AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION
LABORATORY OF FOOD PROCESS ENGINEERING, PROCESSING AND PRESERVATION OF AGRICULTURAL PRODUCTS

"Microbial production of poly(hydroxyalkanoates) and biorefinery development using by-product streams from sunflower-based biodiesel production processes"

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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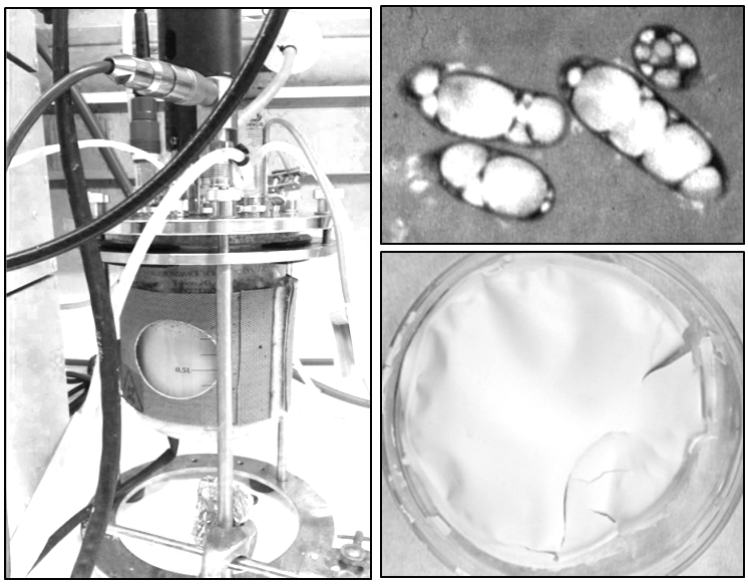

ATHENS

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ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ

ΕΡΓΑΣΤΗΡΙΟ ΜΗΧΑΝΙΚΗΣ, ΕΠΕΞΕΡΓΑΣΙΣ ΚΑΙ ΣΥΝΤΗΡΗΣΗ ΓΕΩΡΓΙΚΩΝ ΠΡΟΙΟΝΤΩΝ

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ΑΠΟΣΤΟΛΟΣ ΚΟΥΤΙΝΑΣ, Επίκουρος Καθηγητής του Τ.Ε.Τ.Δ.Α.-Γ.Π.Α. (Επιβλέπων)

ΣΕΡΑΦΕΙΜ ΠΑΠΑΝΙΚΟΛΑΟΥ, Αναπληρωτής Καθηγητής του Τ.Ε.Τ.Δ.Α.-Γ.Π.Α.

ΙΩΑΝΝΗΣ ΚΟΥΚΟΣ, Αναπληρωτής Καθηγητής του Τμήματος Χημικών Μηχανικών του Παν/μίου Πατρών

ΓΕΡΑΣΙΜΟΣ ΛΥΜΠΕΡΑΤΟΣ, Καθηγητής της Σχολής Χημικών Μηχανικών του Ε.Μ.Π.

ΔΗΜΗΤΡΙΟΣ ΚΕΚΟΣ, Καθηγητής της Σχολής Χημικών Μηχανικών του Ε.Μ.Π.

CHENYU DU, Reader in Chemical Engineering, University of Huddersfield

ΑΘΑΝΑΣΙΟΣ ΜΑΛΛΟΥΧΟΣ, Λέκτορας του Τ.Ε.Τ.Δ.Α.-Γ.Π.Α.

ΑΘΗΝΑ

ΙΟΥΛΙΟΣ 2016

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. 5343/32 αρ. 202 παρ. 2).

ΕΥΧΑΡΙΣΤΙΕΣ

Η παρούσα διδακτορική διατριβή εκπονήθηκε στο Εργαστήριο Μηχανικής Τροφίμων, Επεξεργασίας και Συντήρησης Γεωργικών Προϊόντων και στο Εργαστήριο Μικροβιολογίας και Βιοτεχνολογίας Τροφίμων, του τμήματος Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου στο Γεωπονικό Πανεπιστημίο Αθηνών.

Η εκπόνηση της διδακτορικής διατριβής αποτέλεσε ένα στόχο αλλά και πρόκληση ταυτόχρονα, που τέθηκε και επετεύχθηκε με τη συμπαράσταση και την υποστήριξη πολλών ανθρώπων, στους οποίους το «*ευχαριστώ*» φαντάζει πολύ λίγο. Στο κλείσιμο αυτού του κύκλου, συνεπώς, είναι αναπόφευκτη μια νοερή ανασκόπηση σε όλη τη διαδρομή που μου δίνει το βήμα να εκφράσω τις ευχαριστίες μου σε όλους όσους με το δικό τους τρόπο βοήθησαν, είτε εν γνώσει τους είτε χωρίς, την ολοκλήρωση αυτού του στόχου.

Αρχικά θα ήθελα να ευχαριστήσω τον επιβλέποντα της παρούσας μελέτης, τον Επίκουρο Καθηγητή, κ. Αποστόλη Κουτίνα, ο οποίος διαδραμάτισε μείζονος σημασίας ρόλο σε όλη αυτή τη διαδρομή. Πέραν της άριστης επιστημονικής συνεργασίας, της καθοδήγησης και της μετάδοσης γνώσεων, οι ευκαιρίες, η υποστήριξη και η εμπιστοσύνη που μου έδειξε αποτέλεσε εφαλτήριο δύναμη για την απόδοση των στόχων μου.

Επίσης ευχαριστώ θερμά τον Αναπληρωτή Καθηγητή, κ. Σεραφείμ Παπανικολάου, μέλος της τριμελούς συμβουλευτικής επιτροπής για την άριστη συνεργασία και τη βοήθεια όλα αυτά τα χρόνια.

Τις ευχαριστίες μου θα ήθελα να εκφράσω και προς τον Αναπληρωτή Καθηγητή, κ. Ιωάννη Κούκο, μέλος της συμβουλευτικής επιτροπής για την καίρια τοποθέτηση του και την αμέριστη βοήθεια κατά τη διάρκεια της παρούσας διατριβής.

Ευχαριστώ θερμά και τον Καθηγητή κ. Σταυριανό Γιαννιώτη για την καθοδήγηση και τη συνεργασία στην ολοκλήρωση της διδακτορικής μελέτης.

Επιπλέον, θα ήθελα να εκφράσω τα υπόλοιπα μέλη της εξεταστικής επιτροπής, τον Καθηγητή κ. Γεράσιμο Λυμπεράτο, τον Καθηγητή κ. Δημήτριο Κέκο, το Λέκτορα κ. Αθανάσιο Μαλλούχο και το Λέκτορα, Dr. Chenyu Du. Αποτελεί τιμή μου που δέχτηκαν να συμμετάσχουν στην κρίση της διδακτορικής μου διατριβής, αλλά μεγαλύτερη τιμή αποτελεί το γεγονός ότι συνεργάστηκα με κάθε έναν από αυτούς έστω και για σύντομο χρονικό διάστημα.

Τις ευχαριστίες μου θα ήθελα να εκφράσω επίσης σε όλα τα μέλη του Εργαστηρίου Μηχανικής Τροφίμων, τους υποψήφιους διδάκτορες κα. Χρυσάνθη Πατεράκη, κα. Κατερίνα Παπαδάκη, κα.Σοφία Μάινα, κα. Σοφία Τσάκωνα, κα. Μαίρη Αλεξανδρή, κα. Ερμίντα Τσούκο, κα. Χαρά Δήμου, κ. Δημήτρη Λαδάκη καθώς και τους μεταδιδάκτορες

κ. Ανέστη Βλυσίδη και κ. Χάρη Παπαποστόλου, για την άμεση και άψογη συνεργασία, εντός αλλά και εκτός Εργαστηρίου, καθώς για εμένα είναι πρώτα φίλοι κι έπειτα συνεργάτες. Ένα ακόμα ευχαριστώ για την κα. Ουρανία Καλαντζή που ήταν παρούσα σε όλες τις περιπτώσεις, δίνοντας αντικειμενικές και καίριες συμβουλές και υποστήριξη. Ιδιαίτερες ευχαριστίες θα ήθελα να εκφράσω προς τη Λέκτορα, κα. Αφροδίτη Χατζηφράγκου που ήταν δίπλα μου από την αρχή της διατριβής, παρέχοντας όχι μόνο επιστημονική βοήθεια αλλά κυρίως συμπαράσταση και συμβουλές. Δε θα μπορούσα σε καμία περίπτωση να παραλείψω ένα ακόμα ιδιαίτερο ευχαριστώ προς το μεταδιδάκτορα, κ. Νίκο Κοψαχείλη, με τον οποίο ξεκινήσαμε μαζί από τα «πέτρινα χρόνια» του εργαστηρίου, και ο οποίος πέρα από τη μετάδοση γνώσης, προσέφερε απλόχερα και αμερόληπτα την υποστήριξη του. Τα λόγια είναι λίγα για να εκφράσουν τα συναισθήματα για όλους αυτούς τους ανθρώπους.

Επίσης ευχαριστώ θερμά όλα τα μέλη του Εργαστηρίου Μικροβιολογίας και Βιοτεχνολογίας Τροφίμων, του Εργαστηρίου Χημείας και Ανάλυσης Τροφίμων, και του Εργαστηρίου Ποιοτικού Ελέγχου και Υγιεινής Τροφίμων και ειδικότερα την κα. Αυγή Γαρδέλη, την κα. Νίκη Προξενιά και τον κ. Δημήτρη Δούλτσο για τη βοήθεια τους στην ολοκλήρωση της μελέτης.

Ακόμη, θα ήθελα να εκφράσω τις ευχαριστίες μου προς τον Καθηγητή κ. Ξενοφών Σπηλιώτη, ο οποίος με ώθησε και με ενέπνευσε να θέσω ως στόχο την εκπόνηση της διδακτορικής μελέτης. Ιδιαίτερα ευχαριστώ σε όλους τους φίλους μου, που πολλές φορές παρέλειψα αλλά ποτέ δε με παρέλειψαν και στάθηκαν δίπλα μου σε όλο αυτό το ταξίδι.

Τέλος, τις βαθύτερες ευχαριστίες μου θα ήθελα να εκφράσω προς την οικογένεια μου, του γονείς μου Γιώργο και Γωγώ, την αδερφή μου Ιωάννα και τον αδερφό μου Νώντα, για την υπομονή τους, την κατανόηση, την αμέριστη και την αμερόληπτη υποστήριξη τους όλα αυτά τα χρόνια. Ελπίζω να μπορέσω να ανταποκριθώ στο ελάχιστο στις προσδοκίες τους και να τους ανταποδώσω το μέγιστο. Η παρούσα διατριβή αφιερώνεται σε όλα αυτά τα αγαπημένα πρόσωπα.

Βάσω Καχριμανίδου

Αθήνα, 2016

Table of contents

ABSTRACT

The scope of this thesis is the evaluation of by-product streams deriving from a sunflower-based biodiesel production process, namely sunflower meal and crude glycerol, as renewable feedstocks for the development of a biorefinery concept leading to the microbial production of poly(hydroxyalkanoates) and value-added products. The study was initiated with the utilisation of sunflower meal for the production of fermentation media in a two stage bioprocess. Sunflower meal was utilised as substrate for the production of crude enzyme consortia through solid state fermentation using the fungal strain *Aspergillus oryzae*. Fermented solids were subsequently mixed with unprocessed sunflower meal aiming at the production of a nutrient-rich fermentation feedstock. The highest free amino nitrogen and inorganic phosphorus concentrations achieved were 1.5 g/L and 246 mg/L, respectively, when an initial proteolytic activity of 6.4 U/mL was used. The FAN concentration was increased to 2.3 g/L when the initial proteolytic activity was increased to 16 U/mL.

Sunflower meal hydrolysates were mixed with crude glycerol to provide fermentation media that were evaluated for the production of poly(hydroxybutyrate) (PHB) using the bacterial strain *Cupriavidus necator* DSM 7237. Fed-batch bioreactor fermentations led to the production of 27 g/L PHB with an intracellular content of 72.9% (w/w), that was slightly improved (32.6 g/L PHB) when different bioprocessing strategies were implemented. The effect of levulinic acid as precursor for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) synthesis was evaluated in shake flasks and bioreactor cultures. Continuous feeding of levulinic acid led to the production of up to 23.4 g/L P(3HB-*co*-3HV) with an intracellular content of 66.4% (w/w) and a 3HV content of 22.5 mol%.

Subsequently, under the viewpoint of generating a multitude of end-products, sunflower meal was used for the production of an antioxidant-rich fraction, protein isolate and nutrient-rich fermentation supplements. The ethanolic extract from the sunflower meal presented high antioxidant activity, as it was measured by the $DPPH^{\bullet}$ method. The protein isolate formulated after alkaline treatment followed by acidic precipitation at the isoelectric point had a purity higher than 95% (w/w). The remaining solid and liquid streams from SFM fractionation were employed in the production of nutrient-rich hydrolysates elaborated from the on-site enzyme production for hydrolysis of residual streams. Accordingly, crude glycerol and nutrient-rich hydrolysates were evaluated in fed-

batch bioreactor fermentations for the production of 57 g/L of PHB with an intracellular content of 86.2% (w/w), entailing a conversion yield of 0.47 g/g.

To further extend the sustainability of the proposed biorefinery concept, a preparation rich in crude enzyme produced via SSF using the fungal strain *A. oryzae* was used to lyse cells of *C. necator*, enabling the recovery of intracellular biopolymer. The highest enzymatic lysis of bacterial cells was achieved at 48 °C and uncontrolled pH leading to recovery yield and purity of 98% and 96.7%, respectively. The bacterial cell lysate obtained after the separation of P(3HB-*co*-3HV) granules was evaluated as nutrient-rich supplement together with crude glycerol for the production of PHB in shake flask cultures. Moreover, the bacterial biopolymers were characterised concerning the thermal properties and molecular weight.

A preliminary techno-economic evaluation was performed to evaluate the costs associated with the production of antioxidants, protein isolate and PHB in the integrated sunflower-based biorefinery concept. Overall, it was demonstrated that process economics are significantly affected by the final market value depending on the end-uses of the protein isolate and the crude antioxidant-rich extract.

Keywords: Biorefiney, sunflower, poly(hydroxyalkanoates), crude glycerol

ΠΕΡΙΛΗΨΗ

Ο σκοπός της παρούσας διδακτορικής διατριβής έγκειται στην αξιοποίηση των αποβλήτων της διεργασίας παραγωγής βιοντίζελ με βάση τον ηλίανθο, και ειδικότερα το ηλιάλευρο (ή ηλιόπιτα) και την ακάθαρτη γλυκερόλη, ως ανανεώσιμες πρώτες ύλες για την ανάπτυξη βιο-διυλιστηρίου προς την παραγωγή πολυ-υδροξυαλκανοϊκών εστέρων. Αρχικά πραγματοποιήθηκε η χρησιμοποίηση του ηλιαλεύρου προς την παραγωγή ενός θρεπτικού υποστρώματος για μικροβιακές ζυμώσεις μέσω μιας βιο-διεργασίας δύο σταδίων. Το ηλιάλευρο χρησιμοποιήθηκε ως υπόστρωμα σε ζύμωση στερεάς κατάστασης για την παραγωγή ακα΄τέργαστων ενζύμων με το μυκητιακό στέλεχος *Aspergillus oryzae.* Εν συνεχεία, τα ζυμούμενα στερεά αναμίχθηκαν με ακατέργαστο ηλιάλευρο με απώτερο στόχο την παραγωγή ενός πλούσιου θρεπτικού υποστρώματος για μικροβιακές ζυμώσεις. Η υψηλότερη συγκέντρωση σε άζωτο ελεύθερων αμινομάδων και αμινοξέων (free amino nitrogen, FAN) και ανόργανου φωσφόρου που επτεύχθησαν ήταν 1.5 g/L και 246 mg/L, αντίστοιχα, εφαρμόζοντας αρχική πρωτεολυτική ενεργότητα 6.4 U/mL. Η συγκέντρωση του FAN αυξήθηκε σε 2.3 g/L στην περίπτωση κατά την οποία αυξήθηκε η αρχική πρωτεολυτική ενεργότητα σε 16 U/mL.

