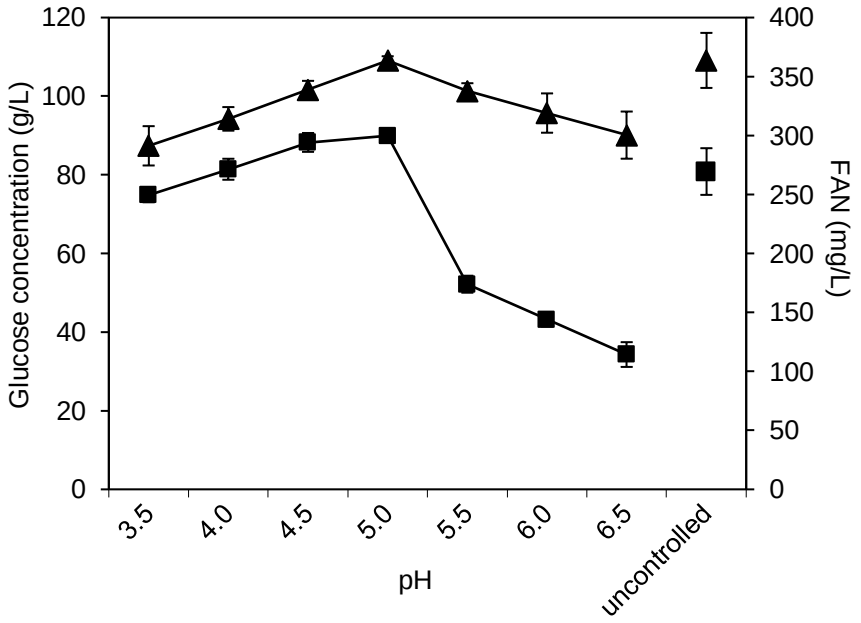

Figure 0-2 Effect of pH on glucose (▲) and FAN (■) production during hydrolysis of 140 g/L initial FRW concentration using crude enzymes produced via SSF of *A. awamori*.

5.3.2. Effect of temperature

Figure 5.3 presents the glucose and FAN production achieved at various temperatures (30, 40, 45, 50, 55, 60 and 65°C) using uncontrolled pH conditions, an initial FRW concentration

of 140 g/L (wb) and initial glucoamylase and protease activities of 0.24 U/mL and 4.3 U/mL, respectively. The optimum temperatures for glucoamylase and protease produced by this strain of *A. awamori* strain are 60 and 55°C, respectively (Wang et al., 2009). Hydrolysis at 60 °C resulted in maximum glucose production, whereas similar FAN production was achieved at a wider temperature range (45 – 55°C). Glucose and FAN production were significantly reduced at temperatures higher than 55°C and 60°C, respectively. Both glucose and FAN production reached maximum values at approximately 24 h hydrolysis. Higher starch to glucose conversion yield can be achieved at 60°C (90.1%) rather than at 55°C (86.6%). However, if hydrolysis of FRW is carried out at 60°C a significantly lower TKN to FAN conversion yield will be achieved (13.8% at 55°C and 7.3% at 60°C). For this reason, all subsequent experiments were carried out at 55°C in order to achieve simultaneously sufficient starch and protein hydrolysis. Producing nutrient-rich fermentation feedstocks requires the production of sufficient nitrogen sources besides glucose.

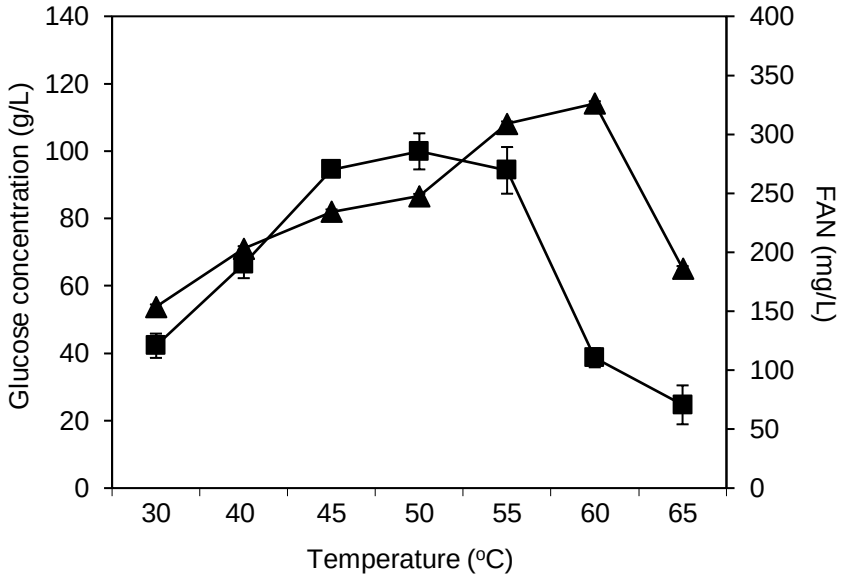

Figure 0-3 Effect of temperature on glucose (▲) and FAN (■) production during hydrolysis of 140 g/L initial FRW concentration with crude enzymes produced via SSF of *A. awamori*.

Industrial hydrolysis of starch or whole cereal flour for the production of fermentation media is carried out using commercial enzymes at high initial concentrations in order to minimise energy costs. Up to 300 g/L and 350 g/L are usually used for whole cereal flour and starch hydrolysis, respectively. Industrial starch hydrolysis is carried following a two-stage process based on liquefaction/gelatinisation and saccharification. Starch liquefaction is nowadays

carried out simultaneously with gelatinisation at temperatures up to 105°C using thermostable bacterial α-amylase, whereas starch saccharification to glucose is carried out using glucoamylase and pullulanase at 60°C. This study aimed to achieve efficient hydrolysis of similar FRW concentrations in a single stage avoiding the use of high temperatures and commercial enzymes as well as the application of a separate stage for liquefaction and gelatinisation.

5.3.3. Effect of initial solids concentration

Table 5.1 presents the effect of five initial FRW concentrations (98, 140, 175, 205 and 235 g/L, wb) and four initial glucoamylase (0.24, 0.49, 0.73 and 0.97 U/mL) and protease (4.03, 8.06, 12.9 and 16.13 U/mL) activities on starch to glucose and TKN to FAN conversion yields. These hydrolytic reactions were carried out by adding the SSF solids containing the crude multi-enzyme mixture and the FRW at the beginning of the process. All experiments were carried out at 55°C and uncontrolled pH conditions. It can be easily observed that increased initial enzyme activities in batch hydrolytic experiments led to increased starch to glucose and protein (expressed as TKN) to FAN conversion yields. For instance, in the case of FRW initial concentration of 235 g/L, a 4-fold increase in crude enzyme activities resulted in substantial improvement of starch to glucose conversion yield from 68% to 84% (w/w). Utilisation of FRW concentrations higher than 235 g/L resulted in low hydrolysis yields with simultaneous operational problems due to the high viscosity of the suspension. It should be stressed that FRW hydrolysis at 60°C would result in approximately 3.5 – 5% higher starch to glucose conversion yields than the values reported in Table 5.1 that were achieved at 55°C. However, the TKN to FAN conversion yields achieved at 60°C were more than 50% lower compared to the respective yields achieved at 55°C.

Table 0-1 Starch and protein (expressed as TKN) conversion yields achieved during batch and fed-batch hydrolysis experiments using various initial FRW concentrations and four initial glucoamylase and protease activities produced via SSF of *A. awamori* cultivated on WMB.

FRW concentration (g/L)	Starch to glucose conversion yield (%)				TKN to FAN conversion yield (%)			
	Glucoamylase activity (U/mL)				Proteolytic activity (U/mL)			
	0.24	0.49	0.73	0.97	4.03	8.06	12.9	16.13
	Batch hydrolysis							
98	89.4 ± 0.3	93.2 ±0.1	93.1 ±0.2	92.8 ±0.3	18.2±0.8	27.2±0.8	42.1±1.3	42.0±1.1
140	86.6 ± 0.6	90.1 ±0.3	91.9 ±0.3	93.1 ±0.1	13.8±1.0	25.9±1.2	38.7±1.2	41.9±0.9
175	85.1 ± 0.2	88.6 ±0.6	89.7 ±0.5	92.9 ±0.4	11.7±1.2	22.7±0.9	35.6±0.9	42.1±1.2
205	82.4 ±0.5	86.0 ±0.3	89.0 ±0.3	93.0 ±0.2	10.1±0.5	19.9±1.1	30.4±0.6	41.2±0.6
235	68.0 ±0.4	70.2 ±0.6	73.5 ±0.2	84.1 ± 0.6	8.4±1.1	17.3±0.9	29.8±1.2	38.5±0.3
	Fed-batch hydrolysis							
235	-	92.1 ±0.2	98.2 ±0.4	-	-	17.3±1.1	30.8±1.0	-
300	-	-	86.1 ±0.1	-	-	-	24.9±0.6	-
350	-	-	73.4 ±0.2	-	-	-	22.7±0.7	-

The starch to glucose conversion yields ($Y_{Glucose}$) could be predicted by a simple empirical equation as follows:

$$Y_{Glucose}(\%) = (0.1148 \times C_{FRW} - 7.874) \times GA + (-0.0019 \times C_{FRW}^2 + 0.456 \times C_{FRW} + 61.97) \quad \textit{(eq. 1)}$$

Initially, linear equations expressing the relationship between starch to glucose conversion yield versus the initial glucoamylase activity (GA) at different initial concentrations of FRW were plotted (Table 5.2) using the results presented in Table 5.1. Subsequently, the set of coefficients α and β of the linear equations presented in Table 5.2 were associated with the respective initial FRW concentration (C_{FRW}). Eq. (1) could be used for the calculation of the starch to glucose conversion yield that could be achieved at various initial FRW concentrations and initial glucoamylase activities. Batch operation led to satisfactory starch and protein hydrolysis when an initial FRW concentration up to 205 g/L (wb) was used. The glucose and FAN concentrations produced were up to 168.9 g/L and 937.2 mg/L, respectively. It should be stressed that this FAN concentration corresponds only to the FAN

produced from FRW hydrolysis. A higher FAN concentration was produced due to hydrolysis of protein present in WMB.

Table 0-2 Linear equations ($Y_{Glucose}$ = α×GA+β) expressing starch to glucose conversion yield versus glucoamylase activity (0.24, 0.49, 0.73 and 0.97 U/mL) at five different initial FRW concentrations.

Initial FRW concentration (g/L)	Coefficients of linear equation	
	α	β
234	22.234	62.993
205	14.817	78
175	10.304	82.99
140	8.24	85.49
98	4.978	88.97

5.3.4. Fed-batch hydrolysis strategy

The operation of fed-batch fermentations requires the utilisation of highly concentrated feeding solutions. Thus, generating as concentrated hydrolysates as possible is essential in order to minimise energy costs. In order to produce more concentrated FRW hydrolysates, a different operational approach was followed in which half of the crude enzyme quantity of known activity was added at the beginning of hydrolysis and the rest after 6 h (this mode of operation is designated as fed-batch hydrolysis in this study). Through this method, the starch to glucose conversion yield was increased up to 98.2% (w/w) at 24 h hydrolysis when an initial FRW concentration of 235 g/L was used. The total glucoamylase and protease activities employed in this experiment were 0.73 U/mL and 12.9 U/mL, respectively (Figure 5.4). Satisfactory starch and protein hydrolysis could be also achieved at initial FRW concentrations of 300 g/L (Table 5.1). Worth mentioning is the fact that this gradual addition of crude enzymes did not seem to positively affect protein conversion yield, since no higher FAN production was noted compared to batch hydrolysis (Figure 5.4b). As shown in Figure 5.4, hydrolysis duration of 24 h was sufficient to achieve 98.2% starch to glucose conversion yield (corresponding to a glucose concentration of 204.5 g/L) and 30.8% TKN to FAN conversion yield (corresponding to a FAN concentration of 803 mg/L). The final FAN concentration corresponding to both FRW and WMB hydrolysis was 1142 mg/L. A higher glucose concentration (228.9 g/L) was achieved when an initial FRW concentration of 300 g/L (corresponding to 86.1% conversion yield) was used that is similar to concentrations

used in traditional industrial processes for hydrolysis of whole cereal flours. Pleissner et al (2014) reported a maximum conversion yield of starch to glucose of 80 – 90% (depending on the waste) during hydrolysis of bakery and food waste from canteens when solid mashes of *A. awamori* and *A. oryzae* were successively added. A glucose concentration of 143 g/L was produced from an initial food waste concentration of 432 g/L containing 0.36 g starch per g solids corresponding to an approximate starch to glucose conversion yield of 0.84 g/g (Pleissner et al., 2014).

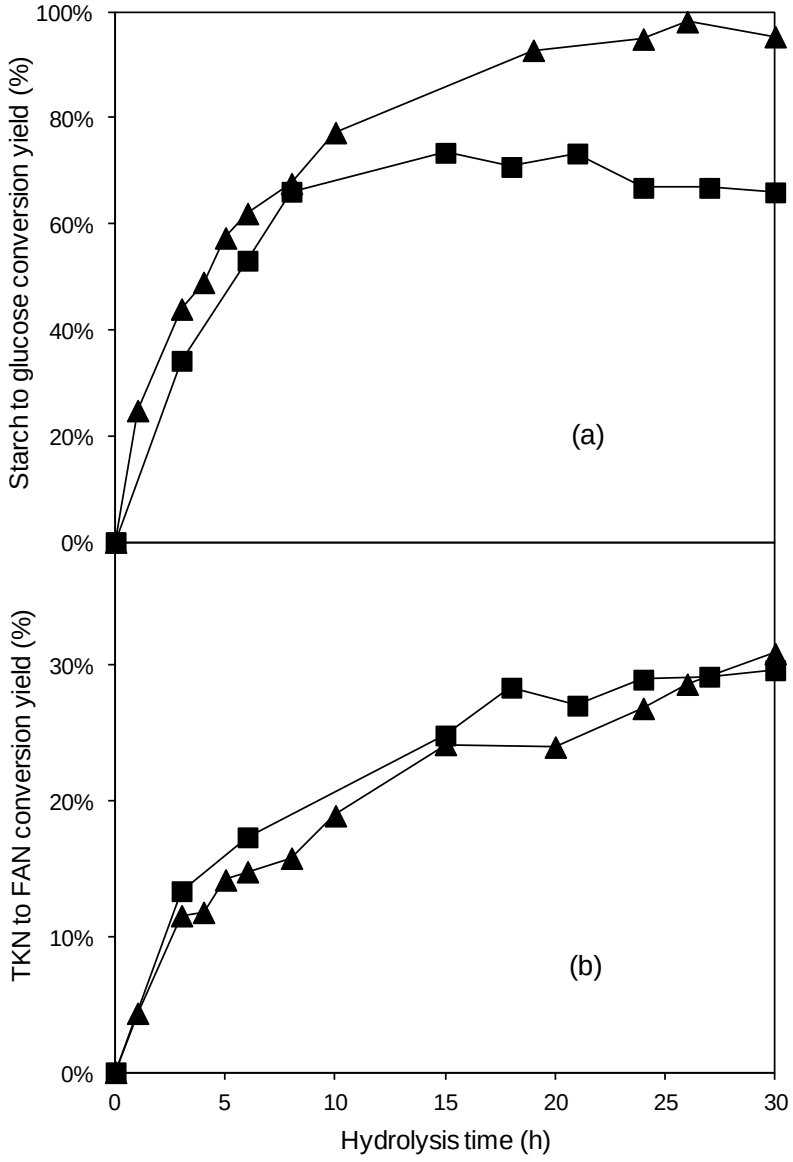

Figure 0-4 Comparison of starch (a) and TKN (b) conversion yields during batch (■) and fed-batch (▲) hydrolysis of 235 g/L initial FRW concentration using a total glucoamylase and protease activities of 0.73 U/mL and 12.9 U/mL, respectively.

5.4. Shake flask cultures of *L. starkeyi* for microbial lipid production

Major objective of the present study was the evaluation of FRW hydrolysates as suitable feedstock for microbial production of lipids by the oleaginous yeast *L. starkeyi*. Preliminary fermentations were carried out in shake flasks using either FRW hydrolysates or glucose-based synthetic media. FRW hydrolysates served as the sole source of carbon, nitrogen and micronutrients. The carbon to FAN ratio of the synthetic medium was adjusted according to the ratio of the FRW hydrolysate used in shake flask fermentations (311 g/g). The C/FAN ratio of the FRW hydrolysate corresponded to the carbon content of the glucose and the nitrogen content expressed as FAN concentration in the hydrolysate. Figure 5.5 presents the production of TDW and lipids as well as the consumption of glucose during cultivation of *L. starkeyi* on either synthetic medium (Figure 5.5a) or FRW hydrolysate (Figure 5.5b).

The initial glucose concentration was around 106 and 109 g/L, respectively. The initial FAN concentration of the hydrolysate was 140 mg/L. In both cases, fermentation was completed in around 200 h after inoculation. However, TDW synthesis and lipid accumulation were substantially enhanced when FRW hydrolysate was employed as the sole substrate. Specifically, maximum TDW reached 30.5 g/L with a lipid content of 40.4% (w/w) when FRW hydrolysate was used. The respective values

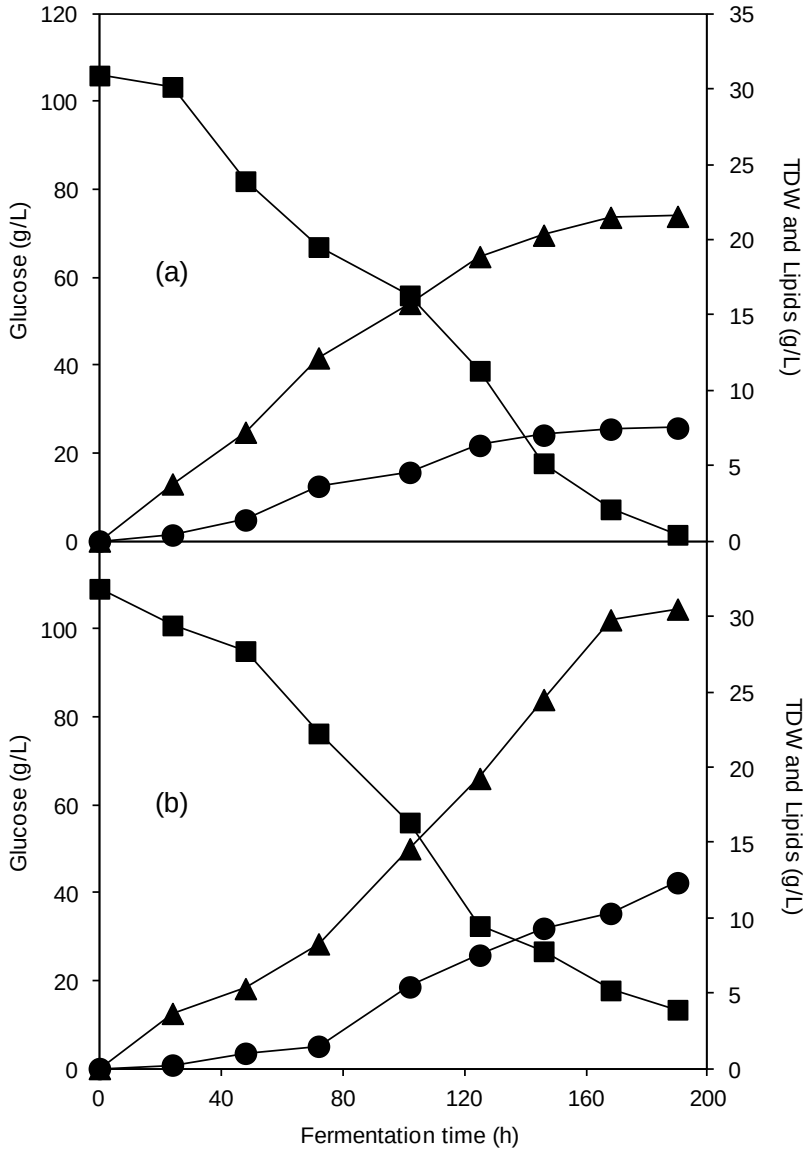

Figure 0-5 Consumption of glucose (■) and production of total dry weight (▲) and microbial lipids (●) during shake flask cultures of *L. starkeyi* on (a) synthetic medium and (b) filter-sterilised FBW hydrolysate.

achieved in synthetic medium were 21.6 g/L and 34.8% (w/w). In both cases, lipid production occurred after FAN depletion from the medium, approximately after 48 h of inoculation (data not shown). These results indicate the suitability of the produced feedstock as a nutrient-complete fermentation feedstock that can sustain both *L. starkeyi* growth and lipid accumulation. FRW hydrolysates seem to contain a balanced composition of glucose, FAN and other nutrients that favour microbial lipid production.

The essential condition that triggers the "*de novo*" accumulation of cellular lipids in oleaginous microorganisms is the presence of nitrogen-limited cultivation conditions (Papanikolaou and Aggelis, 2011; Ratledge and Wynn, 2002). In such case, extracellular nitrogen depletion results in the inhibition of isocitrate dehydrogenase enzyme that regulates

carbon excess channelling towards lipid biosynthesis (Ratledge and Wynn, 2002). However, the presence of organic nitrogen can favour lipid accumulation in a number of oleaginous microorganisms, including strains of *L. starkeyi* (Evans and Ratledge, 1984). The rate of microbial lipid production is influenced by the products of catabolism of the nitrogen source contained in the fermentation medium, rather than being the result of direct stimulation by the nitrogen source (Evans and Ratledge, 1984). Thus, the enhanced lipid accumulation during cultivation on FRW hydrolysates could be attributed to the presence of complex organic nitrogen sources, which was directly assimilable by the microorganism.

Table 5.3 presents literature-cited results regarding microbial oil production by various *L. starkeyi* strains using various carbon sources. Most of the studies employed pure carbon sources and mineral-based media. Gong et al. (2012) investigated the suitability of glucose, xylose, cellobiose and their mixtures as carbon sources for microbial lipid production using the yeast strain *L. starkeyi* AS2.1560, with the most promising results of 31 g/L TDW with an intracellular lipid content of 55%, achieved using a mixture of cellobiose and xylose. The same strain produced 20.3 g/L biomass and accumulated 61.5% (w/w) of lipids in fermentation media containing a mixture of glucose and xylose (Zhao et al., 2008). Lower TDW (12.3 g/L) with a lipid content of 35% have been reported when *L. starkeyi* DSM 70296 was cultivated on xylose (Tapia et al., 2012).

Other studies have focused on lignocellulosic hydrolysates and industrial waste streams including potato starch (Wild et al., 2010) and several hydrolysates of wheat straw (Yu et al., 2011), rice (Probst 2014) and sugarcane bagasse (Xavier and Franco 2014; Xavier et al., 2017). In all cases TDW ranged from 9.6 g/L to 14.7 g/L with microbial oil contents varying between 17.3 – 40.3%. Other studies evaluating fermentative substrates such as hydrolysates of corn (Huang et al., 2011b; Calvey et al., 2016) and wheat bran (Probst & Vadlani 2015) have been proven more efficient reaching TDW of 17.1 - 24.6 g/L and lipid contents of 37.3 – 47.0%. 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 in the current study, when flour-rich waste hydrolysates were used as the sole media in shake flask fermentations. Limited literature-cited studies have reported microbial oil production through supplementation with oilseed meal hydrolysates. 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 5.0 g/L microbial oil.

Table 0-3 Microbial lipid production by various *L. starkeyi* strains cultivated on various carbon and nitrogen sources in shake flask cultures.

Strain	Substrate	Biomass (g/L)	Lipid content (%)	Productivity (g/(L·h))	References
AS 2.1560	Glucose & Xylose	20.5	61.5	0.1	Zhao et al. 2008
AS 2.1560	Cellobiose & Xylose	31.5	55	0.12	Gong et al. 2012
DSM 70296	Xylose	12.3	35	0.03	Tapia et al. 2012
HL	Glucose	17.6	15.3	0.01	Huang et al. 2011a
NRRL Y-11557	Potato starch	10.0	40.3	0.08	Wild et al., 2010
sp.	Wheat straw hydrolysate	14.7	31.2	0.03	Yu et al., 2011
sp.	Rice straw hydrolysate	12.8	35.7	0.06	Probst 2014
CH010	Corncob hydrolysate	17.2	47.0	0.04	Huang et al., 2014
DSM 70296	Sugarcane bagasse hydrolysate	13.3	17.3		Xavier & Franco 2014
ATCC 56304	Wheat bran hydrolysate	17.1	37.3	0.05	Probst & Vadlani 2015
NRRL Y-11557	Corn stover hydrolysate	24.6	38.1	0.05	Calvey et al., 2016
BCRC 23408	Rice bran hydrolysate	13.5	52.6	0.08	Sutanto et al., 2017
DSM 70296	Sugarcane bagasse hydrolysate	9.6	26.1		Xavier et al., 2017
DSM 70296	Crude glycerol	16.5	30.3		Leiva-Candia et al., 2015

5.5. Fed-batch bioreactor culture of *L. starkeyi* on FRW hydrolysate

Although the results of shake flask fermentations were very promising, it was essential to verify the suitability of FRW hydrolysates as medium for microbial oil production in bioreactor cultures. A fed-batch fermentation was carried out in a lab-scale bioreactor aiming to enhance microbial growth and lipid accumulation by *L. starkeyi*. The initial fermentation medium (FRW hydrolysate) was filter-sterilised with initial glucose and FAN concentrations of 90 g/L and 180 mg/L, respectively. Figure 5.6 presents the profile change of glucose, FAN and IP consumption as well as TDW, microbial oil and endo-polysaccharide production during fermentation.

The inoculum employed in fed-batch cultivations with *L. starkeyi* was produced in FRW hydrolysates. This approach had a profound effect on yeast growth and fermentation efficiency. Fed-batch fermentations were also carried out with inocula cultivated in commercial nutrient supplements and the obtained fermentation efficiency was rather low. The physiological state of the inoculum is a critical factor in determining the course of the fermentation. The inoculum production in FRW hydrolysate led to sufficient cells adjustment combined with a high concentration of yeast cells. It was observed that cultivating the inoculum in FRW hydrolysates, a higher yeast cell concentration could be achieved in comparison to commercial nutrient supplements. In this way, a lower lag phase and higher growth and lipid production rate were achieved. Lin et al. (2011) reported that inoculum age and cell density significantly affect lipid accumulation and fermentation efficiency when employing *L. starkeyi* AS 2.1560.

Lipid accumulation started at approximately 40 h when FAN was almost depleted from the fermentation broth. The concentration of IP remained constant at approximately 30 mg/L until the end of the fermentation indicating that IP was not the limiting nutrient that induced lipid accumulation. The initial IP concentration of 161 mg/L was sufficient for microbial growth. This also indicates that the utilisation of WMB provides adequate quantities of IP for bioreactor fermentations. The maximum TDW of 113 g/L was observed at 175 h. Lipid accumulation was determined with two analytical protocols. Figure 5.6 demonstrates that the analytical protocol of Folch et al. (1957) underestimates lipid accumulation. The protocol involving heat treatment with HCl prior to solvent extraction (Tapia et al., 2012) resulted in higher lipid concentrations throughout fermentation. This could be attributed to the sufficient cellular breakage caused during HCl heat treatment, which facilitated an improved release of fatty acids. Due to the presence of thick cell wall, some oleaginous yeasts are often resistant to solvent treatments. Besides, the analytical protocol of Folch et al. (1957) was originally described for lipid extraction from animal tissue and, similarly to other solvent-assisted protocols, its efficiency is best validated in the presence of low lipid contents in the biological sample.

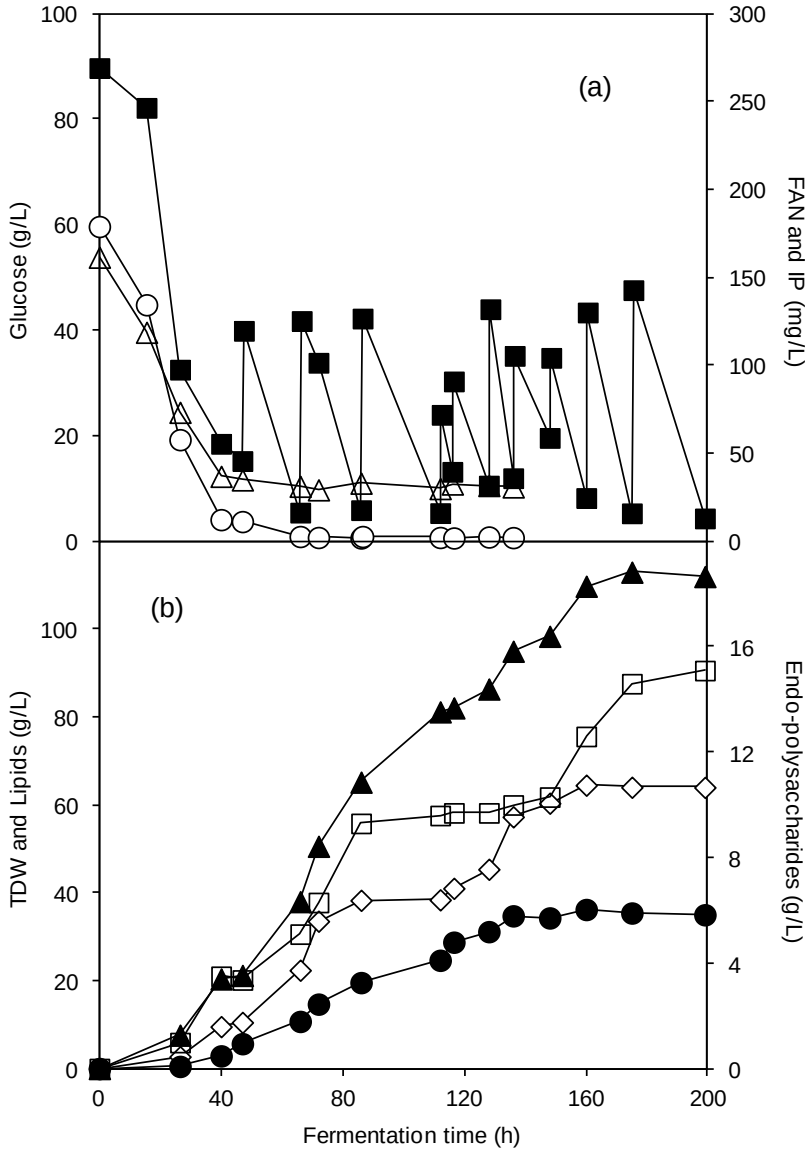

Figure 0-6 Consumption of glucose (■), FAN (○) and IP (Δ) and production of TDW (▲), endo-polysaccharides (□) and lipids extracted either with chloroform:methanol solution (●) or with HCl (◊) during filter-sterilised fed-batch bioreactor culture of *L. starkeyi* using FRW hydrolysate as the sole fermentation medium.