Τα υδρολύματα του ηλιαλεύρου αναμίχθηκαν με την ακάθαρτη γλυκερόλη ώστε να προκύψει το θρεπτικό μέσο ζύμωσης που αξιοποιήθηκε για την παραγωγή πολυ-υδροξυβουτυρικού οξέος (PHB) χρησιμοποιώντας το βακτηριακό στέλεχος *Cupriavidus necator* DSM 7237. Οι ζυμώσεις ημι-διαλείποντος έργου σε βιοαντιδραστήρα οδήγησαν στην παραγωγή 27 g/L PHB με ενδοκυτταρικό ποσοστό βιοπολυμερούς 72.9% (w/w), το οποίο βελτιώθηκε όταν εφαρμόστηκαν διαφορετικές στρατηγικές και σχεδιασμός στη βιοδιεργασία (32.6 g/L PHB). Η επίδραση του λεβουλινικού οξέος ως πρόδρομη ένωση για τη σύνθεση πολυ(3-υδροξυβουτυρικού- *co*-3-υδροξυβαλερικού) P(3HB-*co*-3HV) αξιολογήθηκε κατά τη διάρκεια ζυμώσεων σε αναδευόμενες κωνικές φιάλες και σε βιοανιδραστήρα. Η συνεχόμενη τροφοδοσία με λεβουλινικό οξύ συνετέλεσε στην παραγωγή έως και 23.4 g/L P(3HB-*co*-3HV) με ενδοκυτταρικό ποσοστό 66.4% (w/w) και το ποσοστό του 3HV να ισούται με 22.5 mol%.

Εν συνεχεία, υπό το πρίσμα της παραγωγής μιας πληθώρας διαφορετικών τελικών προϊόντων στα πλαίσια του βιο-διυλιστηρίου, το ηλιάλευρο χρησιμοποιήθηκε για την παραγωγή ενός κλάσματος πλούσιο σε αντιξειδωτικά, απομονωμένης πρωτεινης υψηλής καθαρότητας και θρεπτικών υποστρωμάτων για μικροβιακές ζυμώσεις. Τα αιθανολικά εκχυλίσματα του ηλιαλεύρου παρουσίασαν υψηλή αντιοξειδωτική ικανότητα σύμφωνα με τις μετρήσεις της μεθόδου DPPH$^{\bullet}$. Η απομονωμένη πρωτεϊνη που παρήχθησε έπειτα απο

την επεξεργασία με αλκαλικά διαλύματα ακολοθούμενη απο όξινη κατακρήμνιση, χαρακτηρίστηκε απο καθαρότητα υψηλότερη από 95% (w/w). Τα εναπομείναντα υγρά και στερεά ρεύματα από το διαχωρισμό-κλασμάτωση του ηλιαλεύρου εφαρμόστηκαν σε διεργασία παραγωγής θρεπτικών υποστρωμάτων για μικροβιακές ζυμώσεις, μέσω της παραγωγής ακατέργαστων ενζύμων για την ενζυμική υδρόλυση των υπολοίπων ρευμάτων. Αντίστοιχα, τα πλούσια σε θρεπτικά υδρόλυματα αναμίχθηκαν με την ακατέργαστη γλυκερόλη ως πηγή άνθρακα και εξιολογήθηκαν σε ζυμώσεις ημι-διαλείποντος έργου για την παραγωγή 57 g/L PHB με εσωκυττάριο ποσοστό βιοπολυμερούς 86.2% (w/w), συνεπάγοντας συντελεστή μετατροπής ίσο με 0.47 g/g.

Ακολούθως, με απώτερο σκόπο τη διεύρυνση της βιωσιμότητας του προτεινόμενου βιο-διυλιστηρίου, παρήχθησε μέσω ζύμωσης στερεάς κατάστασης με το μύκητα *A. oryzae* ένα εκχύλισμα ακατέργαστων ενζύμων, το οποίο εφαρμόστηκε στην ενζυμική λύση των κυττάρων του στελέχους *C. necator*, καθιστώντας δυνατή την ανάκτηση του ενδοκυτταρικού βιοπολυμερούς. Το υψηλότερο ποσοστό ενζυμικής λύσης των βακτηριακών κυττάρων επετεύχθη σε θερμοκρασία 48 °C και μη ελεγχόμενη τιμή pH, συνεπάγοντας ποσοστό ανάκτησης και καθαρότητας ίσο με 98% και 96.7%, αντίστοιχα. Το υδρολυμένο βακτηριακό διάλυμα που προέκυωε μετά το διαχωρισμό των μορίων του P(3HB-*co*-3HV) αξιολογήθηκε ως θρεπτικό υπόστρωμα σε συνδυασμό με την ακάθαρτη γλυκερόλη για τη συσσώρευση PHB κατά τη διάρκεια ζυμώσεων σε αναδευόμενες κωνικές φιάλες. Επιπρόσθετα, τα βακτηριακά βιοπολυμερή χαρακτηρίστηκαν αναφορικά με τις θερμικές ιδιότητες και το μοριακό τους βάρος.

Μια προκαταρκτική τεχνο-οικονομική μελέτη πραγματώθηκε προκειμένου να αξιολογηθεί το κόστος που σχετίζεται με την παραγωγή των αντιοξειδωτικών, της πρωτεϊνης υψηλής καθαρότητας και του PHB στα πλαίσια του προτεινόμενου βιο-διυλιστηρίου. Εν κατακλείδι, τεκμαίρεται ότι η οικονομική βιωσιμότητα της διεργασίας είναι άμεσα σχετιζόμενη με την τελική τιμή αγοράς και εξαρτάται απο τις τελικές χρήσεις της απομονωμένης πρωτεϊνης και του εκχυλίσματος των αντιοξειδωτικών.

Λέξεις κλειδιά: βιο-διυλιστήριο, ηλίανθος, πολυ(υδροξυλκανοϊκοί) εστέρες, γλυκερόλη

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1.Introduction

The constantly developing industrialized economies together with the blooming transport sector have intensified the usage of fossil fuels. For the time course of 2010 to 2030, global primary energy consumption is forecasted to increase by 1.6 % per year, expanding 39 % to world consumption by 2030 (Anonymous, 2015). However, the world proven natural gas resources were 187.1 trillion m^3 (tcm) in 2014, adequate only for 54.1 years (Anonymous, 2015). Climatic changes culminating from global warming constitute an imperative concern to be confronted. A reduction of greenhouse gas emissions (GHG) was impelled by the United Nations Framework Convention on Climate Change (UNFCCC) at 1992, entailing the targets acquainted from the Kyoto protocol (1997) which consigned the state parties to GHG emissions reduction.

Under this context, partial substitution of conventional petroleum fuels with renewable fuels will be compulsory until 2030 in EU. Biofuels are liquid or gaseous fuels generated from organic matter (biomass) through chemical or biotechnological processes characterized by reduced GHG emissions (http://ec.europa.eu/energy/en/topics/renewable-energy/biofuels). Legislation and policy frameworks have been established aiming to promote the use of renewable fuels. Apart from reduced GHG emissions that account for 40-80% less than fossil fuels, the European independence on fossil fuels for energy supply should be enhanced while it is argued that job employments should be promoted and novel agricultural market outlets would be also indicated (Skogstad, 2016). *The Directive on the Promotion of the Use of Biofuels or Other Renewable Fuels for Transport* (2003/30/EC) in 2003 established mandates for 2 % substitution of petrol and diesel in road vehicles consumption and 5.75 % in transportation sector by 2010 (Su et al. 2015). To further endeavor the production and use of biofuels, the *Energy Taxation Directive* administered tax reductions and tax exemptions for biofuels. In conjunction with policy support a significant increase in biofuels production within 10 years was observed with global bioethanol expanding from 6.4 to 23.4 billion gallons from 2003-2013 (Anonymous, 2014a).

The *Renewable Energy Directive* (RED) enacted in 2009, inaugurated an obligatory share of 20 % of total European energy demands to derive from renewable resources by

2020. In addition, each EU nation-member separately is committed to integrate a 10 % share of biofuels in the transport sector by 2020 (https://ec.europa.eu/energy/en/topics/renewable-energy/renewable-energy-directive). Transportation sector accounts for the vast percentage of GHG emissions, presenting a massive expansion among any other sector, projecting an increase of 80 % in energy consumption and CO_2 emissions by 2030 (Luque et al., 2008). Nonetheless, it is envisaged that by 2030, 25 % of road vehicle fuels will be supplied by renewable resources (Anonymous, 2006).

Biofuels deriving from biomass, including first generation and second generation biofuels, could help to overcome fossil fuel dependency and cut down CO_2 emissions. Apart from that, production of biofuels combined with value-added products targeting diversified market outlets will endorse business opportunities and job creations while enhancing economic feasibility. World annual ethanol and biodiesel production are expected to increase by 180 billion liters and 42 billion liters by 2021 respectively (Anonymous, 2012a). United States and Brazil represent the dominant producers of ethanol market while EU is unequivocally the leader in production and consumption of biodiesel (Anonymous, 2012a). EU 25 currently generates 2 Mtoe of liquid biofuels, corresponding to less than 1 % of the market (Anonymous, 2016), while the total biofuels consumption in EU transportation was 13.6 Mtoe in 2013, exhibiting an integration rate of 4.7 % (Anonymous, 2014b). Biodiesel share was 79 % of the total biofuels consumption for transport in 2013, with Germany, France and Italy being the leading countries in biodiesel use (Anonymous, 2014b). To counterbalance biodiesel requirements in EU, that are projected to rise to 27 billion liters by 2020 (Anonymous, 2015), it is evident that production rates should be induced, indicatively to meet the target of 7 % final energy consumption to be derived from first generation biofuels until 2020 (Anonymous, 2014b). Oilseed crops comprise the main onset feedstock for biodiesel production, including rapeseed, sunflower, soybean, groundnut etc. Rapeseed oil was the first to be implemented in biodiesel production process and currently constitutes the primary material for biodiesel in Europe followed by sunflower, cultivated in the warmer areas (http://www.eubia.org/index.php/about-biomass/biofuels-for-transport/biodiesel). Indicatively, $5{,}500 \times 10^3$ million tons and 270×10^3 million tons of rapeseed and sunflower oil respectively were utilized in 2015 to yield $11{,}000 \times 10^3$ million tons of biodiesel (Anonymous, 2006; 2012).

On the other hand, apart from fuel production, petroleum refineries contribute to the formation of a vast variety of commodities like polymers and chemicals used in everyday life. In particular, 90 % of the onset feedstock in plastics sector depends on fossilized material, corresponding to a proximate estimation of 6 % of the global oil production (http://www.newplasticseconomy.org/). World annual plastic production rates continuously increase, amounting to 299 million tons in 2013, with 19 % deriving from EU (57 million tons) (Aeschelmann and Carus, 2016). Plastic waste upstream managing encountered only with 25.2 million tons of post-consuming waste with a 38 % share ended up in landfill disposal (http://www.european-bioplastics.org/news/publications/). If the scenario of the constantly emerging plastic utilization does not moderate from the ongoing 3.5 -3.8 % per year, oil consumption in the plastics industry will rise to 20 % of the overall consumption by 2050 (Anonymous, 2016). Correspondingly, the depletion of finite petroleum sources will make inevitable the manufacture of plastic and related products from renewable resources.

Biodegradable polymers, including agro-polymers like polysaccharides and biopolyesters produced via chemical or biological routes, exhibit an alternative to conventional petroleum derived plastics. Conferring to ASTM standard D-5488-94d and European norm EN 13432, ‘‘biodegradable’’ is determined as ‘‘capable of undergoing decomposition into carbon dioxide, methane, water, inorganic compounds, and biomass’’ (Averous and Pollet, 2012). Agro-polymers refer to biopolymers extracted from biomass, consisting of polysaccharides like thermoplastic starch, cellulose, chitin and chitosan as well as polypeptides including whey and soya protein, wheat glucan, casein and collagen among others. On the other hand, biopolyesters are categorized based on their synthesis, which can be carried out via microbial routes like polyhydroxyalkanoates, poly(3-hydroxybutyrate), poly(ε-caprolactone), polylactic acid and bacterial cellulose or can be achieved by synthetic polymerization as in the case of polybutylene succinate and poly(vinyl alcohol). Global biobased plastics production is projected to expand to 3450 thousand metric tons in 2020 entailing a 6 % increase in the time frame from 2013 to 2020, with starch plastics, PLA and PHA demonstrating the most flourishing development based on industry estimates (Shen et al., 2009).

In order to disintegrate global industry reliance on fossilised energy resources it is evident that a transition towards establishing bio-based and sustainable economies that will employ the exploitation of renewable resources like biomass is required. Refining of renewable resources, including various agri-industrial waste streams and by products can

be performed through chemical and biological routes. The implementation of biotechnology enables the conversion of biomass feedstocks to diversified end products including biofuels, platform chemicals and biopolymers. Integrated biorefineries that will implement the production of first and/or second generation biofuels coupled with the synthesis of value added products will contribute to this reconstruction of the present industrial economy.

According to a definition provided by the International Energy Agency (IEA) in the IEA Bioenergy Task 42, a biorefinery is described as "*the sustainable processing of biomass into a spectrum of marketable products and energy*" (Cherubini et al., 2009). Biorefinery processing schemes should be evolved in an equivalent manner that petroleum refineries have done so far, yielding a variety of products like fuels and chemicals. Biorefinery concepts constitute a significant aspect of the future bioeconomy era where renewable raw materials, such as widely available lignocellulosic biomass in conjunction with industrial by-products and waste streams, will be utilized for the production of value-added commercial products, including biofuels, chemicals, biodegradable polymers and antioxidants among others. Implementation of biotechnology enacts as a sustainable pathway for various sectors, exhibiting a substantial tool for the conversion of biomass into an ample range of products. Designing of biorefinery concepts should always aim to enhance productivity and conversion yields in parallel to cost effective upstream and downstream processes and minimization of waste stream generation, under the overall target of sustainability issues. However, the establishment of a new industrial sector is a difficult task not only because of the viability of new technological advances but also because the transition from the non-renewable to the sustainable era should occur smoothly in order to avoid job losses and economic turmoil. A smooth transition can be achieved through the integration of sustainable processing schemes in those conventional industrial plants that generate waste and by-product streams suitable for bioconversion or green chemical conversion into value-added products.

CHAPTER 2

LITERATURE REVIEW

2.1.Introduction

The intensive usage of finite fossil resources intensifies environmental concerns, which when combined with their imminent exhaustion creates the need for developing sustainable feedstocks for the production of fuels and chemicals. Alternative and innovative approaches constitute a prerequisite to sustain industrial cost-effectiveness and establish economic progress entailing the creation of new jobs. Biorefinery concepts constitute a significant aspect of the future bio-economy era where renewable raw materials, such as widely available lignocellulosic biomass in conjunction with industrial by-products and waste streams, will be utilised for the production of value-added commercial products, including biofuels, chemicals, biodegradable polymers and antioxidants among others. The development of sustainable industrial processes necessitates the exploitation of renewable sources of carbon. Koutinas et al. (2014) presented the potential to restructure various conventional industrial sectors (e.g. food industry, pulp and paper industry, 1st generation biofuel production processes) into integrated biorefineries through valorisation of waste and by-product streams.

Under this context, a literature review will be presented in this chapter focusing on valorisation of waste and by-products streams occurring from various industrial and food manufacturing processes targeting the production of poly(hydroxyalkanoates) (PHA). Furthermore, this chapter presents the fundamentals of solid state fermentation and the state of the art regarding the utilisation of glycerol by the microorganism used in this study for the production of PHA.

2.2.Biofuels production: raw materials, processes and perspective

The imminent depletion of fossil raw materials and increasing environmental concerns have paved the way towards the development of a sustainable bio-based economy. Under this context, partial substitution of conventional petroleum derived fuels will be compulsory until 2030 in EU in an attempt to comply with the international agreement of Kyoto protocol aiming to reduce greenhouse gas emissions (GHG). The transportation sector is responsible for the highest GHG emissions, thus in the framework of EU Energy

Policy it is envisioned that by 2030, 25 % of road vehicle fuel will be provided by renewable resources, while each nation-member of the European Union (EU) is committed to integrate biofuels in the transportation sector, replacing conventional fossil fuels by 10 %. It is indisputable that biofuels demand is projected to increase, enhancing the autonomy of EU regarding fuel production and consumption (Anonymous, 2006).