Based on the results of the present study, the applied protocol for lipid extraction in high cell density cultures should ensure the sufficient disruption of the cell wall. The successful recovery of microbial lipids is an issue of major importance for the development of viable microbial oil production processes. Recent literature-cited publications have also reported that the method involving heat treatment with HCl result in more realistic measurements of lipid accumulation (Tapia et al., 2012; Gong et al., 2012; Xu et al., 2012) According to the analytical method reported by Tapia et al. (2012), the maximum lipid concentration achieved was 64.5 g/L at 160 h corresponding to an intracellular oil content of 58.7% (w/w) and a

productivity of 0.4 g/(L·h). The maximum oil content of 61.5% (w/w) was observed at 148 h.

During the final 40 h of the fed-batch culture, lipid production reached a plateau, while TDW increase was due to the accumulation of endo-polysaccharides (Figure 5.6). Endo-polysaccharides are regarded as bioactive compounds and numerous types of mushroom-derived polysaccharides (glucans, galactomannans, proteoglycans) have been reported to possess antitumor, antinflammatory and immunomodulating properties (Wasser, 2002). The events that lead to endo-polysaccharide formation are related to a transition in the intracellular concentration of various metabolites, triggered after nutrient limitation (*i.e.* nitrogen) in the culture medium. When nitrogen depletion occurs, a rapid decrease of intracellular AMP causes alterations in the function of the Krebs cycle resulting in intra-mitochondrial accumulation of citric acid, which after exceeding a critical value, is excreted to the cytoplasm. The presence of ATP-citrate lyase (ATP-CL) in oleaginous microorganisms will cleavage citric acid and generate cellular fatty acids *via* β-oxidation (Ratledge and Wynn, 2002). In the absence of ATP-CL enzymatic complex, citric acid is accumulated in the cytoplasm and will be either excreted into the culture medium or will provoke the inhibition of 6-phosphoro-fructokinase. The latter, in combination with the activity of 6-phosphoglucose isomerase will result in intracellular accumulation of polysaccharides (Papanikolaou and Aggelis, 2011). In the present study, the accumulation of endo-polysaccharides at the late stages of the fermentation could be attributed to the saturation of ATP-CL enzyme, an event which in turn channeled glucose catabolism towards fructose 6-phosphate pathway.

Table 5.4 presents the fatty acid composition of the produced lipids at different cultivation times. The microbial lipids mainly contained oleic acid ($^{\Delta 9}$C18:1) followed by palmitic acid (C16:0), linoleic acid ($^{\Delta 9,12}$C18:2) and stearic acid (C18:0). The distribution of oleic acid, palmitic acid and linoleic acid remained at similar levels throughout the process. The content of saturated fatty acids was higher than 35% (w/w) of total fatty acid content after 86 h fermentation. The oleic acid content was constantly higher than 50% (w/w). Considering the content of saturated fatty acids, the microbial oil produced by *L. starkeyi* is closer to palm oil profile. Similar distribution in fatty acid composition has been reported for other yeast strains of *L. starkeyi* and *R. toruloides* (Li et al., 2007; Zhao et al., 2008; Lin et al., 2011).

Table 0-4 Fatty acid content (%, w/w) of total cellular lipids produced by *Lipomyces starkeyi* during fed-batch cultivation on flour rich waste hydrolysates.

Time (h)	C14:0	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3	C22:0	Other
48	0.8	24.4	-	8.4	52.9	11.3	2.2	-	
66	1.0	25.8	-	7.4	53.1	10.6	2.1	-	
86	1.4	27.6	0.5	7.6	52.4	9.1	1.4	-	
112	0.3	33.1	1.2	6.4	50.6	4.4	0.6	2.3	1.1
136	1.2	27.1	0.6	8.7	52.9	8.5	0.3	0.3	0.4

Fed-batch cultivations offer the advantage of overcoming substrate inhibition obstacles and achieving high density microbial cultures. Indicatively, Li et al. (2007) reported sufficient growth and lipid accumulation (106.5 g/L and 67.5 %, w/w, respectively) during cultivation of the yeast strain *R. toruloides* Y4 on glucose in pilot-scale fed-batch cultures. The overall lipid productivity reported under these culture conditions was 0.54 g/(L·h).

Table 5.5 reports fed batch cultures of *L. starkeyi* on commercial and various renewable substrates for microbial oil production. Mixtures of xylose and glucose were employed in fed-batch cultures for the production of 75.3 g/L of TDW with a microbial oil content of 45.4% (w/w) when a mutant strain of *L. starkeyi* DSM 70296 was used (Tapia et al., 2012). Similar results were obtained by Anschau et al. (2014) when cultivating the same strain on commercial xylose and glucose (70:30), in an attempt to simulate the composition of the hemicellulosic fraction from sugarcane bagasse. Lin et al. (2011) has reported the highest TDW of 104.6 g/L and lipid content of 64.9% at an overall productivity of 1.2 g/(L·h) when glucose and mineral media were used in fed-batch bioreactor cultures. Other studies have reported lower efficiencies with TDW varying between 12.0 – 22 g/L and lipids content of 38.2 – 40.0%. The fermentation results reported in this study are among the most promising in terms of TDW (109.8 g/L) and lipid production (64.4 g/L).

Table 0-5 Fed-batch fermentation for microbial oil production using *Lipomyces starkeyi* cultivated on various carbon sources.

Strain	Substrate	Biomass (g/L)	Lipids (g/L)	Lipid content (%)	Productivity (g/(L·h))	References
NRRL Y-11557	Potato starch	12.0	4.8	40.0	0.096	Wild et al., 2010
AS 2.1560	Glucose	104.6		64.9	1.2	Lin et al., 2011
DSM 70296	G & X	75.3		45.4	0.24	Tapia et al., 2012
DSM 70296	$G^{30}X^{70}$	82.4	38.6	46.9	0.279	Anschau et al., 2014
DSM 70297	Xylose	94.6	37.4	39.5	0.227	Anschau et al., 2015
CBS 1807	X:BP F:BWH	22.0	8.0	38.2		Brandenburg et al., 2016
ATCC 56304	$G_{batch}hX_{lipid}$	60.9	36.6	60.1	0.14	Probst and Vadlani 2017

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

From the results presented (Table 5.5), it becomes obvious that *L. starkeyi* is a highly promising strain for microbial oil production.

5.6. Concluding remarks

A novel biorefinery and bioprocess concept, based on confectionery industry wastes was developed. FRW were successfully converted into a rich nutrient medium that could serve as a generic feedstock for the production of microbial lipids. The integration of such a biorefinery concept into food industries could promote the efficient valorisation of generated waste streams, through their bioconversion into microbial lipids that could be used as feedstock for the production of biodiesel and oleochemicals.

CHAPTER 6

Valorisation of side streams from wheat milling and confectionery industries for consolidated production and extraction of microbial lipids

6.1. Introduction

The concept of the circular economy is emerging as a worldwide strategy to transit from the current linear economy model of production and consumption to efficient resource exploitation (Ghisellini t al., 2016; Maina et al., 2017). Within this framework, bio-economy encompasses the holistic valorization of renewable resources towards the development of biorefinery concepts and bioprocessing schemes to produce high value-added products. Evidently, microbial lipid production constitutes a research area of paramount significance.

This chapter focused on the optimisation of the initial carbon to free amino nitrogen (C/FAN) ratio in fed-batch cultures using the oleaginous yeast *R. toruloides* cultivated on crude FRW hydrolysates. Various feeding strategies were employed at the optimum C/FAN ratio. A consolidated bioprocess is proposed where the crude enzymes produced by solid state fermentation of WMB were employed for the production of FRW hydrolysates and the disruption of yeast cells leading to the release of microbial lipids in the aqueous suspension.

6.2. Effect of sterilisation process

In this section, the development of a two-stage bioprocess for the conversion of FRW streams into a generic fermentation feedstock rich in glucose and directly assimilable amino acids and peptides. Preliminary experiments showed that heat sterilisation was not an ideal process for such a complex fermentation medium. For this reason, heat sterilisation was compared with filter sterilisation in order to identify the optimum sterilisation method. Figure 6.1 presents the consumption of glucose and the production of TDW and lipids during cultivation of *R. toruloides* on FRW hydrolysates subjected either to heat (Figure 6.1a) or filter sterilisation (Figure 6.1b). Initial glucose concentration was around 99 g/L and 94 g/L respectively, whereas the initial FAN concentration was 200 mg/L in both cases. Substrate consumption was notably faster when the yeast cells were cultivated in filter sterilized media, resulting in enhanced TDW formation and lipid accumulation in shorter fermentation duration. The highest TDW in filter sterilised media was 32 g/L with a lipid concentration of 15.2 g/L after 51 h. In the case of heat sterilised media, the highest lipid concentration (10.6 g/L), corresponding to 28.2 g/L of TDW, was observed at 75 h. In the case of liquid media, heat sterilisation is commonly used in conventional fermentation processes, in order to ensure the elimination of endogenous microorganisms prior to media inoculation with pure microbial cultures. However, heat treatment can cause adverse effects to nutrients such

as caramelisation of sugars, denaturation of proteins, inactivation of vitamins and other nutrients of importance for microbial growth, as well as Maillard reactions between sugars and amino groups (Berovic, 2005). In order to avoid such phenomena, separate steam sterilisation of nutrient components is usually performed. However, in the case of crude hydrolysates produced from renewable resources, as in the case of FRW hydrolysates, the latter is not applicable. In the present study, filter sterilisation was proven a suitable practice for media sterilization without compromising nutrient availability, as reflected by *R. toruloides* growth and microbial oil accumulation.

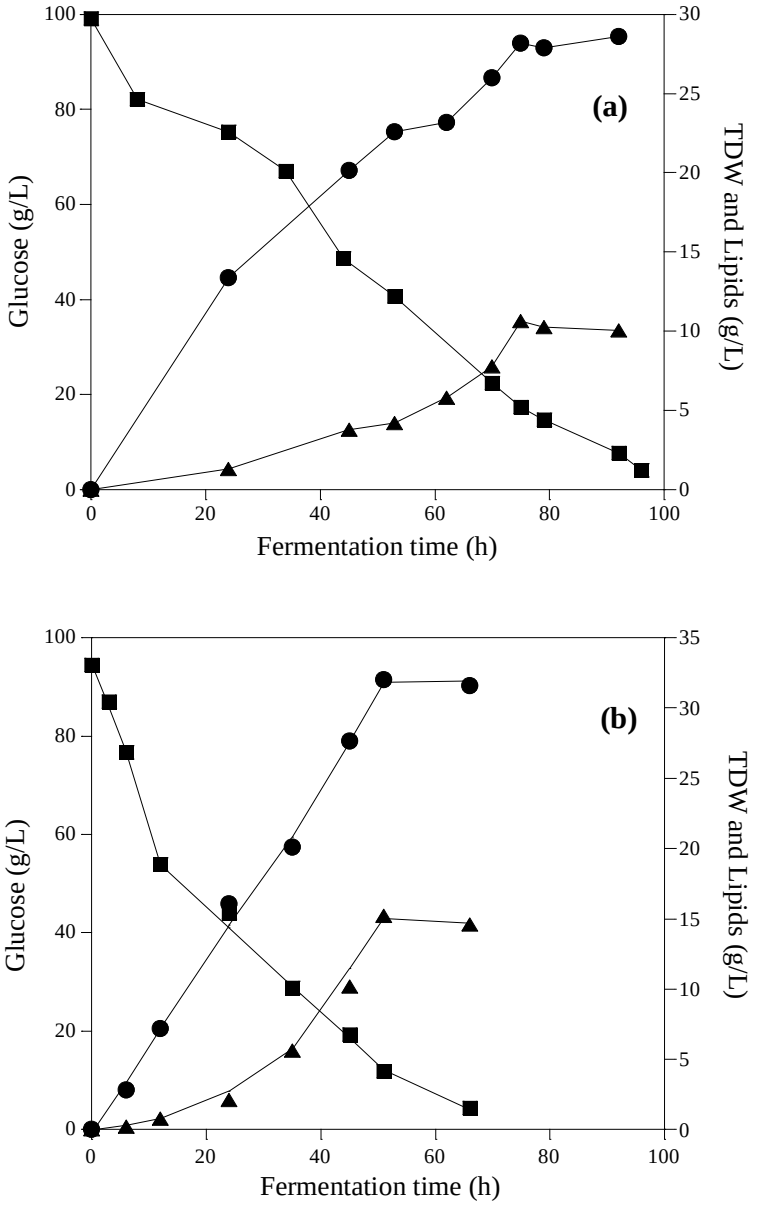

Figure 0-1 Consumption of glucose (■) and production of TDW (●) and microbial lipids (▲) during batch bioreactor cultures of *R. toruloides* cultivated in (a) heat sterilised and (b) filter sterilised FRW hydrolysates.

6.3. Effect of initial carbon to free amino nitrogen ratio in fed-batch bioreactor cultures

One of the major targets of this study was the optimisation of the initial carbon to free amino nitrogen ratio in fed-batch bioreactor cultures cultivating the oleaginous yeast *R. toruloides* on crude FRW hydrolysates. The fed-batch mode was chosen as the most promising cultivation strategy for oleaginous microorganisms promoting high cell density cultures and high lipid production rates (Li and Zhao 2007; Zhang et al., 2011). An essential condition for the initiation of "*de novo*" lipid accumulation in oleaginous microorganisms represents the imposition of nutrient-limited cultivation conditions, with nitrogen often being the limiting factor. The concentration of nitrogen source reflects mainly the quantity of biomass formation, whereas the excess of carbon source concentration largely determines the amount of accumulated lipids (Papanikolaou & Aggelis, 2011). Consequently, the carbon-to-nitrogen (C/N) ratio is considered as a crucial factor towards high cell density and lipid content during cultivation of oleaginous microorganisms. However, optimum C/N is attributed as a strain-dependent regulatory factor and should be determined for individual strains.

In order to obtain different C/FAN ratios, crude FRW hydrolysates were generated through appropriate enzymatic hydrolysis of FRW utilising crude enzyme consortia produced via SSF of *A. awamori*. Cultures were initiated batchwise and after residual glucose concentration dropped to less than 20 g/L, a concentrated glucose feeding solution was introduced intermittently in the bioreactor. Initial glucose concentration was kept at 55 - 60 g/L and FAN concentrations ranged from 220 to 682 mg/L, as shown in Figures 6.2-6.5.

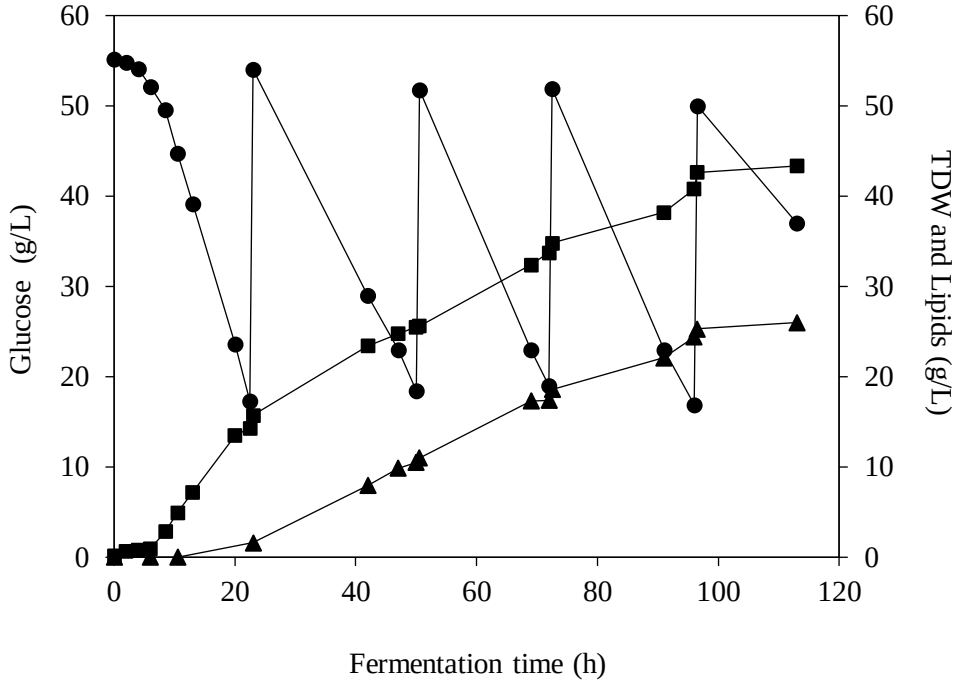

Figure 0-2 Consumption of glucose (●) and production of TDW (■) and microbial lipids (▲) in fed-batch bioreactor culture of *R. toruloides* cultivated in FRW hydrolysate with initial FAN concentration of 200 mg/L.

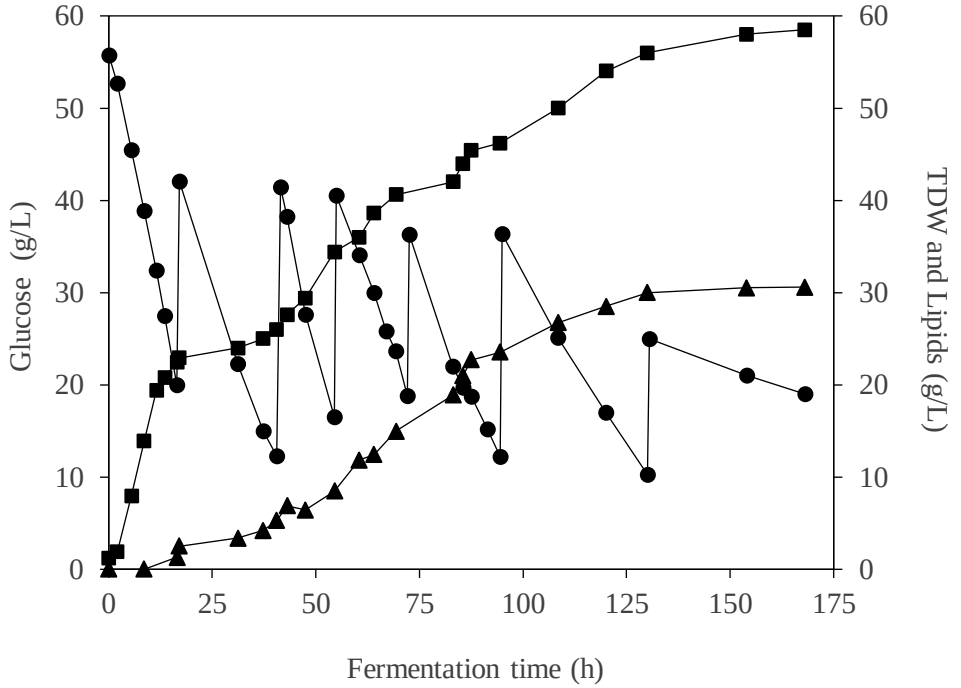

Figure 0-3 Consumption of glucose (●) and production of TDW (■) and microbial lipids (▲) in fed-batch bioreactor culture of *R. toruloides* cultivated in FRW hydrolysate with initial FAN concentration of 400 mg/L.

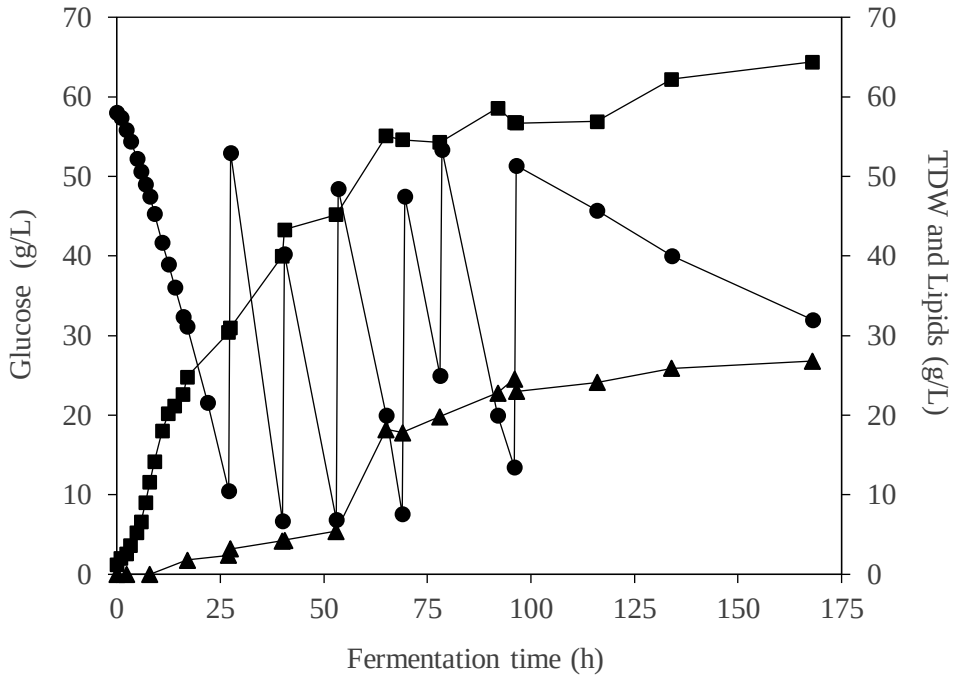

Figure 0-4 Consumption of glucose (●) and production of TDW (■) and microbial lipids (▲) in fed-batch bioreactor culture of *R. toruloides* cultivated in FRW hydrolysate with initial FAN concentration of 500 mg/L.

Table 6.1 summarizes the results obtained from fed-batch bioreactor fermentations of *R. toruloides* using different initial C/FAN ratios. The best results were achieved for initial C/FAN ratio of 80.2 g/g at 142 h corresponding to 61.2 g/L of TDW with an intracellular lipid content of 61.8% (w/w) (Figure 6.5). In this case, the lipid productivity reached 0.27 g/(L·h), with a lipid yield of 0.17 g per g of consumed glucose. In lower initial C/FAN ratios, the lipid content was lower, whereas the residual cell weight (RCW) values were increased. For instance, in cultures with initial C/FAN ratio of 80.2 g/g, the ratio of RCW (23.3 g/L) to TDW (61.2 g/L) was 38%, while the corresponding value in cultures with initial C/FAN ratio of 47.2 g/g was 58%. The higher RCW at decreasing C/FAN ratio could be attributed either to the production of lipid-free yeast cell mass during *R. toruloides* growth (up to approximately 20 - 30 h when FAN and IP were completely consumed) or to the accumulation of endo-polysaccharides. The lowest lipid-free cell concentration (20.9 g/L) reached after the growth stage was observed at the highest C/FAN ratio of 100.4 g/g, whereas the lipid-free cell concentration at C/FAN ratios of 80.2 g/g and 58.2 g/g were 23.3 - 23.7 g/L. In these three cases, the RCW reached a plateau after approximately 30 h, when lipid accumulation initiated. In the case of a C/FAN ratio of 47.2 g/g, the RCW increased up to 36.3 g/L at 43 h, whereas at a C/FAN ratio of 31.9 g/g the RCW increased gradually up to 31.4 g/L at 115 h. In both cases the FAN and IP concentrations were completely consumed

up to 25 h. It has been previously demonstrated that oleaginous microorganisms are capable of accumulating endo-polysaccharides (Tchakouteu et al., 2014). Therefore, the increased RCW values could be also attributed to endo-polysaccharide accumulation (Table 6.1). Another important parameter is the formation of carotenoids by *R. toruloides* strains (Dias et al., 2015), which was indeed observed in this study but it was not quantified. This study identified the optimum C/FAN ratio regarding lipid production. However, a more detailed evaluation of all parameters is required in order to provide the complete capabilities of this strain regarding formation of lipids, endo-polysaccharides and carotenoids. At the highest C/FAN ratio (100.4 g/g), the TDW (49 g/L) was the lowest achieved but the lipid concentration (28.2 g/L) and content (57.5%, w/w) were both second best demonstrating that the low nitrogen content led to lipid accumulation but low cell growth.

The different initial C/FAN ratios had an evident effect on the specific growth rate (μ) of *R. toluloides* (Table 6.1). The highest μ (0.475 h^{-1}) was observed at a C/FAN ratio of 80.2 g/g. At this initial C/FAN ratio, rapid biomass formation is favoured in the yeast growth phase and lipid accumulation is triggered when FAN and IP consumption is terminated. Cultures with lower C/FAN ratios resulted in lower μ values, ranging between 0.286 - 0.18 h^{-1}. A μ of 0.2 h^{-1} was observed in the fermentation that was carried out with the highest C/FAN ratio (100.4 g/g) demonstrating that this is a rather nutrient deficient medium. The supplementation of amino acids and peptides to *R. toruloides* cells in conjunction with the optimum C/FAN ratio of the FRW hydrolysate significantly enhanced lipid accumulation in *R. toruloides*.

Table 0-1 Effect of different initial C/FAN ratios on *R. toruloides* fed-batch bioreactor culture carried out with FRW hydrolysates and intermittent feeding with a glucose-rich solution

C/FAN (g/g)	TDW (g/L)	RCW (g/L)	Lipids (g/L)	Lipid content (%, w/w)	Productivity (g/L·h)	Yield* (g/g)	Specific growth rate (h^{-1})
100.4	49.0 ± 4.3	20.9 ± 4.0	28.2 ± 0.3	57.5 ± 4.5	0.22 ± 0.002	0.14 ± 0.02	0.21
80.2	61.2 ± 1.4	23.3 ± 1.3	37.8 ± 2.7	61.8 ± 3.0	0.27 ± 0.019	0.17 ± 0.01	0.48
58.2	50.2 ± 0.3	23.7 ± 0.1	26.5 ± 0.3	52.7 ± 0.4	0.23 ± 0.003	0.17 ± 0.01	0.29
47.2	62.2 ±1.0	36.3 ± 0.6	25.9 ± 0.4	41.6 ± 0.1	0.19 ± 0.003	0.11 ± 0.01	0.26
31.9	57.8 ± 0.8	31.4 ± 0.3	26.4 ± 0.9	45.7 ± 0.6	0.19 ± 0.003	0.11 ± 0.01	0.18

* g of produced lipids per g of consumed glucose

Besides FAN, the consumption of IP concentration was also analysed during yeast growth (Fig 6.5a). It should be noted that FRW hydrolysates with high initial FAN concentrations, contained similarly increased initial IP concentrations, ranging from 104 to 372 mg/L. In all case, IP consumption was carried out in parallel to FAN consumption (Fig 6.5a), whereas their exhaustion from the medium coincided with the onset of lipid accumulation (Fig 6.5b). Although nitrogen depletion has been mainly studied as the limiting nutrient triggering a series of intracellular biochemical events towards lipid accumulation, the limitation of other micro-nutrients can also lead to efficient lipid production.

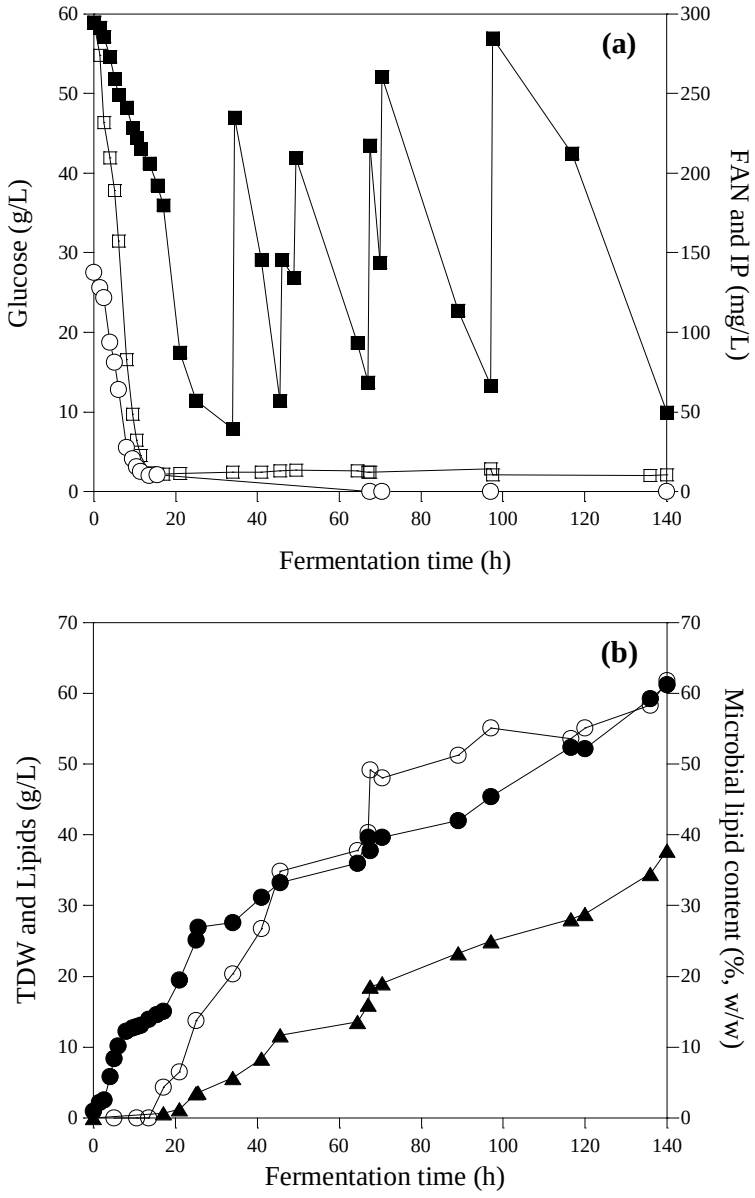

Figure 0-5 a) Consumption of glucose (■), FAN (□) and inorganic phosphorus (Δ) as well as (b) production of TDW (●) and microbial lipids (▲), and evolution of intracellular microbial lipid content (o) in fed-batch bioreactor culture of *R. toruloides* cultivated in filter sterilised FRW hydrolysate with initial FAN content of 294 mg/L.

Phosphorus- or sulphur-limited conditions in carbon- and nitrogen-rich media have been reported as alternative strategies for lipid accumulation in *R. toruloides* (Wu et al., 2010; Wu et al., 2011). Therefore, future studies should evaluate the effect of each one of these nutrients on lipid accumulation when crude hydrolysates are used.

The type of nitrogen source (i.e. inorganic or organic) present in media is acknowledged as a critical factor for oleaginous microorganisms, as it can promote "*de novo*" lipid synthesis in a strain-dependent manner. Lipid accumulation in *R. toruloides* is reported to be favoured in the presence of organic nitrogen sources (Evans & Ratledge, 1984). Evans and Ratledge (1984) concluded that nitrogen source influences lipid production by virtue of its products of catabolism, rather than acting directly as a lipid-stimulating compound itself. In the case of *R. toruloides* CBS 14, cultivation in media supplemented with glutamate as nitrogen source resulted in increased lipid concentrations (up to 50%, w/w) as opposed to cultures with ammonium salts (inorganic source of nitrogen). It was later shown that when organic compounds such as glutamate are utilised as nitrogen sources, metabolic alterations in the extent of NH_4^+ release and accumulation take place that increase the intracellular concentration of NH_4^+. The latter has an impact on the activity of phosphoro-fructokinase enzyme (PFK), responsible for the catabolic flux of carbon, by forming a stable complex with NH_4, less susceptible to the inhibitory effect of citrate on PFK. This particular mechanism found in some oleaginous yeast strains, as in the case of *R. toruloides*, ensures an unrestrained carbon flow towards lipid synthesis (Evans & Ratledge, 1985).