Biofuels are liquid or gaseous fuels generated from organic matter (biomass) through chemical or biotechnological processes characterized by reduced GHG emissions (Naik et al., 2010). The most common biofuels are bioethanol, an alcohol produced mainly from food crops or lignocellulosic biomass and biodiesel, obtained principally from oilseeds and waste animal fats or vegetable oils (http://www.nrel.com). More specifically, different subdivisions or "generations" of biofuels can be distinguished according to the raw material employed for their production.

- *First (1^{st}) generation biofuels*

First generation biofuels are generated from agricultural products, including sugar, starch and oil crops. These feedstocks can also be implemented in food production, thus there is direct competition between fuel production and human consumption. The primary three types of first generation biofuels include bioethanol, biodiesel and biogas. Conversion of raw materials for first generation biofuels implements conventional chemical and/or biotechnological methods and technologies like fermentation and trans-esterification (Naik et al., 2010; Balat and Balat, 2009). Prior to fermentation, chemicals and/or enzymatic pretreatment is required in order to decompose polysaccharides to easily assimilable sugars for yeast and bacteria. Production of biodiesel can be achieved through homogeneous or heterogeneous catalytic reactions, notably transesterification of triglycerides to generate fatty acid methyl esters (FAME).

- *Second (2^{nd}) generation biofuels*

Second generation biofuels are generated by plant and wood biomass and waste vegetable oils and fats. Plant biomass may refer to remaining non-edible parts of crops, such as stems, leaves and husks, as well as plants or crops that are not suitable for human consumption, such as switchgrass and jatropha (Naik et al., 2010, Limayem and Ricke, 2012) Lignocellulosic biomass represents a renewable and sustainable feedstock found around the world in vast quantities. The main components are hemicellulose and cellulose, which can be hydrolyzed to yield mainly C5 and C6 sugars and lignin that occurs as by-product but could be used for energy generation (Luque et al., 2008). Industrial production

of second generation biofuels is not yet cost-competitive since the recalcitrant nature of lignocellulose requires several pretreatment steps in order to decompose lignin and constitute cellulose and hemicellulose susceptible to hydrolysis and subsequent fermentation. The pretreatment steps include physical, thermochemical and/or enzymatic processes. A schematic illustration of pretreatment methods employed for biofuels production is presented in Figure 2.1.

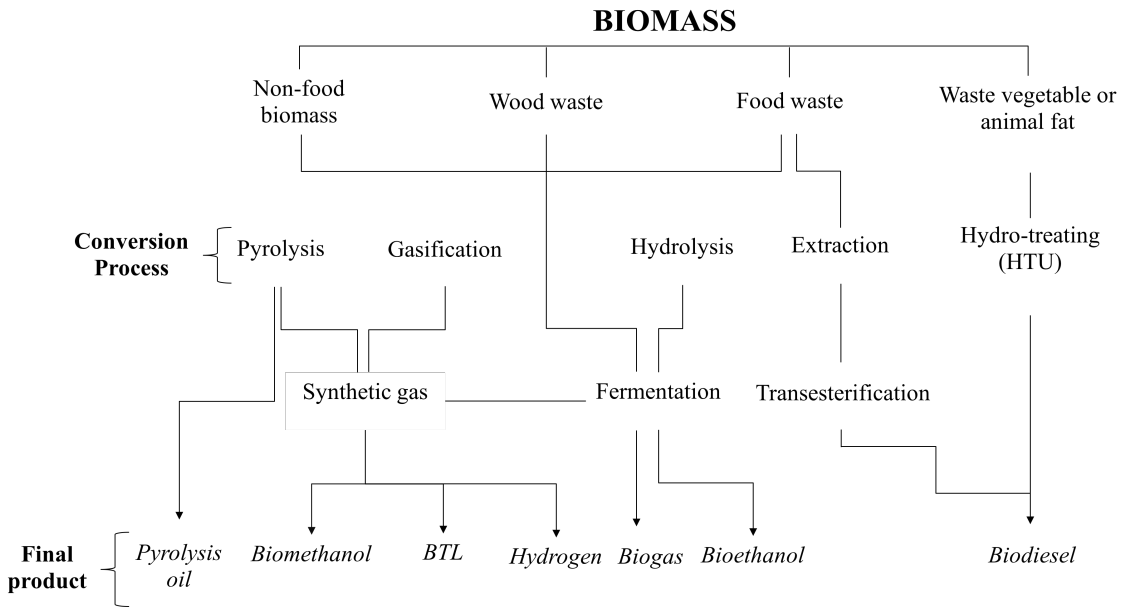

Figure 2.1 Pretreatment methods implemented in biofuels production (Adapted from Stöcker, 2008)

- *Third (3rd) generation biofuels*

Third generation biofuels are defined as the fuel or fuel precursor produced from biomass sources produced on non-arable land, employing integrated processes. For instance, microalgae can be fractionated into various value-added components for the production of biodiesel, ethanol and jet fuel. Furthermore, the microbial oil (or single cell oil) produced from oleaginous yeast has emerged as a potential feedstock for biodiesel production. Microalgae comprise of various photosynthetic microorganisms in aquatic environments, like *Chlorella* sp. or *Spirullina* sp. that utilise solar power and CO_2 through photosynthetic routes to proliferate and accumulate lipids. Microalgae produce 15-300 times more oil for biodiesel production than conventional vegetable oil crops (e.g. soybean), while the simultaneous utilisation of CO_2 entails the potential to mitigate GHG emissions (Dragone et al., 2010). Cultivation of microalgae is established approximately 40 years now for nutrition purposes, pigments or aquaculture, usually on raceway or circular open ponds. Industrial implementation for biofuel production is hindered by the

high cost of manufacture deriving mainly from the relatively low volumetric productivity of lipids and the extraction methods employed. Thus, research has been targeting the development of improved strains, photobioreactor design and extraction methods in order to increase commercialisation of microalgae cultivation (Davis et al., 2011, Dragone et al., 2010).

- *Fourth (4^{th}) generation biofuels*

Fourth generation biofuels could be derived from dedicated engineered plants or biomass, possessing the capability to grow on non-agricultural land, while break-down of biomass in not required to extract the lipids. Continuously on-going developments on plant biotechnology and in carbon capture and storage methods provide the potential to incorporate engineered plants with adjustable characteristics for biofuels production. Consolidating these high yielding energy plants with bioconversion methods for carbon capture and storage it is envisaged to generate carbon-negative biofuels and energy.

2.1.1. Bioethanol

Bioethanol is a liquid alcohol presenting an alternative to gasoline fuel most frequently mixed with gasoline to form the blend E85 (85 % gasoline and 15 % bioethanol). Brazil and United States represent the leading countries in export and production of bioethanol (Table 2.1), corresponding to more than 80 % of the total worldwide production (Luque et al. 2008).

Table 2.1 Distribution of world fuel ethanol production for 2014

Continent	Production capacity (millions of t)
United States	54.1
Brazil	23.4
Europe	5.5
China	2.4
Canada	1.9
Thailand	1.2
Argentina	0.6
India	0.6
Rest of the world	3.3

Sugar cane is the main raw material utilised in Brazil for bioethanol production, while US production relies on corn crops. The manufacture of ethanol is achieved through

a two-step bioprocess, implementing first the hydrolysis of starch into glucose. Subsequently, yeast or bacteria are added in the culture to ferment sugars towards alcohol production. The enzymatic hydrolysis step can sometimes occur in parallel with the fermentation step (simultaneous saccharification and fermentation). Recent research is being directed towards the utilisation of lignocellulosic material for bioethanol production. Apart from improving the bioconversion yield in the pretreatment and fermentation process, one of the major issues also to be considered, is employing yeast and bacteria strain that will be able to metabolize xylose deriving from hemicellulose hydrolysis. Bioethanol separation and purification after fermentation is achieved via distillation followed by a dehydration step to increase the final concentration of fuel grade ethanol (Luque et al., 2008, Balat et al., 2008).

2.2.2. Biodiesel

Biodiesel is an alternative fuel manufactured from renewable feedstocks, mainly vegetable oils, animal fat and waste cooking oils to be further used in diesel engines (Ma and Hanna, 1999) in blends ranging from 6 % to 100 %, with B20 being the most common blend (Knothe, 2015). Biodiesel synthesis occurs from the chemical reaction of triglycerides with an alcohol (usually methanol), in the presence of a catalyst, usually a strong base like sodium or potassium hydroxide. The final products of the transesterification reaction are a mixture of esters and glycerol, as shown in Figure 2.2 (Van Gerpen, 2005).

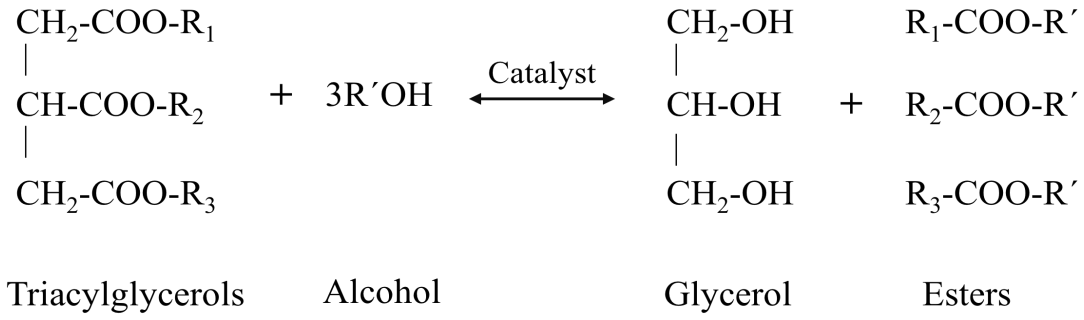

Figure 2.2 Transesterification reaction of triglycerides with base catalyst to produce esters and glycerol

In 2010, the worldwide production of biodiesel reached more than 16×10^6 t (Lamers, 2012) rising up to 26×10^6 t in 2014 (Anonymous, 2015). The United States of America, Brazil and Germany are among the leading producers, while 39 % of global production is located in Europe, yielding 11.5 billion litres (Anonymous, 2015). Germany

remains the predominant biodiesel producer, displaying tax exemptions even up to 100%, followed by France, Italy, Austria and Czech Republic (Anonymous, 2006). Tax reductions and policy mandates entail an increase in biodiesel production and utilisation in the transportation sector aiming to reach the defined targets of the *Renewable Energy Directive*. In particular, 10.75 million t of oil equivalent were consumed for transportation in the EU-28 in 2013 (Anonymous, 2014b).

Biodiesel is mainly composed of fatty acid methyl esters (Anonymous, 2015) deriving from the transesterification of triglycerides with methanol. The raw materials that can be used for the production of biodiesel are both edible (e.g. rapeseed, soybean or palm) and non-edible (e.g. *Jatropha*) oilseeds. Biodiesel production is mainly achieved from soybean in the USA, rapeseed (or sunflower in lower quantities) in Europe and palm oil in South-East Asian countries. Second generation biodiesel can be also achieved from industrial by-products including waste cooking oils and animal fat (Anonymous, 2015) while recently research is focused on the valorisation of lipids extracted from food waste (Canacki, 2007; Karmee et al., 2015). In addition, the development of advanced generation biorefineries for the production of microbial oil by oleaginous microorganisms will generate additional sources of triglycerides for biodiesel and oleochemical production (Koutinas and Papanikolaou, 2011; Meng et al., 2009).

Oilseed-based biodiesel production plants generate oilseed meals and crude glycerol as by-product streams. It is projected that by 2022, the annual worldwide oilseed production will increase by 28%, while the respective oilseed meal production is projected to increase by 23% reaching around 315×10^6 t. Domestic production of oilseed meals in EU-28 reached 29.6×10^6 t in 2014/2015 (Anonymous, 2015b). It is also expected that the annual worldwide biodiesel production from edible vegetable oils will increase to approximately 30×10^6 t by 2022 (Koutinas et al., 2014c), corresponding to the generation of approximately 3×10^6 t of crude glycerol, assuming that 10% (w/w) is the approximate stoichiometric glycerol yield during transesterification of triglycerides with methanol.

Sunflower has attained interest for biodiesel production because its cultivation is favoured by the European Common Agricultural Policy (Panoutsou et al., 2008). Sunflower (*Helianthus annuus*) is an annual plant cultivated either for its seeds or for extracting the oil due to the high oleic acid content found in the seed. The cultivation of sunflower is constantly increasing and is estimated to insignificantly alternate based on market and protein demands in EU-28 (Anonymous, 2015b). Enhanced crop yields entailed an increase in production that reached a capacity of 7.5×10^6 t in 2015/2016,

expected to further increase to 8.4×10^6 t out of a global production of 41×10^6 t in the year 2016/2017 (http://apps.fas.usda.gov/psdonline/psdquery.aspx). Sunflower oil is used either as food or as feedstock for industrial purposes such as biodiesel production. Sunflower is the third most important oilseed regarding annual oil extraction (around 14×10^6 t) for both food and biodiesel production and the fourth most important oilseed regarding annual oilseed meal production (around 15×10^6 t) (Koutinas et al., 2014c).

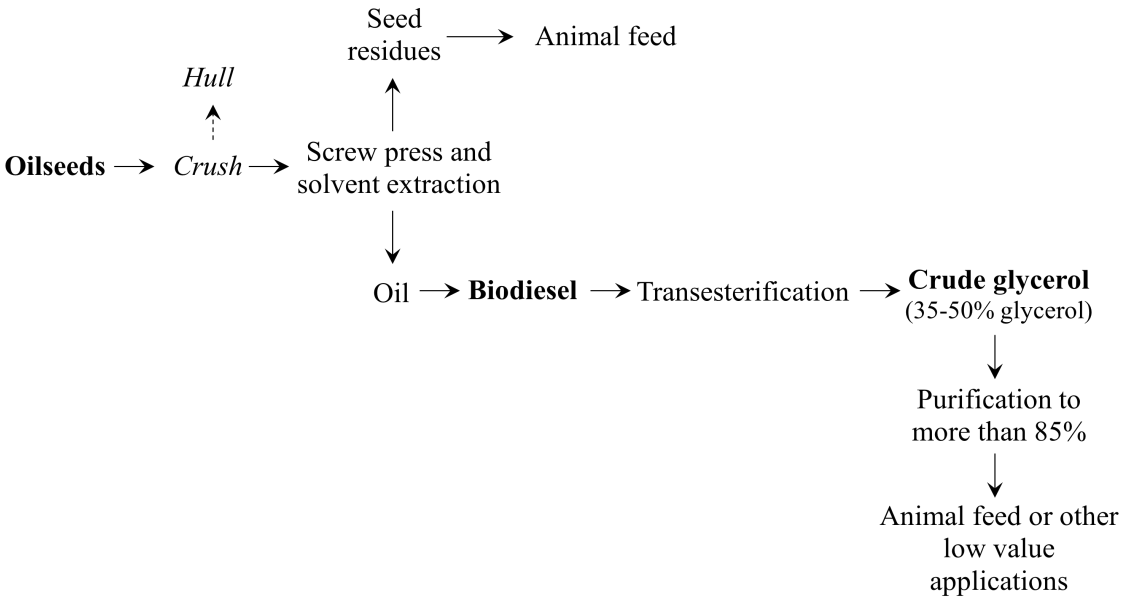

Figure 2.3 Biodiesel production process from oilseeds

The growing demand for biodiesel production from oilseeds is associated with the generation of high quantities of by-products, namely crude glycerol and oilseed meals. Sunflower meal (SFM) is the protein-rich residue obtained after oil extraction from the seed, while crude glycerol is produced (10 % w/w) as a by-product of oil transesterification with methanol (Papanikolaou et al., 2002).