R. toruloides is a promising strain for the production of microbial lipids. This strain has been mainly studied with commercial or crude carbon sources supplemented with commercial nutrient sources such as yeast extract. The potential of *R. toruloides* to produce microbial oil using entirely crude renewable resources as nutrient-complete fermentation media has been reported in limited studies. Yang et al. (2015) reported the production of 16.6, 14.6 and 12.9 g/L of microbial lipids in three successive fermentations carried out with *R. toruloides* Y4 cultivated on recycled yeast cell mass hydrolysates and 70 g/L of initial glucose concentration. The utilization of spent yeast from breweries has been investigated as a crude source of nutrients for the cultivation of *Cryptococcus curvatus* leading to the production of 50.4 g/L of TDW and 37.7% (w/w) of microbial oil content (Ryu et al., 2013). Thiru et al. (2011) reported the production of 69.2 g/L of TDW with an intracellular microbial oil content of 48% (w/w) by replacing baker's yeast autolysate and malt extract with autolysates of de-oiled yeast cells combined with crude glycerol and corn steep liquor.

6.4. Effect of feeding in fed-batch bioreactor cultures

In fed-batch cultivations of oleaginous microorganisms, the choice of the feeding strategy plays a significant role in lipid accumulation and productivity (Zhao et al., 2011). In the fed-batch fermentations presented above, an intermittent feeding strategy of a glucose-rich solution was employed in all cases. Based on the optimum initial C/FAN ratio of 80.2 g/g, two fed-batch fermentations were carried employing two different feeding strategies focusing on either the control of residual glucose in the culture (Figure 6.6) or the addition of a low nitrogen source besides glucose during feeding (Figure 6.6).

In the first feeding strategy, the glucose concentration was maintained in the range of 12.2 – 17.6 g/L throughout feeding that was initiated when the glucose concentration was reduced to less than 15 g/L (Figure 6.6). The time course for glucose consumption as well as TDW and lipid production are presented in Figure 6.6. After 118 h, the TDW reached 62.4 g/L with an intracellular lipid content of 61% (w/w), which were similar to the respective values achieved in the fermentation carried out with intermittent feeding at the same initial C/FAN ratio (Figure 6.2 and Table 6.1). However, the productivity, 0.32 g/(L·h), achieved by keeping an almost constant glucose concentration during feeding was higher than the case that intermittent feeding was used, i.e. 0.27 g/(L·h). Furthermore, the glucose to lipid conversion yield (0.185 g/g) was also higher when an almost constant glucose was maintained during feeding. Zhao et al. (2011) applied a continuous feeding strategy during fed-batch cultures of *R. toruloides* Y4 in defined media and highlighted that low substrate concentration (below 5 g/L) was favourable for TDW and lipid production. In these conditions, the yeast strain *R. toruloides* Y4 produced 127.5 g/L of TDW with lipid content of 61.8% and a high lipid productivity of 0.57g/L h (Zhao et al., 2011).

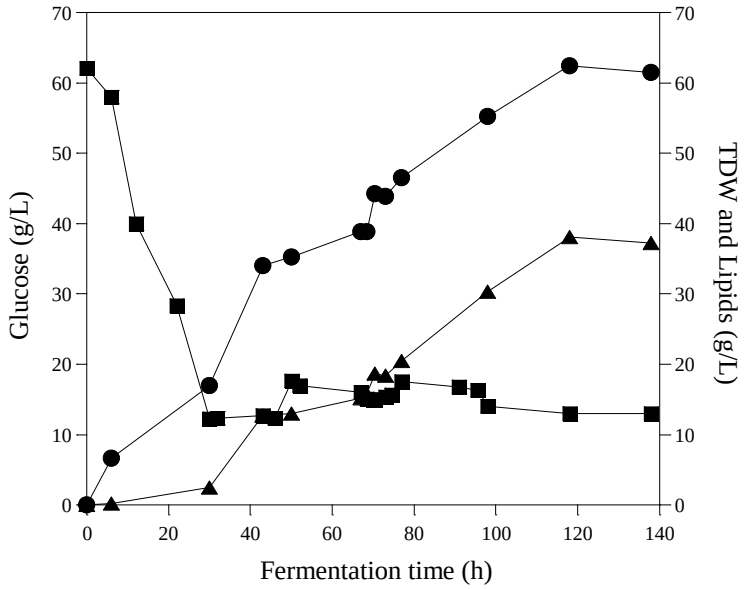

Figure 0-6 Consumption of glucose (□) and production of total dry weight (TDW) (□) and microbial lipids (▲) during fed-batch bioreactor culture of *Rhodosporidium toruloides* following a feeding regime that maintained glucose concentration at 12.2 – 17.6 g/L throughout fermentation.

Although nitrogen-limitation is an essential factor that triggers lipid accumulation, nitrogen source deficiency could decrease the overall lipid productivity in cultures of oleaginous microorganisms (Zhao et al., 2011). To identify the effect of low nitrogen supply during feeding, the second feeding strategy involved the incorporation of 1% yeast extract in the feeding solution. The optimum initial C/FAN ratio of 80.2 g/g was used. The time courses of glucose consumption as well as TDW and lipid production are presented in Figure 6.7.

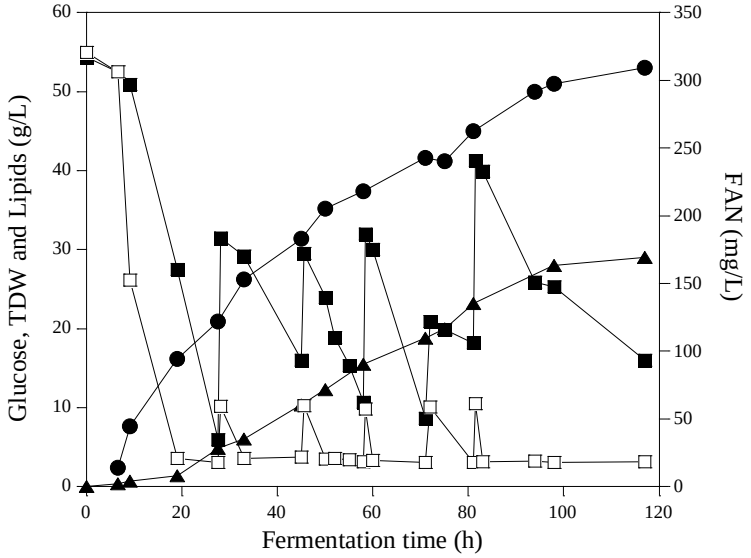

Figure 0-7 Consumption of glucose (□) and free amino nitrogen (FAN) (□) as well as production of total dry weight (TDW) (□) and microbial lipids (▲) during fed-batch bioreactor cultures of *Rhodosporidium toruloides* using intermittent feeding strategy.

The yeast *R. toruloides* produced 51 g/L of TDW containing 55% (w/w) of microbial oil after 98 h. The lipid productivity was 0.28 g/(L·h). Although the highest RCW observed was around 23-24 g/L which was similar to the one observed with feeding containing only glucose (Table 6.1), the final lipid concentration and content were significantly lower. However, the glucose to lipid conversion yield (0.22 g/g) was higher than the fermentation carried out with only glucose feeding. This means that glucose was diverted more efficiently towards lipid accumulation, but this was stopped prematurely compared to the fermentations carried out with only glucose feeding (Table 6.1). Similar results were obtained in fermentations carried out with concentrated FRW hydrolysate containing a glucose concentration of 600 g/L and a FAN concentration of 1.5 g/L using either intermittent feeding or continuous feeding in order to maintain a constant glucose concentration during feeding (results not presented). Similar observations were reported by Saenge et al. (2011a) in fed-batch fermentations of *Rhodotorula glutinis* with feeding containing glycerol and ammonium sulphate. The feeding strategy should be evaluated further in the case of FRW hydrolysates in order to identify the potential to enhance microbial oil production.

6.5. Fatty acid composition of the produced microbial oil

Strain specificity is the major determinant of fatty acid synthesis while other factors i.e carbon and nitrogen sources, micronutrients and the fermentative macro- and micro-

environment could affect fatty acid variations to a lesser extent. Table 6.2 shows the fatty acid profile of *R. toruloides* lipids produced in fed-batch cultures with initial C/FAN ratio of 100.4, 80.2 and 47.2 g/g at different fermentation times (Section 6.3). Oleic (C18:1) and palmitic (C16:0) acids were the major fatty acids followed by stearic (C18:0) and linoleic (C18:2) acids. Minor differences were noticed in fatty acid composition during fermentation, regardless of the initial C/FAN ratio used.

Table 0-2 Fatty acid composition of lipids produced during fed-batch bioreactor cultures of *R. toruloides* in FWR hydrolysate under differenet C/FAN ratios.

Fermentation time (h)	C14:0	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 6,9,15}$C18:3
C/FAN: 100.4							
26	1.0	25.8	-	7.4	53.1	10.6	2.1
44	1.4	27.6	0.5	7.6	52.4	9.1	1.4
96	1.3	27.3	0.6	8.8	52.6	8.3	1.2
114	1.3	27.1	0.6	8.7	52.9	8.5	0.3
C/FAN 80.2							
22	1.0	26.8	-	7.6	51.5	10.7	1.9
42	-	27.4	0.6	8.1	52.0	8.8	1.5
70	0.1	28.5	0.5	8.5	52.0	8.6	1.5
116	1.5	28.3	0.5	8.1	51.1	8.7	1.3
120	1.5	28.7	0.6	7.5	50.3	9.5	1.4
C/FAN 47.2							
134	5.8	26.9	1.0	9.1	53.3	7.0	0.8

At a sequential step, lipids produced from *R. toruloides* 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 6.3). The three lipid fractions mainly consisted of C18:1 (36.9 – 57.6%), followed by C16:0 (18.1 – 27.9%)

and linoleic acid (C18:2) (7.1 – 31.8%). The predominant lipid fraction was NL (82.1%), followed by GL+SL (13.8%) and SL (4.1%).

Table 0-3 Characterization of individual lipid classes of microbial oil produced by *R. toruloides* during fed-batch bioreactor cultures in FWR hydrolysate under a C/FAN ratio of 80.2 g/g.

Lipid classes	(%, w/w)	C14:0	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3
NL	82.1	1.3	25.5	1.4	5.2	57.6	7.1	1.7
SL+GL	13.8	2.6	27.9	-	5.2	54.9	7.8	2.4
PL	4.1	-	18.1	-	4.6	36.9	31.8	8.6

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

6.6. Cell disruption via enzymatic treatment

Davies (1988) has presented detailed information on microbial oil extraction involving drying of oleaginous yeast cell mass and subsequent mixing with hexane, mechanical cell disruption, separation of the lipid-rich hexane phase from the disrupted yeast cells via decanting and purification of lipids via evaporation and recycling of hexane. Similar processing schemes have been presented for the separation of various intracellular products and lipids from microalgal biomass (Molina et al., 2003; Stephenson et al., 2010). Mechanical cell disruption can be achieved via bead milling or high-pressure homogenisation. Jin et al., (2012) employed microwave treatment followed by enzymatic treatment with recombinant β-1,3-glucomannanase for the extraction of lipids from the yeast mass of *R. toruloides* Y4. Cell disruption of microalgal biomass has also been investigated using various methods including mechanical methods, microwave treatment and osmotic shock (Lee et al., 2010).

In this study, the potential to disrupt *R. toruloides* cells via autolysis or by using crude enzyme consortia that were produced via SSF of *A. awamori* cultivated on wheat milling by-products was evaluated. The initial concentration of the yeast cells used was 28.5 g/L with an intracellular lipid concentration of 9.2 g/L. Figure 6.8a shows that around 20% of total lipids were released in the liquid after simple heat treatment of the fermentation broth at 50 °C leading to initiation of autolysis. The process of autolysis is indicated by the increasing FAN concentration that gradually increased up to 45 mg/L after 10 h. The reduction of TDW

during autolysis was only 22.5%. It should be pointed out that further optimisation could improve cell disruption and lipid release in the surrounding liquid. Figure 6.5b shows that around 80% of total lipids were released in the broth when crude enzymes produced by *A. awamori* were used. Around three times higher FAN concentration (145.8 mg/L) was produced than in comparison to yeast cell autolysis. The reduction of TDW during enzymatic cell lysis was around 52%. These results indicate that it is possible to utilise the crude enzymes produced by *A. awamori* for *R. toruloides* cell disruption. The separation of lipids from the aqueous suspension could be achieved by solvent extraction, such as ethyl acetate as was suggested by Jin et al. (2012).

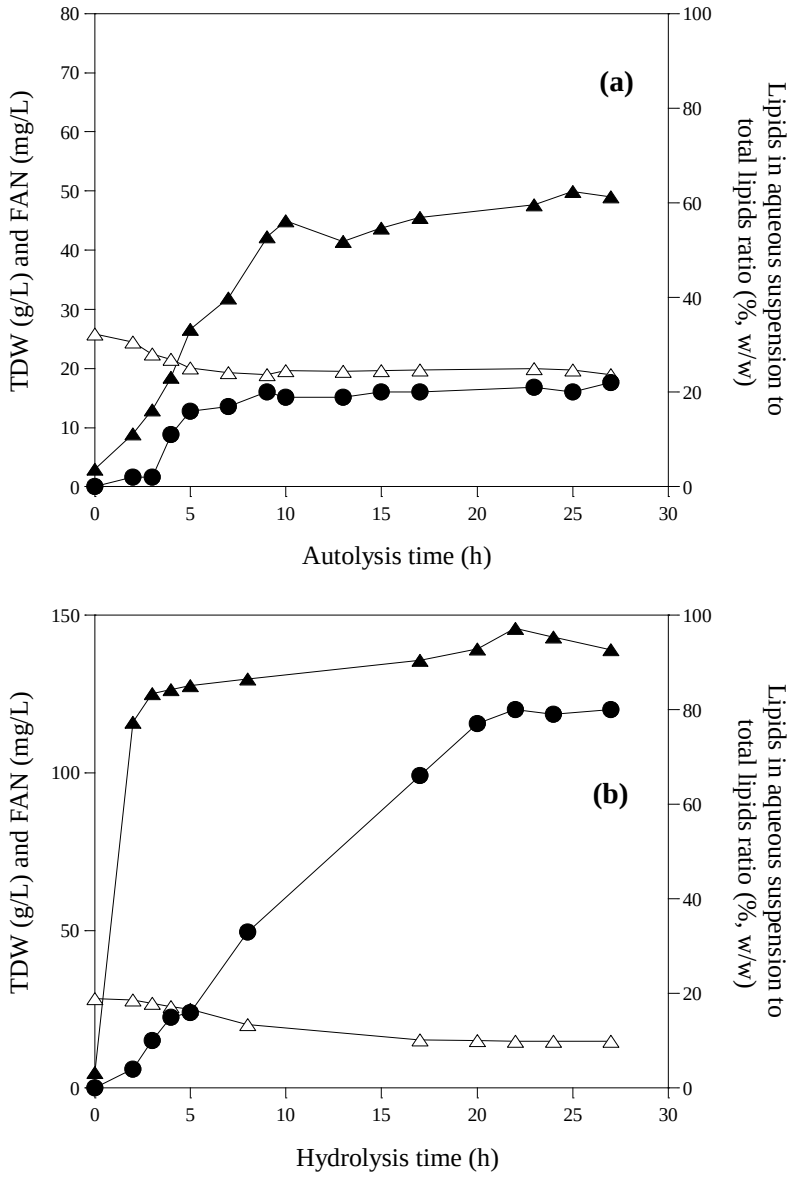

Figure 0-8 Reduction of TDW (Δ), production of FAN (▲) and extraction of lipids in the broth (●) during (a) yeast cell autolysis and (b) hydrolysis of *Rhodosporidium toruloides* cells using crude enzyme produced via solid state fermentation of *Aspergillus awamori*.

The proposed consolidated process could eliminate the need for cell removal from fermentation broth via filtration and drying of the yeast cell mass that is employed in conventional processes. Furthermore, lipid separation is achieved simultaneously with yeast cell hydrolysis producing a nutrient rich suspension that could be recycled in the fermentation stage. In this way, the enzymes produced via SSF could be employed for both the production of FRW hydrolysates, the separation of lipids from the yeast cell mass and the hydrolysis of yeast cells for nutrient recycling. However, the preliminary results presented in this study should be optimised further in order to evaluate the potential of combined yeast cell hydrolysis and lipid removal from the broth via liquid-liquid extraction.

6.7. Concluding remarks

Filter sterilisation has been identified as a preferred sterilisation method of FRW hydrolysates than heat treatment. The initial C/FAN ratio was subsequently optimised regarding microbial oil production in fed-batch cultures of *R. toruloides*. A consolidated bioprocess was developed where the crude enzymes produced by SSF of wheat milling by-products could be employed not only for the production of FRW hydrolysates but also for the disruption of yeast cells and the release of microbial lipids.

CHAPTER 7

Development of a circular oriented bioprocess for microbial oil production using diversified mixed confectionery side-streams

7.1. Introduction

The aim of this experimental section was the valorization of diversified confectionary waste streams, rich in carbohydrates and other micronutrients for the fermentative production of microbial lipids using the oleaginous red yeast *R. toruloides*. It is crucial to configure a bioprocess that will exploit the full potential of the confectionery waste streams, to generate value-added products that can be reintroduced in the food supply chain under the context of zero waste and enhanced sustainability. More specifically, the present study targeted the valorization of all different confectionery waste streams (containing sucrose, starch, and lactose, among others), towards the development of a holistic cascade bioprocess. The effect of the different confectionery side-streams was evaluated on the ability of *R. toruloides* to metabolize confectionary hydrolysates and shift the carbon flux towards lipid synthesis under nitrogen limitation conditions. The fatty acid profile was also evaluated, aiming to identify tailor-made food applications. Interestingly, valorization in a two-stage bioprocess of mixed confectionery waste streams, as described in the present study, entailed modifications in the composition of microbial oil. A higher degree of unsaturated lipids was obtained, advocating the potential to enhance the feasibility of the proposed scheme for further integration in existing facilities, targeting the development of high value-added products, which under the frame of the circular economy might be applied in food formulation.

7.2. Enzymatic hydrolysis of different mixed confectionery waste streams

Chapter 5 presented the production of a nutrient-rich fermentation feedstock deriving from the hydrolysis of FRW using crude enzymatic extracts obtained via SSF with *A. awamori* on wheat milling by-products (WMB). In this study, a similar approach was employed to hydrolyze mixed and diversified confectionery waste streams including mixed food for infants (MFI), mixed confectionery waste streams (MCWS), and mixed waste streams (MWS).

The aforementioned streams contain not only starch, compared to FRW, but also other sources of carbohydrates, e.g., sucrose and lactose. Thus, the study initially targeted the hydrolysis of all carbohydrate sources into their respective monomeric sugars, to be easily assimilated by the yeast strain. Likewise, the hydrolysis of proteins into adequate quantities of amino acids and peptides that can be readily metabolized during a fermentation process was also of high importance. Table 7.1 presents the degree of hydrolysis of starch, sucrose,

and lactose content of all applied waste streams. Conversion of starch to glucose reached more than 95% when an initial concentration of 50 g/L of MFI was applied. The conversion yield of sucrose to glucose and fructose reached 79.4–91.9%, whereas a similar pattern of lactose conversion to glucose and galactose was observed (75.1–89.2%) for all initial MFI solid concentrations. Starch and sucrose hydrolysis yields of MCWS and MWS (91–96.9% and 78.4–92.1%, respectively) were similar to those of MFI, while lactose conversion yields ranged from 70.1–72.3% for MCWS, and from 73.8–88.9% for MWS. The final sugar composition of the MFI and MCWS hydrolysates presented similar composition. MFI consisted of 73.4% glucose, 10.5% fructose, and 16.1% galactose, whereas MCWS contained 74.0% glucose, 11.1% fructose, and 14.9% galactose. MWS hydrolysate contained mainly glucose (83%) and lower amounts of fructose and galactose.

Table 0-1 Degree of hydrolysis (%, w/w) of starch, sucrose, and lactose during hydrolysis experiments, using varying initial solid concentrations of MFI, MCWS and MWS hydrolysates.

Waste stream concentration (g/L)	Composition	MFI	MCWS	MWS
50	Starch	95.6 ± 0.42	96.9 ± 0.89	93.6 ± 0.89
	Sucrose	91.9 ± 1.48	90.9 ± 1.96	92.1 ± 0.67
	Lactose	89.2 ± 0.92	72.3 ± 2.75	88.9 ± 1.28
100	Starch	93.4 ± 1.06	94.5 ± 0.85	91.3 ± 1.62
	Sucrose	83.9 ± 2.26	88.9 ± 1.02	83.6 ± 1.34
	Lactose	81.8 ± 3.54	71.3 ± 0.98	78.1 ± 0.71
150	Starch	91 ± 1.63	93.6 ± 0.56	91 ± 0.99
	Sucrose	79.4 ± 2.19	84.2 ± 1.93	78.4 ± 1.59
	Lactose	75.1 ± 2.83	70.1 ± 1.63	73.8± 2.59

The fungal strain *A. awamori* is of high industrial importance for bioprocesses; hence, it has been widely evaluated, particularly, in solid-state fermentation for the production of hydrolytic enzymes. Smaali et al. (2011) reported on the production of extracellular

thermostable invertase during submerged cultures, triggered by the addition of sucrose. Bertolin et al. (2003) studied the production of glucoamylase via SSF of *A. awamori* on wheat bran. Grape pomace was employed as the sole substrate for the production of cellulase, xylanase, and pectinase with *A. awamori* 2B.361 U2/1 (Botella et al., 2005). The authors reported cellulase activity up to 9.6 ± 0.76 IU/gds during the first 24 h.

The hydrolysis results obtained from our study indicate that the crude enzymatic extracts produced after SSF contain the essential hydrolytic enzymes to break down polysaccharides and oligosaccharides present in MFI and MCWS to generate a rich supplement for bioconversion processes. This will allow for the development of a more consolidated process to exploit all waste streams, under the frame of developing bioconversion processes that can integrate and fit into the circular economy concept.

7.3. Shake flask fermentations for microbial oil production

As it was demonstrated in Table 7.1, the high degree of hydrolysis in MFI entailed the formulation of hydrolysates rich in glucose, fructose, and galactose. The similar composition was obtained in the hydrolysates of MCWS and MWS. Thus, the next step would target the consumption of these sugars sources for the proliferation and microbial oil synthesis by the strain *R. toruloides*. Experiments were initiated by the fermentation of pure commercial sources of each sugar using shake flasks, and the results are presented in Table 7.2. It can be easily seen that after 140 h, substrate consumption was terminated yielding 36–43% of intracellular microbial oil. These experiments were the preliminary step to ensure that *R. toruloides* could consume glucose, sucrose, fructose, and galactose, thus the potential to utilize the hydrolysates obtained from MFI, MCWS, and MSW.

Table 0-2 Consumption of substrate, along with the production of total dry weight (TDW), microbial oil (MO), and intra-cellular oil content during the cultivation of *R. toruloides* using commercial glucose, sucrose, fructose, and galactose at 30 g/L.

Substrate	Fermentation time (h)	Consumed substrate (g/L)	TDW (g/L)	MO (g/L)	Oil content (%, w/w)
Glucose	141	30 ± 0.3	9.4 ± 0.1	3.7 ± 0.3	39.3 ± 2.4
Sucrose	147	30 ± 0.2	10.5 ± 0.3	4.6 ± 0.3	43.8 ± 1.5
Fructose	147	29.4 ± 0.9	9.7 ± 0.4	3.5 ± 0.1	36.1 ± 0.9
Galactose	147	29.6 ± 0.6	9.1 ± 0.1	3.6 ± 0.1	39.6± 1.9

Hence, the next step employed the utilization of these hydrolysates as the sole fermentation supplements for lipid synthesis. Figure 7.1 illustrates the consumption of total sugars along with TDW and lipid production expressed in g/L, using the three different hydrolysates (a: MFI, b: MCWS, c: MWS). In all cases, the initial total sugar concentration was in the range of 80–100 g/L, whereas FAN concentration ranged from 197–243 mg/L. Lipid synthesis started approximately after 44 h, triggered by nitrogen depletion in the medium (Figure 7.1). One of the targets of these experiments was to also identify the better performing substrate as a fermentation feedstock. The hydrolysates from MFI and MCWS resulted in a maximum 42.6% and 52.9% intracellular content of lipids, respectively, whereas in the case of MWS hydrolysate, the microbial oil content did not exceed 37.5%. The highest oil productivity was achieved in the case of MCWS (0.077 g/(L·h)), followed by MFI (0.069 g/(L·h)) and MWS (0.054 g/(L·h)). Interestingly, *R. toruloides* presented higher specific growth rate (0.28–0.29 h^{-1}) when MFI and MCWS were employed as substrates, as compared to MWS (0.12 h^{-1}). Notably, the consumption rate of total sugars was lower in the first hours of fermentation when MCWS hydrolysate was tested compared to MFI hydrolysate. More specifically, the consumption rate was 0.43 and 0.31 g/(L·h) in the case of MFI and MCWS, respectively, at the early phase of the fermentation (44 h).

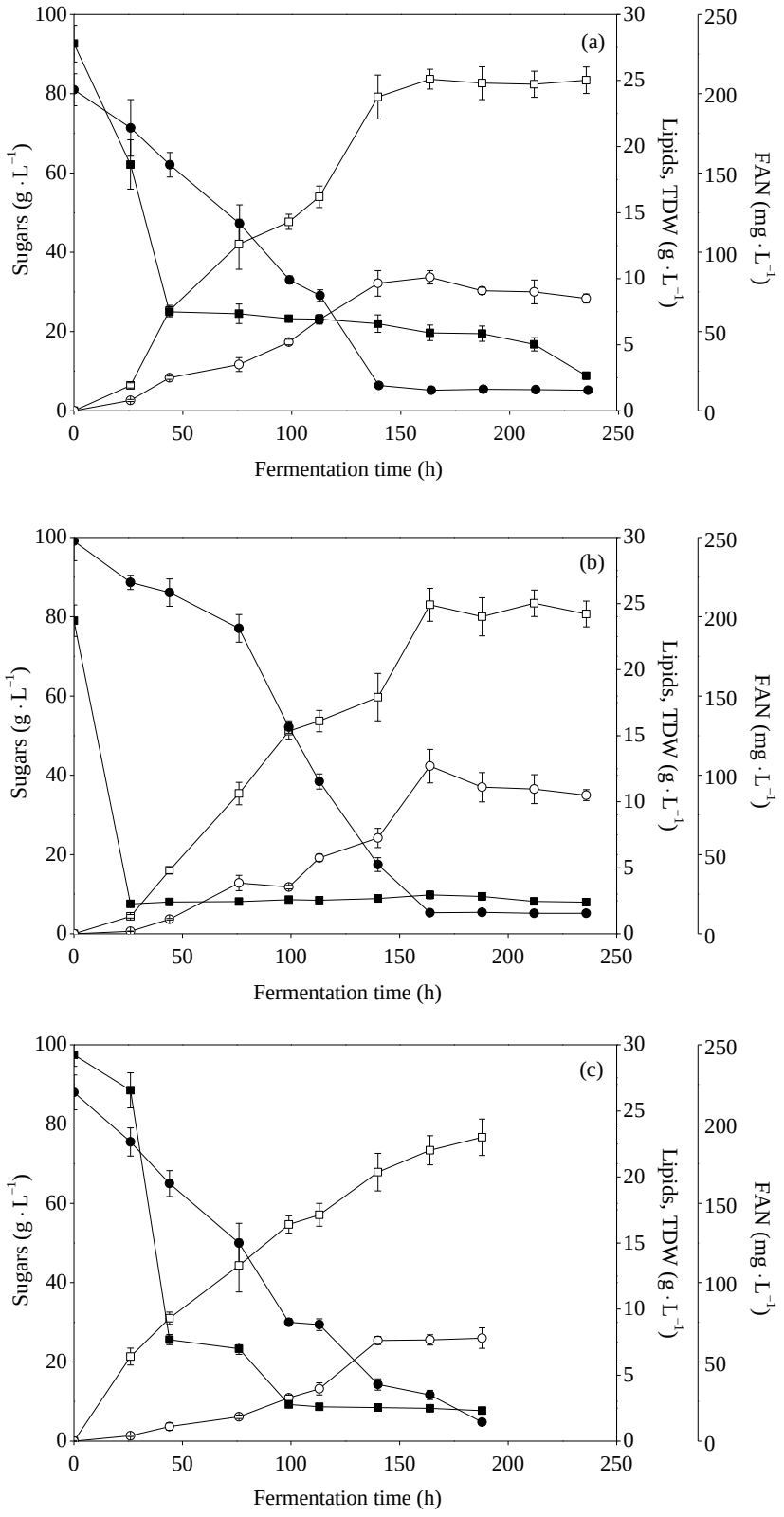

Figure 0-1 Concentration of sugars (●), free amino nitrogen (FAN) (■) and production of total dry weight (TDW) (□) and microbial lipids (○), during shake flask cultures of *R. toruloides* on (a) MFI hydrolysates, (b) MCWS hydrolysates, and (c) MWS hydrolysates.

The strain *R. toruloides* constitutes an industrially important strain for the production of microbial lipids, carotenoids, and various enzymes (Park et al., 2018). *R. toruloides* has the ability to synthesize lipids via cultivation on cassava starch, glucose, xylose, glycerol, and distillery wastewater (Xu et al., 2017). So far, co-cultivation of *R. toruloides* was studied in the viewpoint of exploiting the hydrolysates obtained from lignocellulosic biomass, targeting either lipids or even carotenoids production (Martins el al., 2018). Matsakas et al. (2015) demonstrated the parallel consumption of glucose and fructose using the strain *R. toruloides* CCT 0783 on dried sorghum stalks, achieving a final lipid concentration of 13.8 g/L. In another study, the combination of glucose, xylose, and arabinose resulted in the utilization of xylose after glucose depletion, whereby arabinose was not metabolized (Zhao et al., 2012). The authors employed detoxified sugarcane bagasse hydrolysates, reaching a lipid production equal to 12.3 g/L. Similarly, Martins et al. (2018) evaluated a carob pulp syrup for carotenoids production during fed-batch fermentation of *R. toruloides* NCYC 921, where they demonstrated that glucose was first metabolized, whereas sucrose was not consumed, thus indicating a growth-limiting factor.