2.3 Development of biorefinery concepts

Refining entails the exploitation of all value-added fractions that could be derived from a given raw material for the production of various end-products with diversified market outlets leading to efficient utilisation of the resource with simultaneous maximisation of profit. The deployment of refining principles is the way for successful implementation of industrial processes. For instance, the corn wet milling industry (Peckham, 2001) and petroleum refineries demonstrate the historical evolution of two industrial sectors that

exploited technological innovation and feedstock refining. In an analogous manner, biorefineries should rely on refining of biomass in order to maximise the efficiency of resource utilisation. The development of integrated biorefineries exploiting waste and by-product streams generated by current industrial sectors (e.g. biodiesel production processes from oilseeds) could lead to the production of chemicals, biopolymers, food, feed and high-value ingredients (Koutinas et al., 2014c). Under the target of producing a multitude of end-products, the development of biorefinery concepts entails a comprehensive insight in the fractionation of biomass, bioprocessing, chemical conversion and process economics among others (Naik et al., 2010). Whole-crop, lignocellulosic and green biorefineries represent the initial biorefining concepts demonstrated in the inauguration of the biorefinery era (Kamm et al., 2006). Physical, chemical and biological processes should be combined and incorporated in pretreatment, bioconversion and separation stages in order to design sustainable and cost-effective biorefinery concepts that will be introduced in the future bio-economy era. The current industrial processes used for the production of biofuels or bio-based commodities are comprised of individual production line rather than integrated processing concepts leading to the production of multiple end-products derived from the same feedstock (Cherubini et al., 2010).

A wheat-based biorefinery developed in the Satake Centre for Grain Process Engineering (SGCPE) constitutes an example of cereal-based biorefinery entailing the production of biopolymers, biofuels and chemicals. Wheat fractionation is carried out in order to exploit all wheat components. In this refining process, pearled wheat is initially produced via wheat pearling leading to the separation of bran layers. The pearled wheat that is rich in endosperm is subsequently employed to generate a glucose-based nutrient supplement by enzymatic hydrolysis using amylolytic enzymes. A nitrogen-rich stream was produced via by fungal autolysis exploiting the fungal biomass that was produced during the production of amylolytic enzymes. Creating appropriate mixtures of the glucose and nitrogen-rich streams leads to the production of generic fermentation feedstocks that were further employed in the production of succinic acid, ethanol or PHB (Du et al., 2008; Arifeen et al., 2007; Koutinas et al. 2007; Xu et al. 2010). Whole wheat flour, pearled wheat flour and wheat milling by-products were also implemented as substrates for fungal cultures during solid state or submerged fermentations to generate enzymes for the formulation of fermentation media (Du et al., 2007; Du et al. 2008). Wheat bran deriving from the first fractionation step (pearling) can be used in diversified applications. Accordingly, it is envisaged that oilseed meals, specifically rapeseed meal or sunflower meal could be implemented in the development of an advanced biorefinery concept.

Oilseed meals are nowadays predominantly used as animal feed supplements. The only mature industrial process based on oilseed meals has been developed for the production of protein concentrates or isolates from defatted soybean meals. Soybean protein concentrates (SPC) are produced from defatted soybean meal by three processes: 1) the aqueous alcohol wash process, 2) the acid wash process and 3) the heat denaturation/water wash process (Berk, 1992a). The aqueous alcohol wash process is based on the increase of protein and dietary fibre content via treatment of defatted soybean meal with a 70 – 90% aqueous ethanol solution (Lusas and Riaz, 1995). After ethanol removal and recycling, the liquid fraction is converted into a syrup (known as soy molasses) that contains predominantly carbohydrates and lower quantities of lipids and proteins (Long and Gibbons, 2013). Soy molasses is mainly used as a caloric ingredient and as animal feed additive (Berk, 1992a). Soybean meal is also used for the production of protein isolates after sequential alkaline treatment to dissolve the proteins followed by protein precipitation at the isoelectric region (around pH 4.5) (Berk, 1992b). The residual streams from this process is the solid residue that remains from the alkaline extraction stage and the syrup (known as soy solubles or whey) produced via drying of the liquid supernatant that remains after acidic protein precipitation (Berk, 1992b). The solid residue is used as animal feed or food supplement, while soy soluble is considered a waste stream. Soy molasses and/or soy soluble have been evaluated for the production of fermentation products such as ethanol, butanol, sophorolipids and PHAs (Qureshi et al., 2001; Solaiman et al., 2006, 2007; Siqueira et al., 2008; Long and Gibbons, 2013). Besides food uses, protein isolates have been evaluated for non-food applications such as the production of formaldehyde-free soy adhesives as alternatives to commercial formaldehyde-based wood adhesives (Mo and Sun, 2013).

Research has never focused on the development of biorefinery concepts utilising by-products from sunflower-based biodiesel production processes involving the extraction of value-added co-products followed by the production of fermentation products depending entirely on the original resource for nutrient supply. Consequently, in a subsequent section, the literature review will present the development of integrated biorefinery concepts focusing on the production of PHAs as the primary target product. Furthermore, it will be demonstrated that it is feasible to develop advanced sunflower-based biorefineries leading to the production of PHAs, antioxidant-rich extracts and protein isolates.

2.4 Sunflower meal

Sunflower meal is primarily utilised as animal feed especially for ruminants, or poultry, mainly in Spain, France, Italy and UK (Anonymous, 2015b). The composition of sunflower meal depends on the sunflower variety, the cultivation conditions, as well as the industrial process regarding the removal of the hull and the extraction of oil. The application of dehulling and oil removal via combined mechanical pressing and solvent extraction results in sunflower meals with higher protein contents. Dehulling and complete removal of the oil usually results in sunflower meals with a protein content higher than 30% (https://www.sunflowernsa.com/). Nonetheless, the dehulling process is strongly correlated with the subsequent utilisation of the hull, for example to yield energy via combustion aiming to fulfil the demands of an industrial plant (Koutinas et al., 2014c). Apart from energy production, sunflower hull has been valorised in biobased materials (panels, bricks, tiles etc) and in soil bioremediation (Koutinas et al., 2014c). Besides the high protein content, sunflower meal contains significant amount of carbohydrates (e.g. pectins, pentosans, cellulose), phenolic compounds and numerous micro- and macronutrients. Compared to soybean and rapeseed meals, sunflower meal has higher fibre and lower protein contents (nat. sunflower assoc.) and its application as animal feed is constantly increasing (Anonymous, 2015b). Sunflower meals have also been evaluated as fermentation feedstock mostly in solid state fermentations using filamentous fungi (Lomascolo 2012). Biotechnological applications of sunflower meal resulted in the production of antibiotics (Sircar 1998, Sarada 1998), polyunsaturated fatty acids (Jacobs 2010), biosurfactants (Jadhav 2011) and enzymes, e.g. endoxylanase (Rajoka 2005).

Integrated oilseed-based biorefineries could be developed through combined microbial bioconversion of sunflower meal and crude glycerol and extraction of value-added ingredients contained in oilseed meals. Besides conventional uses as animal feed supplements, oilseed meals could be fractionated into various value-added products (Coats et al., 2001; Das Purkayastha et al., 2013; Kannan et al., 2012; Long and Gibbons, 2013) such as:

- protein concentrates, isolates or respective hydrolysates with a wide spectrum of end-uses (e.g. feed and food additives, adhesives, bioactive peptides preventing chronic diseases),
- antioxidant-rich formulations for non-food applications, carbohydrate extraction (e.g. pectin from rapeseed meal) or hydrolysis,
- glucosinolate-derived biopesticides

- fermentation products derived from molasses (e.g. soy-based) generated during protein extraction processes.

Ren et al. (2010) proposed "reactive extraction", where biodiesel is produced via direct contact of macerated seeds with methanol and catalyst, as an intensified process for both biodiesel production and extraction of value-added by-product streams (e.g. antioxidants). The production of protein concentrates and isolates has been investigated from all oilseed meals but industrial implementation has occurred only in the case of soybean meal. Villanueva et al. (1999) presented a process employing alkaline extraction followed by isoelectric precipitation for the production of a protein isolate fraction from sunflower meal. The production of enzymatic hydrolysates of oilseed protein isolates could increase protein separation from the original oilseed meal and diversify industrial applications due to improved functional properties of the hydrolysates (Vioque et al., 2000). Preliminary results have shown that sunflower protein isolates could be used for the production of biodegradable films (Rouilly et al., 2006).

Oilseed meals could be also used for the extraction of value-added ingredients, such as antioxidant-rich fractions with food or non-food applications. De Leonardis et al. (2003) reported that phenolic compounds from sunflower could be used as antioxidant additives to improve the oxidative stability of sunflower oil. Das Purkayastha et al. (2013) successfully employed an antioxidant-rich fraction extracted from rapeseed press-cake as an additive in biodiesel produced from waste cooking oil in order to increase its storage stability. The main phenolic compound in sunflower seeds and sunflower meal is chlorogenic acid (5-caffeoylquinic acid) (Weisz et al., 2009). CGA possesses both nutritional functions (e.g. improvement of body's resistance against diseases, prevention of oxidative damage, reduction of fat) and pharmacological activities since it can act as antioxidant. It also possesses antidiabetes, antihypertension and chemo-preventive properties (Zhao et al., 2012). Chlorogenic acid could be also used for non-food applications such as the production of sunscreen compositions (Cheetham and Banister, 2000). Ethanolic plant extracts containing chlorogenic acid show anti-fungal properties with potential use as fungicides (Navarro et al., 2012).

The polysaccharides contained in oilseed meals could be hydrolysed for the production of sugars that could be used as carbon sources in fermentation processes. Camargo et al. (2014) reported that acid hydrolysis of sunflower cakes and meals could lead to the production of hydrolysates rich in glucose, xylose and arabinose, whereas the remaining solid stream was rich in cellulose.

2.5 Crude glycerol

Glycerol, also known as 1,2,3-propanetriol or glycerine, constitutes an environmentally benign molecule that can serve as a renewable resource in diversified applications. Glycerol is a trihydroxyalcohol, non-toxic, viscous, odourless and colourless hygroscopic liquid. Traditionally dating back to 1940s, glycerol was synthetically manufactured, exhibiting as primary end-uses the production of nitro-glycerine and alkyd resins, while the market price of pure glycerol was as high as 2500-4000$/t for pharmaceutical industries (Ciriminna et al., 2014). Apart from pharmaceuticals, glycerol top market outlets are also directed in food, cosmetic and tobacco industry (Chatzifragkou and Papanikolaou, 2012; Ayoub and Abdullah, 2012). Worldwide glycerol production was reckoned at 2,444 t in 2015, projecting an annual increment of more than 6.5 % from 2015 to 2022 that will contribute to an analogous development in glycerol market size (Radiant Insight Inc., 2015). This growth is attributed mainly to the upcoming demand for glycerol in countries like Southeast Asia and Africa, that until now it constituted a luxury commodity.

Glycerol is generated as a by-product of biodiesel and soap manufacturing industry; hence production of glycerol is strongly associated with market demand and the production of these products. During biodiesel production, crude glycerol (raw glycerol, crude glycerine or bioglycerol) is produced (10 %, w/w) as a by-product of oil transesterification with methanol (Papanikolaou et al., 2002; da Silva et al. 2009). Crude glycerol will also be generated via the transesterification of other sources of triglycerides including cooking oil, animal tallow and jatropha (Chatzifragkou and Papanikolaou, 2012) that are projected to enter the biodiesel manufacturing plants. The upcoming boost in biodiesel production will induce a proportional increase in crude glycerol synthesis. Crude glycerol production increased three fold from 2003 to 2006 and is estimated to expand up to 6 million t in 2025 (Ciriminna et al., 2014). Noteworthy, more than 67 % of the pure glycerol available in the international market in 2014 was obtained from crude glycerol occurring from the biodiesel industry (Luo et al., 2016). The outcome of the increasing production of biodiesel was a reduction in the glycerol market price that reached almost zero or negative values. The industrial production of pure glycerol by Dow Chemical was terminated in 2007 and by Procter & Gamble Chemicals was terminated in 2006 also highlighting the saturation of the glycerol market (Anitha et al., 2016). Hence, it is evident

that new outlets for crude glycerol surplus should be established, focusing on the utilisation of glycerol in biorefineries.

Crude glycerol streams produced from biodiesel plants have purities in the range of 18 – 90% (w/w) (Luo et al., 2016). The purification of crude glycerol is an expensive process. For this reason, the application of crude glycerol refining is closely dependent on the industrial volume capacity, imposing a bottleneck for small to medium producing plants that cannot sustain the high cost of refining (Thompson and He 2007; Anitha et al., 2016). The worldwide refined glycerol market is rather limited achieving an approximate production of 2 billion pounds per year and a market value of $1 billion (Anonymous, 2005, Anitha et al. 2016). Refined glycerol has a purity of 99.5 – 99.7 % and can be divided into three different types, namely technical grade, USP glycerol and Kosher glycerol (Ayoub et al., 2012).

The composition of crude glycerol varies significantly depending on the initial feedstock (e.g. type of vegetable oil, tallow) as well as the alcohol and catalysts utilised during the transesterification process. Crude glycerol refining entails the following major steps: 1) neutralisation and washing with water to remove soaps and salts, 2) flash or vacuum distillation to recover methanol and water (Luo et al., 2016). Several purification methods have been previously presented in the literature and comprise of ion-exchange resins, membrane separation technology, vacuum distillation under reduced pressure and activated carbon adsorption (Nanda et al., 2014; Luo et al., 2016). The initial crude glycerol stream that results from the biodiesel separation stage, usually achieved via decanting, contains 40 – 50 % (w/w) glycerol (Leung et al., 2010; Chatzifragkou and Papanikolaou 2012), while evaporation of methanol and water increases the glycerol purity up to 77 – 90 % (w/w) (Mothes et al., 2007, Ciriminna et al., 2014). The main contaminants in crude glycerol are water, methanol, residual fatty acids and corresponding esters, mono- di- and triglycerides, and salts (NaCl or K_2SO_4) in varying proportions depending on the extent of glycerol purification (Mothes et al., 2007).

The partial purification of glycerol is essential in order to recycle the surplus methanol in the biodiesel production stage and also produce a crude carbon source for the production of various metabolic products via microbial bioconversion. However, the impurities present in crude glycerol could be inhibitory for the growth of microorganisms. Nevertheless, glycerol exhibits an easily assimilable carbon source for a plethora of microorganisms and has been widely evaluated in bioconversions for the production of various chemicals (da Silva et al. 2009), including 1,3-propanediol (Chatzifragkou et al,. 2011; Vivek et al., 2016), 2,3-butanediol (Metsoviti et al., 2012) succinic acid (Lee et al.,

2001; Vlysidis et al., 2011), ethanol (Ito et al. 2005; Clomburg and Gonzalez, 2013), citric acid (Papanikolaou et al., 2002) and poly(hydroxyalkanoates) (Ashby et al., 2004, Solaiman et al., 2006; Cavalheiro et al., 2012; Kachrimanidou et al., 2014; Dimou et al., 2015). Consequently, given the fact that crude glycerol is nowadays available in surplus quantities, biodiesel plants could be restructured into novel biorefineries through the integration of glycerol-based bioconversions in the existing processing lines for the production of various chemicals. The prospect of integrating the production of poly(hydroxyalkanoates) in biodiesel production facilities has been addressed in various literature-cited studies that will be presented in a subsequent section.

2.6 PHA structure and properties

Under the frame of constructing a sustainable bio-based economy, the global manufacturing capacity of bio-based polymers is projected to increase three-fold from 5.7 million t in 2014 to 17 million t in 2020 with poly(hydroxyalkanoates) expected to show the second most dynamic development (Aeschelmann and Carus, 2016) Poly(hydoxyalkanoates) is a group of polyesters produced intracellularly as carbon and energy reserve granules. Intracellular accumulation of PHAs is usually observed when one nutrient (e.g. nitrogen, phosphorus, oxygen) is present in the fermentation broth in limiting concentration, while, in the same time, there is available an excess source of carbon. Most bacterial strains, such as *Cupriavidus necator* (formerly classified as *Ralstonia eutropha* and *Alcaligenes eutrophus*), accumulate PHAs as secondary products under nutrient limiting conditions. However, there are some bacterial strains, such as recombinant *Escherichia coli* and *Alcaligenes latus*, that synthesise PHAs as a primary metabolite during microbial growth.

The generic chemical formula of PHAs is presented in Figure 2.4, where *R* represents alkyl side groups of varying chain lengths. The most common monomers are 3-hydroxybutyrate (3HB, m = 1 and R = CH_3), 3-hydroxyvalerate (3HV, m = 1 and R = CH_2CH_3) and 3-hydroxyhexanoate (3HHx, m = 1 and R = $CH_2CH_2CH_3$). The simplest and most widely studied member of the PHA family is called poly-(3-hydroxybutyrate) (PHB). Three of the most widely studied copolymers are poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV), y = 1], poly-(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx), y = 2] and poly-(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB]. Recent research has also focused on the production of terpolymers, such as P(3HB-*co*-4HB-*co*-3HV) and P(3HB-*co*-3HV-*co*-3HHx) (Bhubalana

et al., 2010; Cavalheiro et al. 2012). PHAs can be categorized according to the number of carbon atoms into short-chain-length (monomers containing 3-5 carbon atoms) and medium-chain-length (monomers containing more than 6 carbon atoms) (Ashby et al. 2011; Du et al., 2012). A historical overview of PHA research and industrial applications are presented in previous publications (Verlinden et al., 2007; Castilho et al., 2009; Du et al. 2012).

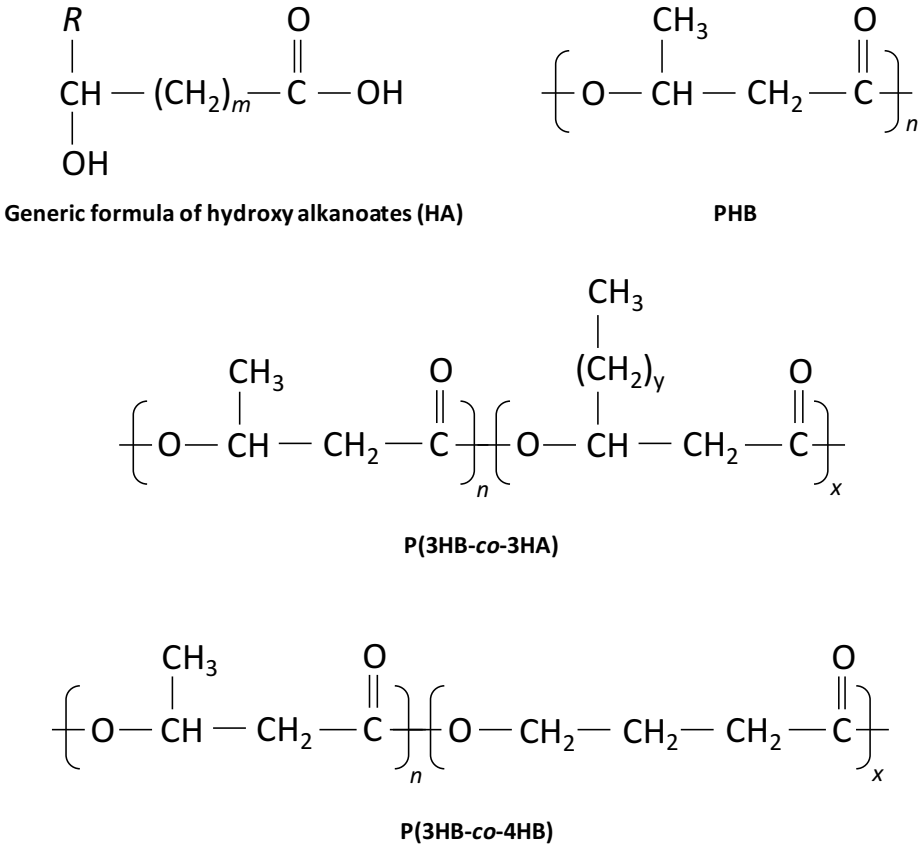

Figure 2.4 Generic chemical formula of HA monomers and common PHA copolymers

As previously mentioned, one of the most important advantages of PHAs is their biodegradability and biocompatibility. Under aerobic conditions, PHAs are degraded to carbon dioxide and water, while under anaerobic conditions, methane and water are the final products. Hence, these compounds can be utilised from various microorganisms living in soil and water as carbon source for their growth, without toxic effects to the environment.

PHB was the first member of the PHA family that was identified after isolation from *Bacillus megaterium* (Lemoigne, 1926). It can be produced by many bacterial strains (especially various strains of *C. necator*) in high concentrations (more than 150 g/L) and intracellular content (more than 80% on a dry weight basis) from commercial carbon sources (mainly glucose as well as fructose and sucrose) and starch hydrolysates (Ryu et

al. 1997; Yu et al. 2003). In addition, the physical properties of PHB are similar to polypropylene. However, the brittle and thermally unstable nature of PHB limits its commercial applications and constitutes one of the major reasons that have prevented its production in large scale operations. The high crystallinity of PHB (55 – 80%), associated with the formation of large spherulites, is the main reason that causes the brittle nature of PHB. It should be stressed though that the application of appropriate processing methodologies could reduce the undesirable mechanical properties of PHB, which could be used for the production of ductile films (Barham et al., 1992). Furthermore, the molecular weight of the PHB homopolymer produced by many bacterial strains, under varying fermentation condition and utilisation of different feedstocks may also result to a biopolymer with improved characteristics (Kusaka et al., 1999).

P(3HB-*co*-3HV) was the first copolymer of the PHA family that was identified and subsequently produced on industrial scale by Imperial Chemical Industries (ICI) using a *Ralstonia eutropha* strain. The incorporation of 3HV units in different proportions in the copolymer by *R. eutropha* was achieved by the addition of propionic acid as a carbon source precursor that induced the metabolic synthesis of 3HV units. The production of P(3HB-*co*-3HV) also demonstrated that it is feasible to alter the properties of PHAs by controlling fermentation conditions. For instance, the addition of increasing propionic acid concentrations during PHA accumulation results in increasing proportions of 3HV units (expressed as mol %) in the P(3HB-*co*-3HV) copolymer. In this way, it was demonstrated that the incorporation of 3HV units in the P(3HB-*co*-3HV) copolymer results in improved mechanical properties (Byrom 1987; Choi and Lee 1997).

Since the identification and commercial production of P(3HB-*co*-3HV) copolymers, research has focused on the identification or modification of microbial strains capable of producing PHA copolymers without addition of carbon source precursors or the production of different PHA copolymers, with addition of carbon source precursors, containing two, three or four monomers that demonstrate desired properties (Madden et al., 1999; Loo et al. 2005; Koller et al. 2007a). For instance, the archeon *Haloferax mediterranei* accumulates (72.8 %, w/w) of P-(3HB-*co*-3HV) that contains 6 mol% 3HV units directly from whey sugars, while it produces the terpolymer P(3HB-*co*-3HV-*co*-4HB) when it is supplemented with 3HV and 4HB precursors.

Table 2.2 presents specific properties of various PHAs compared with major petroleum-derived plastics. Nowadays, it is widely accepted that the physical properties of PHAs can vary from brittle PHB homopolymers with high crystallinity to flexible PHA copolymers with lower crystallinity, such as P(3HB-*co*-3HV) and P(3HB-*co*-3HHx), to

elastic PHA copolymers, such as P(3HB-*co*-4HB) and P(3-hydroxyoctanoate-*co*-3-hydroxydecanoate) (Wolf et al., 2005; Whitehouse et al., 2006). In the last 30 years, PHAs have been identified as potential biopolymers for a wide spectrum of end-uses including food packaging, flushable hygiene products, tissue engineering applications, adhesives, agriculture and biocomposites (Wolf et al., 2005).

A plethora of organic molecules have been evaluated as precursors for the copolymer P(3HB-co-3HV) synthesis, including sodium propionate, levulinic acid and valerate among others. In particular, levulinic acid has drawn significant attention. Levulinic acid is likely to become a cost-efficient precursor for P(3HB-*co*-3HV) production as it will eventually be produced in bulk quantities in future biorefineries utilising a variety of lignocellulosic resources or waste streams (Bozell et al., 2000). The significant production of by-products leading to a reduction of the maximum theoretical yield, the high cost of equipment and difficulties with catalyst recycling currently hinder the commercialisation of levulinic acid production. The Biofine process is the most known procedure that has been developed for levulinic acid production (Hayes et al., 2006). The cost of levulinic acid production could be reduced from its current high price of 6 – 9 €/kg, at an annual production level of 450 t, to less than 0.2 $/kg depending on the production capacity (Bozell et al., 2000). This unitary cost is much lower than the unitary cost of other precursors (e.g. 0.95 $/kg to more than 2 $/kg for propionic acid in the period 2003-2009) employed for the production of 3HV co-monomer units in P(3HB-*co*-3HV) fermentations. The combination of crude glycerol with levulinic acid could provide low cost carbon sources for P(3HB-*co*-3HV) synthesis. Ashby et al. (2012) reported P(3HB-*co*-3HV) production with the strain *Pseudomonas oleovorans* NRRL B-14682 using levulinic acid and crude glycerol as carbon sources, but the reported concentrations and intracellular contents were rather low to support industrial implementation. Furthermore, although other carbon sources (e.g. glucose, fructose, xylose, hemicellulosic hydrolysates) have been used together with levulinic acid for the production of P(3HB-*co*-3HV), the reported concentrations and intracellular contents achieved in most studies are not satisfactory to warrant industrial implementation (Jang and Rogers, 1996; Chung et al., 2001; Keenan et al., 2006). A relatively high P(3HB-*co*-3HV) concentration of 12.6 g/L and a significantly high intracellular content (81.2 %, w/w) were reported by Wang et al. (2013).

Table 2.2 Thermophysical properties of various PHAs

Polymer	Co-monomer (mol%)	T_g [a] (°C)	T_m [b] (°C)	ΔH_m [c] (J/g)	$\%X_c$ [d]	$T_{d(50\%)}$ [e] (°C)	Reference
PHB	-	4	177	84	60±5	-	Shimamura et al., 1994
PHB	-	4	180	-	-	-	Akaraonye et al., 2010
P(3HB-*co*-3HV)	4.1 [f]	2.3	163	-	-	252	Han et al., 2010
P(3HB-*co*-3HV)	8 [f]	-1.2	169	49.00	33	-	Lee et al., 2008
P(3HB-*co*-3HV)	12 [f]	-	153	66.40	45	-	Gunaratne and Shanks 2005
P(3HB-*co*-3HV)	12 [f]	-1.9	155	72.61	49	271	Garcia et al., 2013
P(3HB-*co*-3HV)	30 [f]	2.1	111	-	-	352	He et al., 2001
P(3HB-*co*-3HHx)	3.5 [g]	-1	140 & 151	44	-	-	Tsuge et al., 2004
P(3HB-*co*-3HHx)	5 [g]	-1 to -3	125-138 & 142-155	38-47	-	-	Loo et al., 2005
P(3HB-*co*-3HHx)	11 [g]	-1	136	60	40±5	-	Shimamura et al., 1994
	17 [g]	-2	130	39	29±5	-	
P(3HB-*co*-4HB)	12 [h]	-4.3	124	56	38.2	270 [i]	Luo et al., 2009
Polypropylene	-	-10	176	-	70	-	Akaraonye et al., 2010
Polystyrene	-	100	240	-	-	-	

[a] Glass transition temperature; [b] Melting temperature; [c] Enthalpy of fusion; [d] Percentage of crystallinity; [e] Degradation temperature; [f] 3-hydroxyvalerate; [g] 3-hydroxyhexanoate; [h] 4-hydroxybutyrate; [i] $T_{d(5\%)}$

2.7 The strain Cupriavidus necator

Cupriavidus necator, formerly classified as *Ralstonia eutropha* or *Wautersia eutropha*, is a Gram-negative, rod-shaped, non-spore forming, soil bacteria. *C. necator* is an aerobic bacterium demonstrating an optimum temperature at 27 °C (Makkar and Casida, 1987), and pH value within the range of 7.0 - 8.0. According to Makkar and Casida (1987), the described *C. necator* strain proliferated on acetate, fructose, citrate, oxalacetate and succinate but did not consume glucose, glycerol, lactose, mannose, rhamnose and xylose. However, the ability of numerous wild-type or adapted *C. necator* strains to grow on glucose and glycerol has been demonstrated in the literature (Xu et al. 2010; Cavalheiro et al. 2012). The significance of *C. necator* strains is attributed to the fact that under nutrient limitation, such as nitrogen, phosphorus, magnesium, sulphur or oxygen, in parallel with surplus presence of carbon source, they accumulate polyhydroxyalkanoates intracellularly as energy and carbon reserve source in the form of granules or inclusions (Solaiman et al., 2006; Ashby et al., 2011; Koutinas et al., 2014c).

Polyhydroxybutyrate synthesis in the majority of bacteria, including *C. necator*, constitutes a tree-step reaction regulated at enzymatic level, when carbohydrates or acetate are utilised as substrates. The reaction is catalysed by three enzymes (Figure 2.5), initiated by the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA by β-ketothiolase. Susbequently, acetoacetyl-CoA is reduced by NADPH-dependent reductase acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA, while finally PHB is formulated by the polymerisation activity of PHB synthase on (R)-3-hydroxybutyryl-CoA monomers (Witholt and Kessler, 1999; Koutinas et al., 2014c). The acetyl-CoA and free CoA interact significantly with the regulation of PHB synthesis (Haywood et al., 1980). High intracellular concentrations of NADH and NADPH are also claimed to inhibit citrate synthase and trigger PHB accumulation by shifting metabolic flux to PHB synthesis (Witholt and Kessler, 1999).

Besides the homopolymer PHB, *C. necator* strains can utilise diversified co-substrates, together with the main carbon source, that act as precursors for the synthesis of copolymers, such as P(3HB-*co*-3HV) and P(3HB-*co*-3HHx), or terpolymer, such as P(3HB-*co*-4HB-*co*-3HV) (Jaremko and Yu, 2011; Cavalheiro et al., 2012; Wang et al., 2013). In particular, propionic acid and valeric acid have been employed during fermentations with glucose to generate P(3HB-*co*-3HV). Correspondingly, condensation of

one acetyl-CoA from glucose and one propionyl-CoA from propionic acid is catalysed by β-ketothiolase to form 3-ketovaleryl-CoA that is reduced to 3-hydroxyvaleryl-CoA, the precursor of 3-hydroxyvalerate (Jaremko and Yu, 2011). Polymerisation of P(3HB-*co*-3HV) is similarly conducted by PHA synthase. When valerate is employed instead, valeryl-CoA is synthesized, that is further converted to 3-hydroxyvaleryl-CoA directly via the β-oxidation pathway (Yu et al., 2009; Jaremko and Yu 2011).

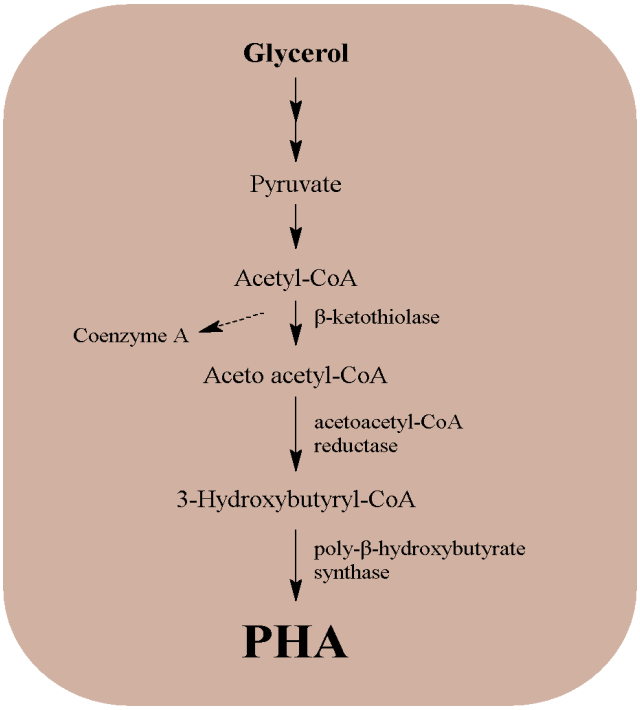

Figure 2.5 PHB biosynthetic pathway of *Cupriavidus necator* from glycerol

2.8 PHA production integrated in biorefinery concepts

It is nowadays widely recognised that successful implementation of industrial PHA production will only be achieved through the development of sustainable processes coupled with the production of biodegradable polymers with desirable properties. Sustainability aspects include cost-competitiveness, environmental benignness and production of biodegradable polymers that serve certain market and societal needs. Additional advantages will be provided through the ability to produce PHAs with adjustable properties that could be used in different end-uses by simple modification of fermentation conditions. For instance, the production of different types of PHAs that could be used for both commodity (e.g. food packaging) and speciality (e.g. scaffolds for tissue engineering applications) end-uses by simple modification of fermentation parameters could provide process flexibility.