Table 7.3 shows the fatty acid profile of *R. toruloides* lipids generated during shake flask cultures on commercial sugars (glucose, sucrose, fructose, and galactose). The major fatty acids were oleic (C18:1) and palmitic (C16:0), whereas lower quantities of stearic (C18:0) and linoleic (C18:2) acids were observed. Oleic and palmitic acids corresponded to more than 75% (w/w) of the total amount of fatty acids synthesized by *R. toruloides*. This is in accordance with the results reported by Tchakouteu et al. (2016), whereby oleic and palmitic acid reached 76.7% of the total fraction of lipids produced. Slininger et al. (2016) reported that cultivation of *R. toruloides* NRRL Y-1091 using lignocellulosic hydrolysate resulted in 72.9% of oleic and palmitic, whereas in another study, *R. toruloides* Y4 reached 66.9%, after 134 h of fed-batch fermentation (Li et al., 2007).

Table 0-3 Fatty acid composition of lipids produced during shake flask cultures of *R. toruloides* on commercial sugars (glucose, sucrose, fructose, galactose).

Fermentation time (h)	C14:0	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3
Glucose							
60	1.8	34	0.3	7.6	43.2	8.4	4.13
92	1.3	27.4	0.8	8	46.9	9.5	3.5
140	0.9	24.5	0.8	5.9	51.5	12.3	4
Sucrose							
23	1.9	34.7	-	7.9	42.8	8.6	4.1
103	1.2	27.1	0.8	8.5	48.9	9.7	3.7
147	0.9	24.9	0.8	6.1	50.7	12.4	4.1
Fructose							
45	1.6	28.1	1.1	7.5	46.8	10.9	2.7
103	1.4	27.9	0.7	7.9	48.7	9.5	3.6
147	0.9	24.5	0.9	5.7	51.5	12.3	3.7
Galactose							
24	1.2	26.2	0.9	6.8	45.3	11.1	2.7
105	1.6	27.5	0.9	7.5	49.7	8.9	3.8
140	0.9	24.9	0.9	6.8	50.3	11.3	4.6

7.4. Fed-batch bioreactor cultures using MFI And MCWS hydrolysates

One of the major targets of this study was to identify the potential of MFI, MCWS, and MWS hydrolysates as sole fermentation substrates for lipids production. Following the results from shake flask cultures, the next step employed the evaluation of the best performing substrates (MFI and MCSW) in bioreactor cultures.

In this study, the initial C/N ratio was adjusted to ~70–80 g/g according to Chapter 6. Figure 7.2a presents the time course consumption of total sugars (glucose, fructose, and galactose), along with FAN utilization, whereas in Figure 2B, the production of TDW, microbial lipids, and IPS are presented. Initial total sugar concentration was ~50 g/L, with glucose being the main sugar, whereas fructose and galactose were present in low concentrations (5.4 and 6.4 g/L, respectively). Intermittent additions of the feeding solution were performed, when sugars' concentration was lower than 20 g/L. Maximal concentration of microbial lipids was

observed after 92 h of fermentation (16.6 g/L) with an intracellular content of 43.3% (w/w) and a productivity of 0.18 g/(L·h).

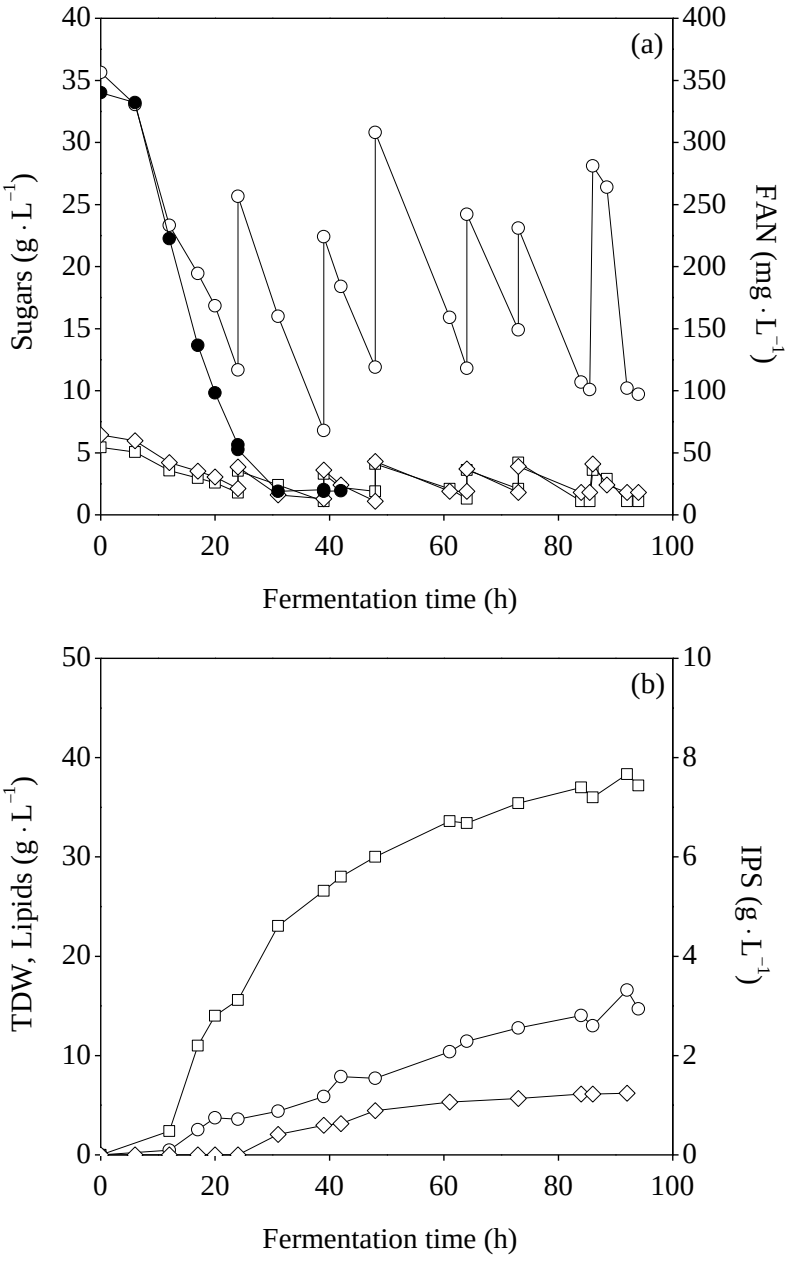

Figure 0-2 (**a**) Concentration of glucose (○), fructose (□), galactose (◊), free amino nitrogen (FAN) (●) and (**b**) production of total dry weight (TDW) (□), microbial lipids (○), and intracellular polysaccharides (IPS) (◊) during fed-batch bioreactor fermentations of *R. toruloides* on MFI hydrolysates.

Similarly, Figure 7.3 depicts the results obtained when MCWS hydrolysate was evaluated by applying a fed-batch strategy. In this case, lipid production reached 17 g/L after 98 h, resulting in productivity of 0.17 g/(L·h) and a lipid content of 45.6% (w/w).

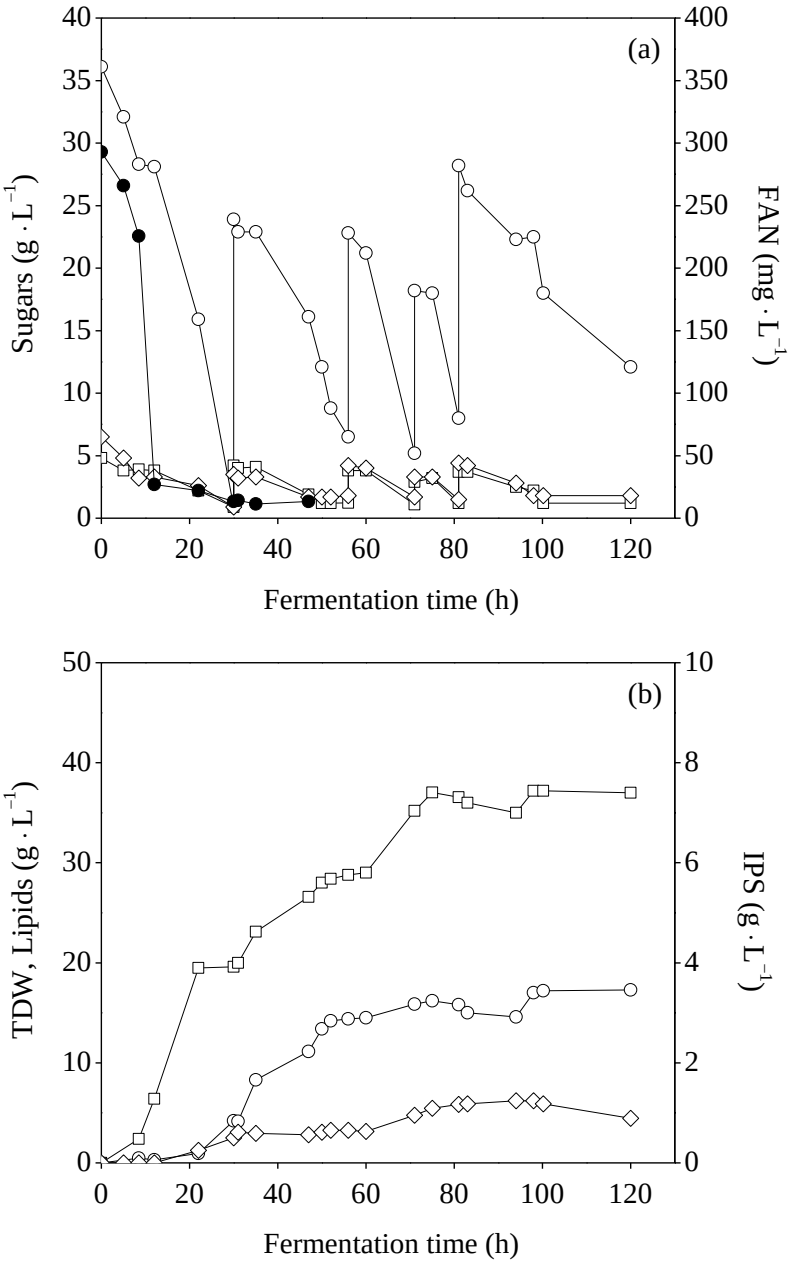

Figure 0-3 (A) Concentration of glucose (○), fructose (□), galactose (◊), free amino nitrogen (FAN) (●) and **(B)** production of total dry weight (TDW) (□), microbial lipids (○), and intracellular polysaccharides (IPS) (◊) during fed-batch bioreactor fermentations of *R. toruloides* on mixed confectionery waste streams (MCWS) hydrolysates.

It is easily observed that when both MFI and MCWS hydrolysates were employed, FAN depletion from the media channeled carbon flux to lipid overproduction. It is also interesting to note that consumption of glucose, fructose, and galactose occurred at the same time in both cases, without indicating carbon catabolite repression phenomena in the presence of glucose.

Studies on the simultaneous consumption of carbon sources have been recently initiated, and significant work is undertaken on transcriptomic and proteomic levels to understand the metabolic network (Slininger et al., 2016; Bommareddy et al., 2017). Bommareddy et al. (2017) presented a metabolic model of *R. toruloides* by using different carbon sources. When glucose was compared with glycerol during bioreactor fermentations, increased biomass was noted in the case of glucose, regardless of the final production of lipids, which was almost equal in both cases. Following flux distribution analysis, the same authors showed that when glucose comprised the sole carbon and energy source, 63% of NADPH derived from the pentose phosphate (PP) pathway, complimented by the cytosolic malic enzyme (Bommareddy et al., 2015). In the case that glycerol alone was employed, NADPH was supplied by the cytosolic malic enzyme, indicating a reduction in PP pathway. Similarly, using the pentoses xylose and arabinose, high PP pathway was observed to meet the demand for NADPH uptake (Bommareddy et al., 2015). Combination of glucose with glycerol entailed a diauxic growth; however, glycerol addition improved lipid synthesis. Wiebe et al. (2012) evaluated C5 and C6 sugars, particularly glucose, xylose, arabinose, and their combinations, during batch and fed-batch cultures. They stated that lipid production in the mixture was lower compared to individual application of glucose or xylose, regardless of the proportions of glucose and xylose in the mixture.

Table 7.4 presents the fatty acid profile of the microbial lipids produced during fed-batch experiments using MFI and MCWS hydrolysates. Oleic (C18:1), stearic (C18:0), palmitic (C16:0), and linoleic (C18:2) were the major fatty acids identified. Oleic acid (C18:1) was the major fatty acid produced; however, it was increased from approximately 52.5–61.4% when MFI and MCWS were used. When the latter substrates were employed, the fractions of palmitic acid (C16:0) and linoleic (C18:2) were significantly reduced, whereby the fractions of stearic acid (C18:0) increased almost 2-fold compared to FRW hydrolysates. Similarly, the fraction of palmitic acid was decreased when MFI and MCWS were used in fed-batch experiments compared to shake flask cultures, where individual carbon sources were used. On the other hand, oleic acid was increased when MFI and MCWS were applied for lipid bioconversion. The differences found in fatty acid composition when different substrates (FRW, MFI, and MCWS) were employed may be attributed to their different sugar composition. In fact, FRW contains only glucose, whereas MFI and MCWS contain also fructose and galactose. The effect of substrate composition on the fatty acid profile of *R. toruloides* microbial oil has been highlighted also by previous studies (Xu et al., 2017; Boviatsi et al., 2019). Further research studies focused on proteomics and transcriptomics

have demonstrated that gene expression and substrate specificity have a key role in lipid synthesis (Chen et al., 2014). Specifically, Fillet et al. (2017) mentioned that the final chain length of the fatty acid relates to the substrate preference of the elongase 3-ketoacyl-CoA. Furthermore, Zhu et al. (2012) pointed that the yield of polyunsaturated fatty acids depends on the number of an acyl carrier protein (ACP), which is part of the fatty acid synthase system, exhibiting a significant role in the chain-elongation process.

Table 0-4 Fatty acid composition of plant-derived oils compared with the composition of the microbial lipids produced during fed-batch bioreactor cultures of *R. toruloides* using MFI and MCWS hydrolysates.

Oil source	**Fatty acids (%)**							**Reference**
	C14:0	**C16:0**	**$^{\Delta 9}$C16:1**	**C18:0**	**$^{\Delta 9}$C18:1**	**$^{\Delta 9,12}$C18:2**	**$^{\Delta 9,12,15}$C18:3**	
Soybean	–	6–10	0.1	2–5	20–24.9	50–60	4.3–11	(a-e)
Rapeseed	–	2.8–14		0.9–2	13.6–64.1	11.8–26	7.5–13.2	(a-e)
Cottonseed	–	27–28.7	–	0.9–2	13–18	51–58	8	(a-e)
Sunflower	–	4.6–6.4	0.1	2.9–3.7	17–62.8	27.5–74	0.1–0.2	(a-e)
Palm oil	0.7	36.7–44	0.1	5–6.6	3–46.1	8.6–11	0.3	(a-e)
Olive oil	<0.1	7.5–20	0.3–3.5	0.5–5	55–83	3.5–21.0	≤1.0	f
Microbial lipids from bioreactor cultures								
FRW	1.5	28.7	0.6	7.5	50.3	9.5	1.4	Chapter 6
MFI	1.4	10.3	0.7	14.5	61.2	5.3	0.4	This study
MCWS	0.9	15.2	0.9	13.8	61.7	6.1	0.1	

[a]Van Gerpen et al., 2004; [b]Ratledge et al., 1993; [c]Singh, 2010; [d]Thompson et al., 2006; [e]Ramos et al., 2009; [f]Boskou et al., 2006.

The applied feeding strategy during fed-batch cultures, oxygen saturation, and primarily carbon source constitute key factors on the final profile analysis of TAGs produced. The effect of feeding strategy and dissolved oxygen was beyond the scope of the current study; thus, the emphasis was given on the carbon source. It is generally accepted that *de novo* synthesis of lipids in *R. toruloides* results mainly in unsaturated fatty acids (e.g., oleic and linoleic acid) (Xu et al., 2017). Fei et al. (2016) used corn stover hydrolysates and reported that oleic acid (C18:1) was the major fatty acid produced in all applied strategies, followed by palmitic (C16:0). Wiebe et al. (2012) reported that stearic, linoleic, and palmitic fractions

were affected in the presence of mixed carbon sources compared to pure glucose fermentation experiments, whereby xylose and arabinose addition induced an increase in C16:0 and C18:2 fatty acids. Bommareddy et al. (2015) showed a content of 57% in saturated fatty acids when glucose alone was employed, whereas, during shake flask cultures in our study, the corresponding amount reached approximately 37.6% (Table 7.3). This could be attributed to the conditions during fermentation (bioreactor compared to shaking flasks), thus indicating future research. Zeng et al. (2018) undertook the utilization of food waste hydrolysates containing glucose as the major carbon source during flask experiments, demonstrating that oleic (75.8%) was the predominant fatty acid, followed by palmitic, linoleic, and lower quantities of stearic.

It should be stressed that the results obtained in the present study highlight that the valorization of the whole confectionery waste streams potential through the proposed process enhance the feasibility for further integration in existing facilities. Likewise, the proposed scheme leads to the production of lipids with a higher degree of unsaturation, a result that could be exploited under the frame of the circular economy towards the development of high value-added products for targeted food formulations within the initial industry.

7.2. Concluding remarks

The potential of generating nutrient-rich fermentation supplements deriving from diversified confectionery waste streams has been well established. All the evaluated hydrolysates performed well both in shake flask cultures and fed-batch bioreactor cultivations using *R. toruloides* to produce microbial lipids. A consolidated bioprocess previously developed to valorize mixed confectionery waste streams could be further expanded to also target future food applications of microbial lipids under the context of the bio-economy era. Likewise, the proposed process conforms unequivocally to the principles of the circular economy, as the entire quantity of confectionery by-products are implemented to generate added-value compounds that will find applications in the same original industry, thus closing the loop.

CHAPTER 8

Evaluation of microbial oil for biodiesel production

8.1. Introduction

Substitution of fossil diesel fuel by biodiesel is a realistic alternative due to its compatibility with commercial diesel engines. However, traditional raw materials used for biodiesel production may compete with food production (edible vegetables oils), land and water. Biodiesel production using microbial oil derived from food waste could provide a sustainable replacement for diesel fuel. The fermentation media and the type of the microorganism has an important effect on the fatty acid composition of the produced microbial oil. For this reason, it is important to study the effect of each parameter, as they influence both the amount of oil and, more importantly, the optimum oil quality to produce biodiesel. This part of the study focused on the evaluation of microbial oil (obtained in Chapter 6) as a potent substrate for biodiesel production and its subsequent characterization.

8.2. Determination of microbial oil properties from *R. toruloides*

The composition of intracellular lipids in fatty acid methyl esters gives strong indication for its suitability as feedstock for biodiesel production. Microbial oil produced with the yeast strain *R. toruloides* on fed-batch bioreactor cultures using FRW was initially characterized in terms of peroxides ($meqO_2/kg$), density at 20 °C (kg/m^3), viscosity at 40 °C (mm^2/s), water content (mg/kg), calorific value (MJ/Kg) and acid value (mg KOH/g). The aforementined properties are the main contributors to the biodiesel final quality. As shown in Table 8.1 properties of microbial oil produced with *R. toruloides* were quite comparable to those of the fungal strain *Mortierella* sp. K11 and six vegetable oils that have been used for the production of biodiesel. It should be noted that microbial oil from *R. toruloides* presented a relatively high acidity, which could be due to the relatively high concentration of free fatty acids contained in microbial oil. Due to the relatively high acidity of the microbial oil produced (2.58 mg KOH/g), it was essential to perform the esterification of fatty acids under acidic conditions followed by transesterification of triglycerides under alkaline conditions in order to achieve a high conversion to fatty acid methyl esters.

Table 0.1 Properties of microbial oil produced in fed-batch bioreactor cultures with *R. toruloides* grown in FRW and comparison with other oil sources.

Properties	Microbial oil *R. toruloides* [1]	Microbial oil Mortierella sp. K11 [2]	Jatropha oil [2]	Rapeseed oil [2]	Corn oil [3]	*Jatropha curcas* oil [4]	*Sterculia foetida* oil [4]	*Ceiba pentandra* oil [4]
Peroxides ($meqO_2/kg$)	6.4 ± 0.60	-	-	-	-	-	-	-
Density 20 ^oC (kg/m^3)	965 ± 10	920	940	911	919	915	937	905
Viscosity 40 ^oC (mm^2/s)	20.7 ± 0.10	54.81	24.54	37.3	36.27	28.35	63.5	34.45
Water content (mg/kg)	615 ± 15	-	-	-	749	-	-	-
Calorific value (MJ/Kg)	30.23±0.5	32.05	38.65	39.7	39.8	38.96	39.79	39.59
Acid value (mg KOH/g)	2.58 ± 0.2	28.22	28.71	197	0.26	12.78	5.11	11.99

[1]This study**,** [2]Kumar et al., 2011; [3]Mata et al., 2012; [4]Onga et al, 2013

8.3. Production of biodiesel using microbial oil from *R. toruloides* and properties determination

The biodiesel produced in this study was evaluated based on the standard methodology of the European biodiesel standard EN 14214. Table 8.2 includes the properties of biodiesel produced with microbial oil from *R. toruloides* (this study) as well as biodiesel derived from other plant-based oils presented in literature cited publications. Limits of each value measured, based on the EN standard 14214 as well as the protocols applied for each property determintation are also included. The obtained results are encouraging as the majority of the biodiesel properties were found within the limits set by the European biodiesel standard EN 14214. Additionally, the results of this study are quite comparable to other literature cited studies that evaluate biodiesel production using plant-based oils, i.e *Jatropha, Sterculia, Ceiba*.

The conversion efficiency of triglycerides to FAMEs in relation to the percentages of monoglycerides, diglycerides, triglycerides, free glycerol and total glycerol determines the efficiency of the transesterification process. In case that the transesterification is incomplete, the biodiesel produced contains high levels of mono-, di- and triglycerides. As shown in Table 8.2, the mono-, di- and triglycerides content of the produced biodiesel was very low (lower than the corresponding limits set in EN 14214.). The content of free glycerol (0.22%, w/w) was higher than the maximum permissible value (0.02%, w/w) which consequently resulted in an increased content of total glycerol (0.31%, w/w). The reduction of glycerol content of biodiesel could be easily achieved via the optimization of the biodiesel purification process. The low conversion of microbial lipids into FAMEs (80% w/w) is probably attributed to the fact that apart from triglycerides, microbial oil consists also of phospholipids, glycolipids and sphingolipids. The conversion efficiency of these lipid classes to FAMEs is much lower than the transesterification efficiency of triglycerides. Nagle and Lemke (1990) reported that biodiesel transesterification yield from phospholipids was 30% less than the corresponding one from triglycerides. They stated that the lower conversion yields from the phosphatidyl choline, phosphatidyl glycerol, and mono- and digalactosyl diglyceride esters can be explained by the substitution of fatty acid groups by carbohydrate and phosphate groups.

Table 0.2 Properties of biodiesel derived from microbial oil produced in fed-batch bioreactor cultures with *R. toruloides* grown in FRW and comparison with the properties of different biodiesel produced from vegetable oils

Properties	Method	Biodiesel					
		R. toruloides[1]	*Jatropha curcas* [2]	*Sterculia foetida* [2]	*Ceiba pentandra* [2]	*Jatropha curcas* [3]	Limits EN 14214
Content of biodiesel in FAMEs (%, w/w)	EN14103	80	96.8	96.0	96.4	-	min 96.5
Monoglycerides (% w/w)	EN 14105	0.36	-	-	-	-	0.80
Diglycerides (% w/w)	EN 14105	0.01	-	-	-	-	0.20
Triglycerides (%, w/w)	EN 14105	0.01	-	-	-	-	0.20
Free glycerol (%, w/w)	EN 14105	0.22	-	-	-	-	0.02
Total glycerol (%, w/w)	EN 14105	0.31	-	-	-	-	0.25
Ash (%, w/w)	UNE-EN ISO 10370	0.015	0.020	0.015	0.029	0.025	max 0.3
Viscosity (mm^2/s)	EN ISO 3104	4.3	3.91	4.92	4.15	4.87	3.5 – 5.0
Density (kg/m^3)	EN ISO 3675	895	839	873	857	879	860 - 900
Calorific value (MJ/Kg)	ASTM D240	38.43	40.427	40.179	40.490	-	min 35
Water content (mg/kg)	ISO 12937	437.17	-	-	-	-	max 500
Oxidative stability (h)	UNE-EN 14112	7.2	9.4	3.44	4.22	-	min 6
Flash point (ºC)	ISO 3679	174	161	160.5	163.5	191	>120

[1] This study, [2] Onga et al, 2013; [3] Mofigur et al, 2012.

The viscosity of biodiesel (4.3 mm^2/s) was determined within the limits of EN 14214. Viscosity value of biodiesel is directly related to fuel injection during combustion. High viscosity of vegetable oils and animal fats reduces combustion efficiency and create operational difficulties in the diesel engine as the latter require low viscosity fuels (Keskin et al., 2008). The minimum calorific value required by EN 14214 is 35 MJ/kg. Therefore, the obtained calorific value (38.43 MJ/kg) is above the minimum threshold. Calorific value indicates the amount of energy that could be transferred during the combustion and its value is directly related to the fuel's elemental composition (carbon, nitrogen and oxygen) (Pinzi et al., 2011). Biodiesel presents lower calorific values compared to conventional fuels, due to its high oxygen content and thus larger volume of biodiesel is required for injection.

Oxidation stability is a critical property to evaluate the storage profile of biodiesel. Microbial oil undergoes several degradation stages during the transesterification process as it is exposed to high temperatures and solvents (Carmona et al., 2021). Additionally, the presence of water, and an increased fraction of linolenic acid could reduce storage time of biodiesel and cause several damages to the applied engine. Water is involved in the transesterifaction process in order to cease the reaction after it is completed. Water content in biodiesel samples was 437.17 mg/kg which is lower than the maximum threshold of EN 14214, while the oxidation stability (6 h) did not meet the limits set by the Standard. The oxidation stabitly could be improved via the supplementation of biodiesel with antioxidant compounds, i.e pyrogallol and butylated hydroxytoluene, in concentrations between 1000 ppm and 8000 ppm (Rashed et al., 2015). Linolenic methyl ester was also checked due to triple bonds that lead to autooxiadtion reactions; in this case, it was in compliance with the European standard. The flash point which is very important for safe storage and handling of the biodiesel was equal to 174 °C and thus higher than the required minimum threshold of 120 °C. The flash point depends on the boiling point and therefore the chemical structure of biodiesel. In general, a low flash point implies the presence of methanol in biodiesel.

8.4. Concluding remarks

This study demonstrated that food industry waste with respect to flour-rich waste streams can be used for biodiesel production via microbial oil production by *R. toruloides*. Most of biodiesel properties that were determined complied with the European biodiesel standard EN 14214. FAMEs could be improved through modification of the fatty acid composition of the MO by selecting the appropriate oleaginous yeast strain, by modifying fermentation parameters (i.e. temperature, C/N ratio, glucose loading and aeration) or through genetic engineering.

CHAPTER 9

Conclusions and future perspective

The current situation concerning the generation of food wastes giving a particular focus on flour-rich, bakery and confectionery wastes was reviewed in Chapter 2. The necessity to establish biorefinery concepts aiming at the integrated valorization of these wastes and the production of bioeconomy products was also described. The biotechnological production of microbial oil was reviewed highlighting the renewable resources that have been used up to date. Finally, emphasis was given on the DSP processes for cell lysis and microbial oil extraction.

In Chapter 5, a novel biorefinery and bioprocess concept, based on confectionery industry wastes was developed. In particular, an enzymatic hydrolysis process of WMB and FRW was developed for the production of a nutrient-rich feedstock suitable for microbial fermentations. Firtsly, the crude enzyme consortia production by *A. awamori* was studied through the solid state fermentation on WMB. Enzyme-rich SSF solids were mixed with FRW suspensions for the production of fermentation feedstocks that were subsequently evaluated for microbial oil production using the oleaginous yeast strain *L. starkeyi*. The multi-enzyme production and FRW hydrolysis were optimised in order to achieve highly concentrated hydrolysates similar to those produced in traditional saccharification processes of starch and cereal flours. The obtained results concerning *L. starkeyi* showed that it is a highly promising strain for microbial oil production. These results are among the highest reported (maximum oil content 61.5% (w/w) reported in this PhD thesis) in the literature regarding microbial oil production from glucose-based media with the strain *L. starkeyi*.

In Chapter 6, the initial C/FAN ratio in fed-batch cultures was optimized, using the oleaginous yeast *R. toruloides* cultivated on crude FRW hydrolysates as the substrate. A consolidated bioprocess was proposed where the crude enzymes were produced by solid state fermentation of WMB. The resulted FRW hydrolysates, were used as the substrate for microbial oil production and different feeding strategies were studied at the optimum C/FAN ratio. Finally, crude enzymes were also studied for the disruption of yeast cells leading to the release of microbial lipids in the aqueous suspension. As a conclusion of this chapter, filter sterilisation revealed as the preferred sterilisation method of FRW hydrolysates than heat treatment. The yeast *R. toruloides* produced 51 g/L of TDW containing 55% (w/w) of microbial oil with a lipid productivity of 0.28 g/(L·h). The enzyme-assisted cell lysis was effectively employed, resulting in high oil recovery.

The microbial oil production from the oleaginous yeast *R. toruloides* using diversified mixed confectionery side-streams was studied in Chapter 7. The effect of the different confectionery side-streams was evaluated on the ability of *R. toruloides* to metabolize confectionary hydrolysates and shift the carbon flux towards lipid synthesis under nitrogen limitation conditions. Maximal concentration of microbial lipids was 16.6 g/L with an intracellular content of 43.3% (w/w) and a productivity of 0.18 ·g/(L·h). Interestingly, valorization of mixed confectionery waste streams entailed modifications in the composition of microbial oil and resulted in higher degree of unsaturated lipids. In particular, oleic acid (C18:1) was the major fatty acid and it was shown to be increased up to around 61% when MFI and MCWS were used.

The microbial oil produced by *R. toruloides* using FRW hydrolysates in fed-batch cultures has been evaluated for biodiesel production. The properties of the obtained biodiesel complied with the the European biodiesel standard EN 14214.

This PhD thesis presented a novel integrated biorefinery concept using flour-rich, bakery and confectionery waste streams for the production of high concentrations of microbial oil using two different oleaginous yeasts. This study highlighted the perspective to valorise the whole confectionery waste streams through the proposed processes, enhancing the feasibility for further integration in existing facilities. Likewise, the proposed scheme leads to the production of high concentrations of lipids showing high degree of unsaturation, a result that could be exploited under the frame of the circular economy towards the development of high value-added products for targeted food formulations within the initial industry. Future studies could focus on the potential applications of microbial oils as supplements in the food, chemical and nutraceutical products.

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