An important innovation on future PHA-based processes will be the creation of cascade processing schemes in order to increase resource efficiency (Anonymous, 2012b). Cascade processing is based on the re-utilisation of packaging material after its use (also called post consumer plastics) for other commercial purposes. For instance, hydrolysis into monomers could create value-added platform molecules for the chemical industry. In addition, bioplastics could be used as replacements for coal and heating fuel due to their high calorific value (Anonymous, 2012b). Re-utilisation of PHA-based packaging materials is strongly dependent on the development of suitable recycling technologies.

Despite their significant advantages, industrial production of PHAs is hindered by high production cost. Previous attempts to produce PHAs in large scale had to rely on conventional fermentation technologies that cannot compete with low cost petroleum-derived plastics. As mentioned earlier, raw material supply is one of the most important factors that should be optimised in order to reduce processing costs. For this reason, recent research focuses on the utilisation of low-cost feedstock for PHA production (e.g. molasses, crude glycerol, whey, animal fats, waste cooking oils among others) aiming to substitute for conventional and expensive carbon sources. Table 2.3 presents results regarding PHA production from various waste and by-product streams. However, even if waste or by-product streams are used as fermentation feedstocks, aerobic cultivation for PHA production in industrial scale operations is still an expensive unit operation. For this reason, integration of PHA production into existing industrial plants or the development of

Table 2.3 PHA production from various crude renewable resources, waste and by-product streams

By-product or waste stream	Strain	PHA type	TDW (g/L)	PHA concentration (g/L)	PHA content (%)	Productivity (g/L/h)	Reference
Bagasse hydrolysates	*Ralstonia eutropha*	PHA	10.1		60.2		Yu and Stahl, 2008
Wheat-derived media (shake flask cultures)	*Cupriavidus necator* NCIMB 11599	PHB	73.2	51.1	70	0.3	Koutinas et al., 2007b
Wheat-derived media (bioreactor cultures)	*Wautersia eutropha* NCIMB 11599	PHB	175.2	162.8	93	0.89	Xu et al., 2010
Soybean oil	*Ralstonia eutropha* H16	PHB	126	95.8	76	0.99	Kahar et al., 2004
Oleic acid	*Ralstonia eutropha* PHB$^-$4 (DSM 541)	P(3HB-*co*-3HHx)	138	102.1	74	1.06	Marsudi et al., 2007
	Pseudomonas putida PGA1	PHAs-mcl	30.2	13.52	44.8	0.19	
Hydrolyzed whey	*Haloferax mediterranei* DSM 1411	PHA	11	5.5	50	0.05	Koller et al., 2007b
Hydrolyzed whey permeate	*Pseudomonas hydrogenovora* DSM 1749	PHB	10.83	1.3	12	0.03	Koller et al., 2008
	Hydrogenophaga pseudoflava DSM 1034		6.75	2.7	40	0.05	
	Pseudomonas hydrogenovora DSM 1749		10.58	1.27	12	0.03	
Hydrolyzed whey permeate and valerate	*Methylobacterium* sp. ZP24	P(3HB-*co*-3HV)	12	1.44	12	0.05	Nath et al., 2008

Cheese whey		PHB	5.53	3.54	64	0.09	
Saccharified waste potato starch	*Ralstonia eutropha* NCIMB 11599	PHB	179	94	55	1.47	Haas et al., 2008
Extruded rice bran and extruded corn starch	*Haloferax mediterranei* ATCC 33500	PHB	140	77.8	55.6	0.65	Huang et al., 2006
Wheat straw hydrolysates	*Burkholderia sacchari* DSM 17165	PHB	105	75.6	72	1.6	Cesário et al., 2014
Sugarcane molasses and corn steep liquor	*Bacillus megaterium*	PHB	3.6	2.2	59.4	0.045	Gouda et al., 2001
Sugarcane molasses and urea	*Bacillus megaterium* BA-019	PHB	72.6	30.5	42	1.27	Kulpreecha et al., 2009

new industrial plants for PHA production should be combined with the production of value-added co-products. This can be achieved through fractionation of agricultural residues and industrial waste and by-product streams into value-added co-products.

PHA production cost increases further due to downstream separation and purification of PHAs from residual microbial mass. Several methods have been reported for the recovery of PHAs based on the utilisation of organic solvents such as acetone, chloroform or dichloroethane. However, these methods are unfavourable for large scale production since solvents increase operational cost and additional equipment for solvent recovery is often needed. Alternative extraction methods have been also proposed including enzymatic lysis of residual microbial mass (Kapritchkoff et al., 2006; Verlinden et al., 2007), supercritical fluid extraction (Hejazi et al., 2003), mechanical disruption of bacterial cells coupled with chemical treatment, autolysis of bacterial cells, and chemical treatment under acidic or alkaline conditions (Yu and Chen, 2006; Verlinden et al., 2007).

Several studies have also focused on the estimation of PHA production costs from different feedstocks (Choi and Lee, 1997; Van Wegen et al., 1998; Posada et al., 2011). However, there are limited studies on the evaluation of integrated biorefineries focusing on the fractionation of the initial raw material combining the production of PHAs with the extraction or production of value-added co-products. In addition, future costing studies should also focus on the evaluation of the potential to integrate PHA production in existing industries to minimise logistics and decrease the cost of infrastructure.

In recent years, several studies focused on the production of PHAs from low-cost renewable resources (Akaraonye et al., 2010; Koller et al., 2010; Du et al., 2012). Accordingly, in the following section the presentation of representative biorefinery concepts targeting the production of PHAs and other value-added products will be presented. In particular, PHA production could be combined with biofuel and bioenergy production, leading to enhanced sustainability. Furthermore, solid state fermentation could be employed for the production of crude mixtures of enzymes that could be employed for different purposes (e.g. production of nutrient-rich hydrolysates) within a biorefinery utilising renewable resources.

2.8.1 Solid State Fermentation

Solid state fermentation is defined as the fermentation of a solid, insoluble substrate performed in the absence or near-absence of free water. Nonetheless, adequate quantity of

water should be supplied to sustain microbial proliferation and metabolic activity (Pandey, 2003). The solid substrate exhibits the source of nutrients for microbial entities or provides a supporting matrix for microbial growth. Solid state fermentation comprises a heterogenous process where solid, liquid and gas phases correlate and interact (Thomas et al., 2013). Several aspects that should be undertaken for the efficient development of solid state fermentation procedures, implement the selection of micro-organism and the respective substrate, the particle size and moisture content of the substrate, process parameters like pH value and temperature during fermentation, and product recovery among others (Thomas et al., 2013).

The optimum moisture content of the substrate relates to the microbial strain as well as the type of the substrate. Most frequently, fungal and yeast strains are employed in SSF based on the water activity (a_w) requirements (approximately 0.5 – 0.6) while, on the other hand, bacterial strains exhibit lower moisture demands at water activities of 0.8 – 0.9 (Thomas et al., 2013). In particular, fungi require lower moisture content in the substrate, within the range of 40 – 60 %, compared to yeast (Shingania et al., 2009). The selection of the appropriate substrate entails a major concern in order to maximise the efficiency of solid state fermentations. This is a rather difficult task because there are many options due to the existence of numerous agri-industrial waste streams and by-products. Generally, solid state fermentation is distinguished by lower energy demands and generation of lower volumes of processing waste (Pandey, 2003) when compared to submerged fermentations. Higher productivities and lower risk for contamination outline additional advantages of solid state fermentation over submerged fermentations (Couto and Sanromán, 2006). An ample variety of agro-industrial waste and by-products have been evaluated via solid state fermentation processes including crop residues, industrial by-products, food waste and by-product streams as well as vegetable oil production side streams (Thomas et al., 2013).

Solid state fermentation has been successfully applied to yield diversified bulk chemicals and products or speciality chemicals and commodities. These include mainly enzymes (e.g. proteases, phytase, lipases, pectinases, cellulases, xylanases), organic acids, and pigments directed towards industrial food and feed applications. Moreover, solid state fermentation has been implemented in bioremediation, bioleaching, biopulping and biological delignification (Thomas et al., 2013).

2.8.2 Valorisation of biodiesel industry by-products

The continuous growth of biodiesel production coincides with proportional production of by-products streams. As previously demonstrated, oilseed meals and crude glycerol constitute the main by-products during biodiesel production process, entailing the necessitation for valorisation routes. Glycerol represents an easily assimilated carbon source for many microorganisms, thus has been widely evaluated in PHA bioconversions. Table 2.4 presents various literature-cited publications on PHA production from crude glycerol based media.

Table 2.4 Outline of PHA production from crude glycerol from various microorganisms

Fermentation media	Strain	PHA type	Total dry weight (g/L)	PHA concentration (g/L)	PHA content (%)	Productivity (g/L/h)	Reference
Crude glycerol [a]	*Cupriavidus necator* DSM 545	PHB	76.2	38.1	50	1.1	Cavalheiro et al., 2009
Pure glycerol with NaCl [a]	*Zobellella denitrificans* MW1	PHB	81.2 ± 2.5	54.3 ± 7.9	66.9 ± 7.6	1.09 ± 0.16	Ibrahim and Steinbuchel, 2009
Crude glycerol (85%) [a]	*Burkholderia cepacia* ATCC 17759	PHB	23.6	7.4	31.4	0.062	Zhu et al., 2010
Crude glycerol (47-77%) [a]	*Pseudomonas oleovorans* NRRL B-14682	PHB	3.0	1.14	38	0.016	Ashby et al., 2011
Crude glycerol (40%), fatty acid soaps and residual fatty acid methyl esters [b]	*Pseudomonas oleovorans* NRRL B-14682	PHB	1.3	0.17-0.35	13-27	0.002-0.005	Ashby et al., 2004
	Pseudomonas corrugata 388	*mcl*-PHA	2.1	0.88	42	0.012	
Crude glycerol (85%) and terephtalic acid [a]	*Pseudomonas putida* GO16	*mcl*-PHA	19.1 ± 0.26	6.25 ± 0.23	35.63 ± 1.31	0.13	Kenny et al., 2012
Crude glycerol and γ-butyrolactone [a]	*Cupriavidus necator* DSM 545	P(3HB-*co*-4HB)	30.19	10.9	36.1	0.17	Cavalheiro et al., 2012

Crude glycerol, γ-butyrolactone and propionic acid [a]	*Cupriavidus necator* DSM 545	P(3HB-*co*-4HB-*co*-3HV)	45.25	16.7	36.9	0.25	
Crude glycerol (81%) and rapeseed meal hydrolysates [b]	*Cupriavidus necator* DSM 545	P(3HB-*co*-3HV)	19.6	10.9	55.6	0.12	Garcia et al., 2013
Crude glycerol [b]	*Halomonas* sp. KM-1	PHB	4.1	1.6	39	0.034	Kawata and Aiba, 2010
Crude glycerol (60.05%) [a]	*Bacillus megaterium*	PHB	7.7	4.8	62.4	0.114	Naranjo et al., 2013
Wine lees hydrolysates and crude glycerol	*Cupriavidus necator* DSM 7237	PHB	42.2	30.1	71.3	0.56	Dimou et al., 2015

[a] experiments conducted in bioreactor
[b] experiments conducted in shake flasks

Besides crude glycerol, biodiesel production from oilseeds leads to the production of oilseed meals as a valuable by-product stream. Oilseed meal is the protein and carbohydrate rich fraction that remains after the extraction of oil. The main conventional commercial outlet for oilseed meals is as animal feed. In the period 2012-2021, biodiesel production from edible vegetable oils will still rely mainly on rapeseed and sunflower. Based on recent estimates, approximately 315×10^6 t of oilseed meals are expected to be produced by 2021, corresponding to an increase up to 23% based on current production capacities (Anonymous 2012c).Future biodiesel industries could be converted into novel biorefineries through valorisation of crude glycerol and oilseed meal streams leading to the production of biodiesel, chemicals, food and feed ingredients and biopolymers such as PHAs.

Ashby et al. (2004; 2011) evaluated the production and properties of PHAs accumulated by the bacterial strains *Pseudomonas oleovorans* NRRL B-14682 and *P. corrugata* 388 cultivated on crude glycerol. Ashby et al. (2011) reported that the molecular weight of PHB was decreased with increasing methanol concentration in crude glycerol. Mothes et al. (2007) and Garcia et al. (2013) evaluated the effect of NaCl and K_2SO_4 on PHA production during fermentation with the bacterial strains *Paracoccus denitrificans*, *Cupriavidus necator* JMP 134 and *Cupriavidus necator* DSMZ 545. These salts are present in crude glycerol depending on the catalyst (NaOH or KOH) employed during transesterification of triglycerides. The inhibition caused by NaCl on PHA production is more pronounced at significantly lower concentrations than K_2SO_4. On the other hand, Passanha et al., (2014) studied the effect of sodium on *Cupriavidus necator* cultivation, stating that NaCl addition at concentrations higher than 6 g/L caused stress conditions that reduced PHA accumulation. Mothes et al. (2007) reported that bioreactor fermentations with *C. necator* JMP 134 cultivated on crude glycerol and inorganic chemicals as additional nutrients could lead to the production of PHB contents up to 70% (w/w). Recent studies reported higher PHA content using crude glycerol from biodiesel industry. In particular, Hermann-Krauss et al. (2013) reported a higher PHB content up to 75.4% using the strain *Haloferax mediterranei*, while Dimou et al. (2015) achieved a PHB content of 71.3% during fed-batch cultivations of *C. necator* on crude glycerol and wine lees hydrolysates. Rodríguez-Contreras et al. (2015) also evaluated glycerol streams during bioreactor fermentations of *C. necator* DSM 545 and *B. sacchari* DSM 17165. Crude glycerol has also been employed in bioreactor fermentations for the production of PHB using the bacterial strain *C. necator* DSM 545 leading to 50 % (w/w) PHB content and 1.1

g/L/h PHB productivity (Cavalheiro et al., 2009). Tanadchangsaeng and Yu (2012) stressed that the productivity (around 0.92 g/L/h) of glycerol fermentation towards PHB synthesis is lower than the one achieved from glucose.

Crude glycerol could be also combined with other carbon sources that could be used as precursors for the production of PHA co-polymers (Cavalheiro et al., 2012). The production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyvalerate) was reported when *C. necator* DSM 545 was cultivated on crude glycerol, propionic acid (stimulator of 4HB accumulation and 3HV precursor) and *γ*-butyrolactone (4HB precursor). In all studies presented above inorganic chemicals were used as nutrient supplements.

Apart from fermentation efficiency of PHA production, it is also crucial to assess the properties of the polymer produced and the associated production cost. Tanadchangsaeng and Yu (2012) reported that although the thermal and physical properties of the PHB produced from glycerol is similar to the one produced from glucose, the molecular weight of the glycerol-derived homopolymer is lower than the molecular weight of the PHB produced from glucose. This is usually attributed to glycerol end-capping during the polymerisation process that inhibits the activity of PHA synthase (PHAc), thus leading to the production of PHAs with low molecular weights (Madden et al., 1999; Ashby et al., 2012; Koller and Marsalek, 2015).

Posada et al. (2011) presented a comparative techno-economic evaluation of PHB production from crude glycerol using two different bacterial strains, *C. necator* and *Bacillus megaterium*, and three different downstream separation strategies. Fermentation of *C. necator* resulted in the production of 81.6 g/L of which 57.1 g/L was PHB. The fermentation efficiency of *C. necator* was significantly higher than the one achieved by *B. megaterium.* The most cost-competitive process involved PHB production in fed-batch fermentations with *C. necator* followed by PHB separation and purification with heat pretreatment, enzymatic-alkaline digestion, centrifugation, washing, evaporation, and spray drying. Posada et al. (2011) reported also that glycerol purification to 98 % (w/w) contributes approximately 6% of the overall PHB production cost, thereby slightly affecting the total cost. In this study, it was concluded that the PHB production cost from crude glycerol could be as low as 2 US$/kg depending on the downstream process utilised.

PHA production from crude glycerol could be combined with the valorisation of oilseed meals or residues remaining after extraction of the oil. For instance, rapeseed meal could be utilised for the production of various value-added fractions including protein isolates, carbohydrates, hulls, phenolic compounds and glucosinolates with various applications such as animal feed, pesticidal agent, bioactive proteins, glues and adhesives, paper coatings and ingredients for cosmetics among others (Anonymous, 2011; Egues et al., 2010). Another alternative application of oilseed meals is based on the production of complex nutrient supplements for fermentation processes including PHA production. In this way, commercial inorganic chemicals will be replaced improving the sustainability of the whole biorefinery concept. Oilseed meals contain significant quantities of protein, minerals and other necessary nutrients for microbial growth. Enzymatic hydrolysis of protein to amino acids and peptides, and phytic acid to phosphorus could provide a hydrolysate suitable for PHA production. Crude enzymes could be produced via solid state fermentation employing appropriate fungal strains and oilseed meals as substrates (Wang et al. 2010; Kachrimanidou et al. 2013). Wang et al. (2010) reported the production of a nutrient-rich hydrolysate from rapeseed meal with a free amino nitrogen content of 2016.2 mg/L and inorganic phosphorus of 304 mg/L that was subsequently used successfully as nutrient supplement combined with glucose as carbon source for the cultivation of *Saccharomyces cerevisiae*. Kiran et al. (2012; 2013) also evaluated rapeseed meal hydrolysates as a nutrient rich supplement for microbial oil production using the oleaginous yeast *Rhodosporidium toruloides* Y4. A generic feedstock deriving from rapeseed meal was also valorised for 1,3-propanediol production during repeated batch fermentations using the strain *Clostridium butyricum* VPI 1718 (Chatzifragkou et al., 2014). Garcia et al. (2013) investigated the generation of a microbial feedstock through hydrolysis of rapeseed meal, which was combined with crude glycerol as the sole fermentation medium for PHA production. Fed-batch fermentations resulted in a production of 10.9 g/L P(3HB-*co*-3HV) without addition of any precursor. The properties of the biopolymer produced were also examined, leading to the conclusion that this bioprocess could be incorporated in rapeseed-based biodiesel plants contributing to the sustainability of biodiesel biorefineries.

Figure 2.6 presents a biorefinery concept developed during this study in which sunflower meal is utilised only for the production of fermentation feedstock through a two-stage bioprocess. Initially, the production of crude enzymes via solid state fermentation is involved with the fungal strain *Aspergillus oryzae*, followed by hydrolysate production via

enzymatic hydrolysis. The obtained hydrolysate was implemented in bacterial bioconversions with the strain *C. necator* to evaluate its ability to produce PHAs. However, this processing scheme does not take advantage of the full potential of sunflower meal that contains value-added ingredients that could be isolated contributing to the development of a more sustainable biorefinery approach.

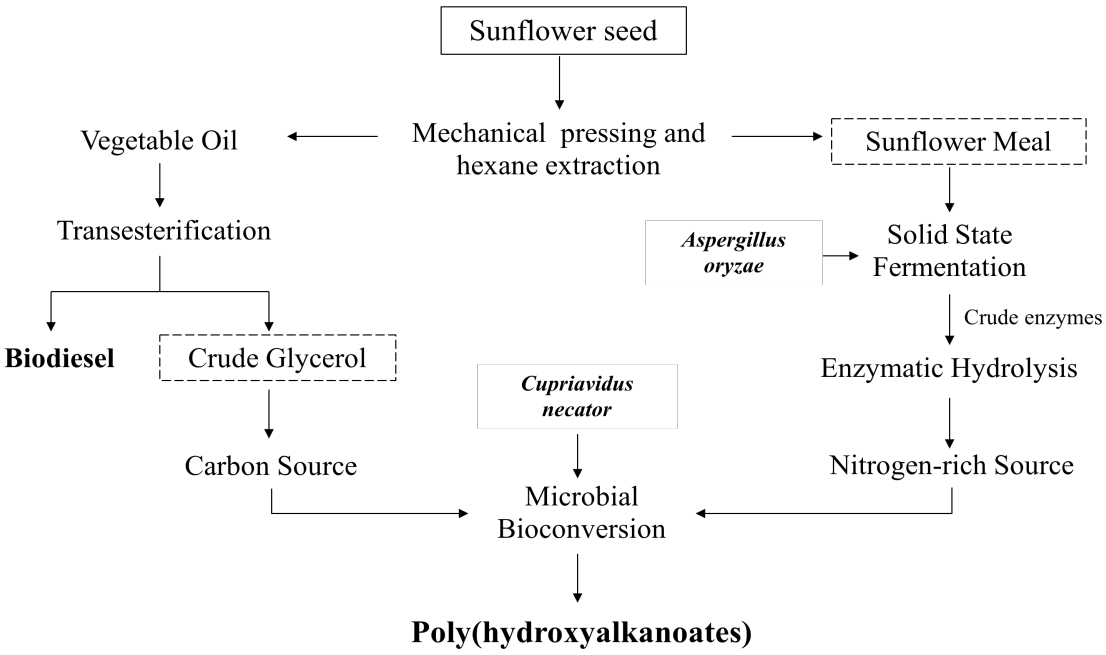

Figure 2.6 Utilisation of sunflower-derived biodiesel industry by-products for PHA production

Correspondingly, Figure 2.7 presents a sunflower-based biorefinery where besides fermentation feedstock, sunflower meal is also used for the production of an antioxidant-rich stream and a protein isolate product. The sunflower seed is covered by the hull that could be removed before oil removal by mechanical pressing and solvent extraction in biodiesel production processes. The protein content in sunflower meals can be increased via dehulling and complete oil extraction. The composition of sunflower meal is variable and is highly dependent on cultivation conditions, sunflower variety and the industrial process used for biodiesel production. Dehulling or partial dehulling of sunflower seeds could provide a by-product that could be used for the production of energy, hemicelluloses, organic amendment for the soils, and biomaterial (Anonymous, 2011). The sunflower meal that remains as a by-product after (partial) dehulling and complete oil extraction was fractionated into three different fractions (i.e. a protein-rich fraction, a lignocellulose-rich fraction and a liquid fraction) by a simple sedimentation/flotation

process based on the formation of an aqueous suspension (Bautista et al., 1990; Parrado et al., 1991). This separation is based on the different densities of major components in sunflower meal. Subsequently, antioxidants can be removed from the protein-rich fraction, as well as from the lignocellulosic fraction. The most important of the phenolic compounds found in sunflower is chlorogenic acid.

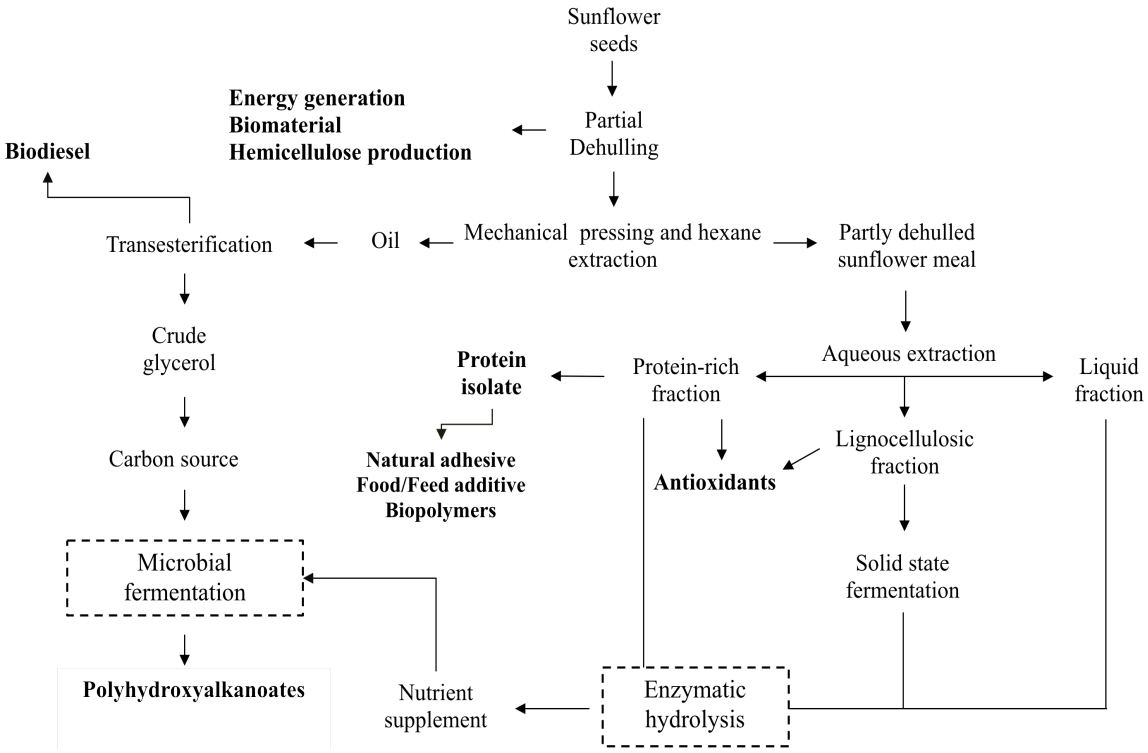

Figure 2.7 Advanced sunflower-based biorefinery concept

The protein isolate extracted from the protein-rich fraction, after treatment with acid and alkaline solutions, could be utilised for the production of biopolymers, edible films or as a substitute of formaldehyde in resins that are used in wood-based applications. Yust et al. (2003) suggested that the protein of sunflower meal could be used as animal feed. Remaining fractions from the process presented in Figure 2.7 were used as substrate in solid state fermentation with a fungal strain of *Aspergillus oryzae*, a well known producer mostly of proteolytic enzymes. The solids at the end of solid state fermentation were further implemented as enzyme-rich medium for hydrolysis of macromolecules contained in remaining sunflower fractions. The liquid fraction from sunflower meal fractionation was also used as suspension liquid in enzymatic hydrolysis, aiming at the generation of a nutrient rich supplement. At the end of hydrolysis, remaining solids were separated from the hydrolysate by centrifugation, and could be possibly used for combustion to generate heat or as a carbohydrate-rich resource for the production of

hydrolysates for other fermentations. The nutrient-rich supplements formulated with the different combinations of residual streams were valorised as feedstock for the production of PHB. The advanced biorefinery concept results in the manufacture of 3 end-products (antioxidants, protein isolate and PHB) from the same raw material presenting a high potential of improved process economics.

2.8.3 Valorisation of 2nd generation bioethanol industry by-products

Cellulose, hemicellulose and lignin are the main components found in lignocellulosic raw materials and the corresponding composition is dependent on the biomass resource. Production of sugar-rich hydrolysates from lignocellulosic biomass requires treatment with combined thermochemical treatment and enzymatic hydrolysis. Previous studies on utilisation of lignocellulosic resources have focused on hydrolysis of cellulose and hemicellulose fractions to simple sugars for microbial fermentation mainly aiming to bioethanol production. Nonetheless, given the interest arising in biopolymer production, bioethanol production could be combined with PHA production. Cellulose could be utilised for the production of bioethanol, while sugars from hemicellulose could be utilised for the production of PHAs. In this way, a conventional process employed for the production of bioethanol from lignocellulosic biomass could be upgraded into an advanced biorefinery concepts.

Silva et al (2004) screened 55 strains as potential PHB producers from xylose and identified *Burkholderia sacchari* IPT 101 and *B. cepacia* IPT 048 that were subsequently evaluated via cultivations on xylose and bagasse hydrolysates. Intracellular PHB content reached 62% and 53% for the two strains, respectively, when cultivated on bagasse hydrolysates. Keenan et al. (2006) utilised detoxified hemicellulose hydrolysates from lignocellulosic resources for the production of P(3HB-*co*-3HV) with *Burkholderia cepacia* through supplementation with levulinic acid (0.25 – 0.5 %) to achieve a P(3HB-*co*-3HV) concentration of 2 g/L, a P(3HB-*co*-3HV) content of 40% (w/w) and 3HV composition of 16 – 52 mol%. When xylose and levullinic acid were used in microbial bioconversions with *Burkholderia cepacia*, the P(3HB-*co*-3HV) concentration and 3HV composition achieved were up to 4.2 g/L and 61 mol%, respectively. Sugarcane bagasse hydrolysates were evaluated for PHA synthesis via fermentation of *Ralstonia eutropha* (Yu and Stahl, 2008). The effect of inoculum concentration, dilution of hydrolysate and implementation of an adapted strain was studied regarding PHA accumulation, which reached up to 57%

(w/w) polymer content. PHB was the major polymer accumulated, whereas copolymers could be also produced that presented high ductility. Wheat straw hydrolysates were evaluated by Cesário et al. (2014) with the bacterial strain *B. sacchari* DSM 17165 achieving remarkable total dry weight concentration up to 105 g/L with an intracellular content equal to 72% and volumetric productivity of 1.6 g/L/h.

PHA production could be incorporated in existing bioethanol production facilities from both sugar cane in Brazil and cereals, such as wheat and corn, in other countries worldwide. Sugar cane utilisation for bioethanol production generates significant quantities of bagasse, a lignocellulosic raw material that could be used for combined production of ethanol from cellulose and PHAs from hemicellulose sugars (mainly xylose). Integration of PHA production in existing cereal-based facilities used for bioethanol production could be achieved by incorporating straw utilisation as raw material for combined production of bioethanol and PHAs. Such integrated biorefinery concepts could improve the sustainability of 1st generation bioethanol production plants.

2.8.4 Valorisation of food supply chain wastes and by-products

Food supply chain waste and by-product streams are produced throughout the whole value-chain starting from the production of the raw material followed by the processing into edible products by the food industry and the final disposal by consumers, restaurants or catering services among others. Valorising the waste and by-product streams derived from the food industry sector would result in the creation of novel biorefineries leading to restructured and advanced industrial plants that will not only satisfy the traditional market of food production but also other markets that are nowadays depend on petroleum to provide the necessary feedstocks. Food processing waste and by-product streams constitute renewable resources enriched in carbohydrates, proteins, oils and fats, phenolic compounds and various micronutrients.

2.8.4.1 PHA production from winery by-products

Wine production constitutes an important industrial sector in many countries around the world, such as the South European countries, United States, Chile and Australia. Wine making generates both solid and liquid by-products. Residues from wine production involve mainly trimming wastes, grape stalk, grape pomace or marc, wine lees and winery wastewater. These by-products are currently supplied to ethanol distilleries (in

the case of wine lees), used (if possible) as fertilizers or processed as wastes in order to reduce the environmental impact caused by their disposal to the environment. However, given the fact that environmental policies are changing, new practices should be applied aiming at valorisation of winery by-product streams.

On-going research focuses on valorisation of residues from wine making. Trimming wastes are rich in cellulose, hemicellulose and lignin. Combined thermochemical treatment with enzymatic hydrolysis can be applied to convert cellulose and hemicelluloses into C5 and C6 sugars that can be assimilated by microorganisms. Delignification steps are usually required since the complex structure of lignin prevents hydrolysis of polysaccharides. Bustos et al. (2005) evaluated the use of trimming wastes and wine lees aiming at the production of lactic acid through simultaneous saccharification and fermentation with *Lactobacillus pentosus*. Trimming wastes could be also used as solid support in solid state fermentations for the production of various enzymes (Sanchez et al., 2002).

Grape pomace or marc is the solid fraction remaining after the extraction stage and it consists of skins, pulp, seeds and stems of grapes. Research has focused on efficient utilisation of this waste stream, since it contains lignocellulosic fractions that can be hydrolyzed and further used in microbial bioconversions. Solid state fermentation for production of hydrolytic enzymes has also been reported using grape marc as solid support (Botella et al., 2005).

Wine lees is the remaining residue after the end of the fermentation stage. It is a rich source of ethanol, tartaric acid, phenolic compounds and yeast cells. Wine lees can be used for the production of potable alcohol (wine lees produced by large wineries are mainly used for the production of ethanol), as nutrient supplement for fermentation (Bustos et al., 2004; Salgado et al., 2010), for the production of tartaric acid (Versari et al., 2001; Rivas et al., 2006) and as raw material for composting (Diaz et al., 2002; Nogales et al., 2005). A novel process has been developed by Dimou et al. (2015) targeting the creation of a novel biorefinery concept based on wine lees valorisation (Figure 2.8). The process starts with centrifugation of wine lees in order to separate the liquid stream that can be used for ethanol production via distillation. The ethanol produced can be used as potable or fuel ethanol depending on the purity. Current processes produce potable ethanol. Ethanol could be also used as a platform chemical to supply the future sustainable chemical industry. Alternatively, ethanol could be also utilised as carbon source for

microbial fermentation aiming to PHB production by the bacterial strain *C. necator* NCIMB 12080 (Senior et al., 1986). This, however, may not be a cost-competitive alternative when compared to the traditional potable ethanol market. The remaining liquid after ethanol extraction can be used in subsequent hydrolysis stages to increase the presence of nutrients.

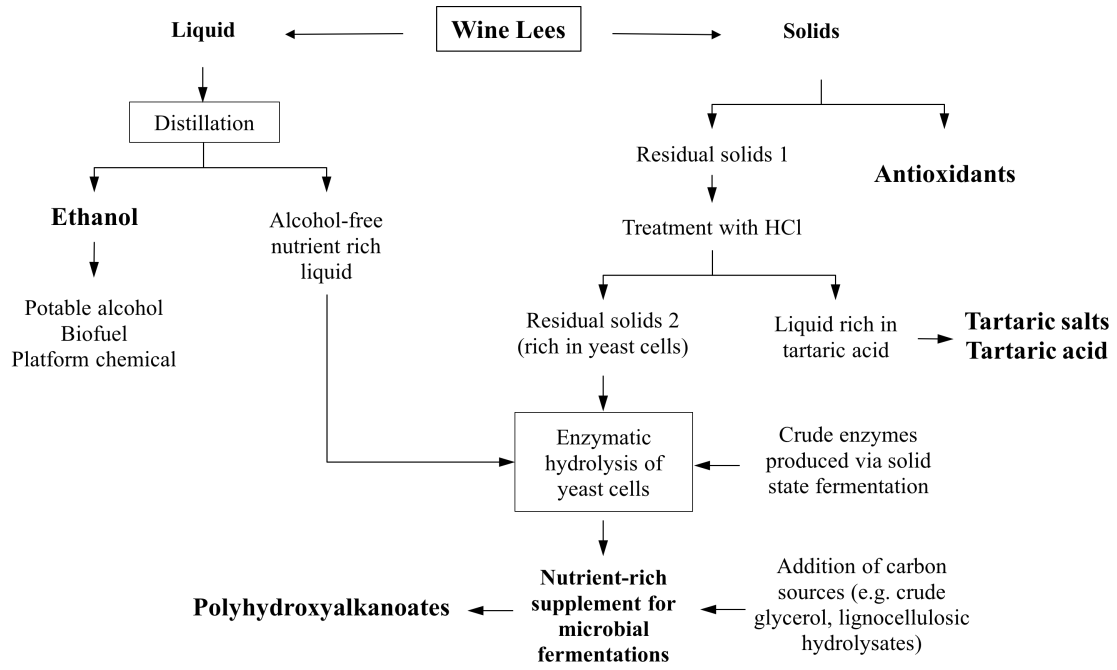

Figure 2.8 Valorisation of wine lees for biorefinery concept development

The solid fraction that remains after centrifugation of wine lees contains phenolic compounds with antioxidant properties, tartrate salts and yeast cells. A phenolic-rich fraction can be easily isolated via solvent extraction. Tartrate salts can be subsequently separated from yeast cells via treatment with hydrochloric acid. Versari et al. (2001) extracted tartaric acid with purity up to 99% from three different winery by-product streams, including wine lees. Moreover, Nurgel and Canbas (1998) investigated the production of tartaric acid from grape pomace. The use of tartaric acid is well established in wine making in order to adjust the pH of the must prior to fermentation. Tartaric acid could be also used as food additive.

After the extraction of phenolic compounds and tartrate salts, residual wine lees solids are subjected to enzymatic hydrolysis with the addition of crude enzymes produced via solid state fermentation of a fungal strain of *Aspergillus oryzae* on wheat bran. The ethanol-free medium that remains after the distillation step is used as liquid in the

hydrolysis stage. In this stage, yeast cells are lysed and converted into a nutrient rich supplement similar to yeast extract. This supplement is rich in various sources of nitrogen (e.g. amino acids and peptides), phosphorus and various trace elements. This nutrient supplement can be combined with a carbon source (e.g. crude glycerol from biodiesel industries) as fermentation media for the production of PHB with *C. necator*. Dimou et al. (2015) demonstrated that PHB production by the strain *C. necator* DSM 7237 is feasible when mixtures of crude glycerol and wine lees hydrolysates are used. However, supplementation with a low quantity of minerals is necessary showing that this nutrient supplement is deficient in some minerals. The wine lees hydrolysate could be combined with a sugar-rich hydrolysate derived from treatment of lignocellulosic streams derived during wine production.

2.8.4.2 PHB production from confectionery and bakery industry waste-streams

Significant quantities of waste streams are generated annually from confectionery industries and bakeries. These industrial sectors produce flour, starch or sugar rich waste streams generated either during processing or as end-of-date products returned from the market. Confectionery waste streams are currently used as animal feed, for composting or are discarded to landfills. However, these low cost materials constitute renewable feedstocks that could be used for the development of novel biorefinery schemes. Anaerobic digestion from various food waste streams and biodiesel production from cooking oils are predominant alternatives that have been proposed for the utilisation of various food waste streams. Dorado et al. (2009) utilised hydrolysates derived from wheat milling by-products as fermentation media for the production of succinic acid (50.6 g/L). Leung et al. (2012) developed a two-stage bioprocess involving solid state fermentation and enzymatic hydrolysis of waste bread to produce a fermentation feedstock for the production of succinic acid (47.3 g/L at a conversion yield of 0.55 g SA/g bread) using the bacterial strain *Actinobacillus succinogenes*.

A potential biorefining concept for the production of PHAs and biodiesel from confectionery industry waste streams is presented in Figure 2.9. In the case of confectionery wastes that contain high oil content, this could be removed via solvent extraction and subsequently converted into biodiesel. Remaining fractions will be rich in directly assimilable sugars such as glucose, fructose, sucrose and lactose as well as starch and protein. Utilising starch and protein rich waste streams as sources of carbon and

nitrogen in fermentation processes demands the conversion of starch into glucose and protein into amino acids and peptides. The amylolytic and proteolytic enzymes required for the hydrolysis of these macromolecules could be produced via solid state fermentation using the fungal strain *Aspergillus awamori* cultivated on wheat milling by-products. The fermented solids, rich in amylolytic and proteolytic enzymes, are subsequently combined with confectionery wastes to produce hydrolysates that can be used in fermentation processes for the production of PHAs. The production of PHAs from confectionery industry wastes could be employed for the production of biodegradable packaging materials for the same industry.

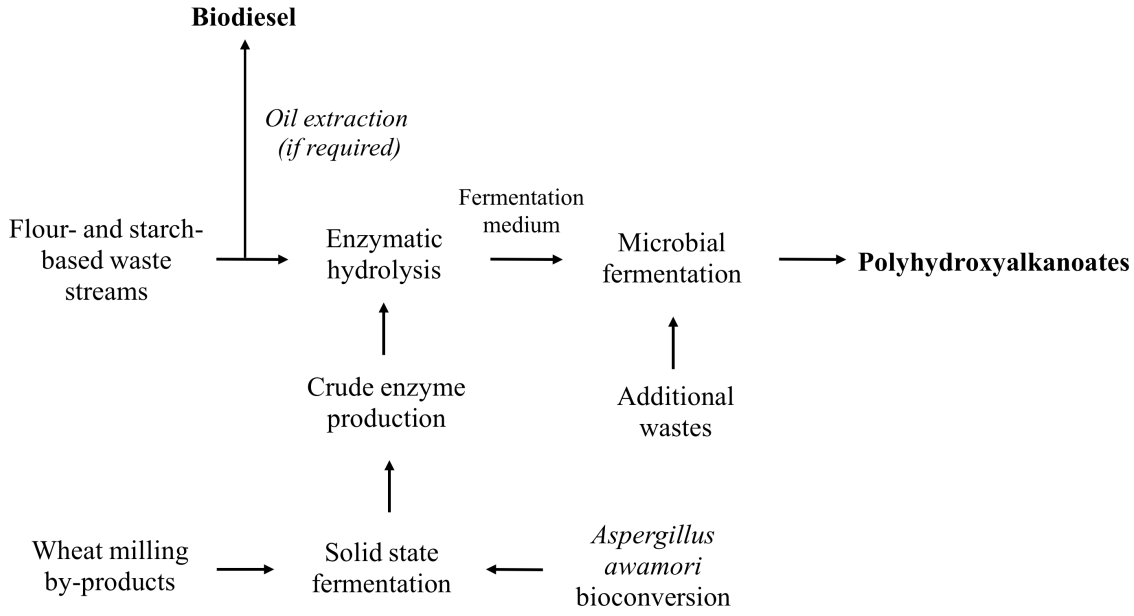

Figure 2.9 Valorisation of confectionery industry waste streams

Whole wheat grains have been used as the sole raw material for the production of PHB along with various value-added products (Koutinas et al., 2007a; Koutinas et al., 2007b; Xu et al., 2010). In this biorefinery concept, wheat is fractionated into bran and gluten as value-added co-products, while remaining fractions are used for the production of fermentation media suitable for the production of PHB via fed-batch cultures using the microbial strain *Wautersia eutropha* NCIMB 11599. Xu et al. (2010) developed a fermentation process for the production of PHB from wheat-derived fermentation media during fed-batch cultures in a bioreactor. The highest PHB concentration achieved was 162.8 g/L. However, wheat is regarded a food resource and should not be used for

chemical production. Starch or flour rich food wastes could be used, instead of wheat, as a renewable resource for the production of PHB.

2.8.4.3 PHB production from whey

Whey is the main by-product occurring from cheese manufacture and lactose is one of its primary components. Current whey valorisation processes mainly focus on the production of whey powder, whey protein concentrate or whey protein isolate. Utilisation of whey in fermentation processes has been widely investigated, given the fact that it is produced in many countries in significant quantities. Furthermore, whey valorisation will also contribute to the improvement of the environmental impact of the cheese industry because whey disposal is a notorious environmental burden.

Future cheese industries could incorporate integrated processing schemes for the production of whey protein and PHAs. Koller et al. (2010) reviewed various bioconversions that employed whey permeate as carbon source aiming at the production of PHAs. Different strategies were proposed concerning uses of whey permeate, including direct conversion as substrate or hydrolysis of lactose to glucose and galactose. Moreover, Wong and Lee (1998) presented PHB production from whey powder with recombinant *E. coli* in pH-stat cultures. In fed-batch cultures with additions of concentrated whey solution the corresponding dry cell weight and PHB concentrations were 87 g/L and 69 g/L, respectively. The PHB content reached up to 80% (w/w). These results established that PHB fermentation process from whey could be industrially employed, increasing the sustainability and market alternatives of traditional cheese producing plants.

Whey protein concentrate and isolate that could be extracted from whey by ultrafiltration and evaporation steps can be applied as food additives targeting to improve the functional properties of food products. Hence, keeping that in mind, biorefinery schemes based on whey utilisation could be easily proposed.

2.9 Downstream separation and purification of PHA

As previously stated, industrial implementation of PHA production is hindered by the high cost of manufacture which is mainly attributed to the fermentation media employed and the downstream separation and purification of PHA. The first bottleneck could be overcome by substituting the expensive and purified components used in the formulation of fermentation medium (e.g. yeast extract) with nutrient rich supplements deriving from renewable resources. Crude nutrient supplements could be produced via enzymatic hydrolysis of renewable resources, providing a complete fermentation medium, including nitrogen, phosphorus and micronutrients, for the production of PHA as well as other metabolic products, such as 1,3-propanediol, microbial oil and bacterial cellulose (Chatzifragkou et al., 2014; Tsakona et al., 2014; Tsouko et al., 2015).

The second impediment on the industrial production of PHAs relates to downstream separation of intracellular polyesters. Isolation and purification of PHAs have been extensively studied regarding the development of environmentally benign and cost competitive processes for industrial implementation (Jacquel et al., 2008; Gumel et al., 2012; López-Abelairas et al., 2015). Solvent extraction of PHB has been widely employed using various solvents, including non-chlorinated industrial solvents such as anisole, cyclohexanone and phenetole (Rosengart et al., 2015). PHA separation has also been achieved via digestion of non-PHA bacterial cell mass using chemical (using acids or alkalis) or enzymatic treatment (Gumel et al., 2012; López-Abelairas et al., 2015). Mechanical disruption of non-PHA bacterial cell mass can also be carried out combined with subsequent treatment using surfactants (e.g. anionic sodium dodecyl sulfate) or chemicals (Jacquel et al., 2008; Gumel et al., 2012). PHA recovery has also been carried out with processes involving supercritical fluid extraction, air classification and dissolved-air flotation (Jacquel et al., 2008; Gumel et al., 2012).

Enzymatic cell lysis has many advantages including low energy requirements, biological specificity, mild operating conditions and low capital investment (Harrison, 1991). However, the high cost of enzymes is a major disadvantage regarding their industrial implementation. In the case of PHA recovery, commercial proteolytic enzymes have been mainly applied (Kapritchkoff et al., 2006; Yasotha et al., 2006; Holmes and Lim, 1990). Enzymatic digestion of non-PHA cell mass has been previously demonstrated as the sole method of purification or coupled with the utilisation of surfactants to decolorize and deodorize the polymer or to remove cell debris (Suzuki et al., 2008;

Lakshman et al., 2006; Horowitz and Brennan, 2010). Holmes and Lim (1990) reported the utilisation of proteolytic enzymes and surfactant treatment to disintegrate bacterial mass of *Alcaligenes eutrophus* NCIB 11599 for PHB separation. The potential valorisation of hydrolysed cell mass was also proposed as a potential approach for the re-utilisation of nutrients released during enzymatic lysis (Holmes and Lim, 1990). Cost competitive production of enzymes could be achieved through solid state fermentation of fungal strains using diversified agri-industrial waste and by-product streams (Melikoglu et al., 2013; Diaz et al., 2013). Fungal strains, such as *Aspergillus oryzae*, can produce various enzymes including protease, phosphatase, pectinase and lipase (Toida et al., 1998; Wang et al., 2010; Heerd et al., 2012). The *A. oryzae* strain used in this study is a well known producer of numerous enzymes that could assist in the hydrolysis of bacteria cell wall leading to the recovery of PHAs.

The cell wall of gram-negative bacteria is composed of a peptidoglycan layer surrounded by an outer membrane, consisting mainly of proteins, phospholipids, lipoproteins and lipopolysaccharides (da Silva et al., 2012). Gram-negative bacteria are resistant to enzymatic lysis by lytic enzymes that hydrolyse only the peptidoglycan layer, such as lysozymes and murein hydrolases. Therefore, the effective lysis of the cell wall of gram-negative bacteria can be accomplished by the combination of various enzymes, such as amidase, lipase and protease. Filamentous fungi can produce a combination of enzymes required for the lysis of the cell wall of gram-negative bacteria (da Silva et al., 2012). Tsakona et al. (2016) showed that crude enzymes preparation produced via solid state fermentation of *Aspergillus awamori* could be used for the lysis of oleaginous yeast cells leading to the release of microbial oil that is originally produced as an intracellular product. Therefore, during the present study and the development of a complete biorefinery concept based on sunflower meal, an environmental benign downstream process was also established, aiming to facilitate biopolymer recovery avoiding the usage of organic solvents.

2.10 Conclusion

According to the literature review presented in this chapter, it can be deduced that the necessity to reduce our dependence on fossil resources will inevitably lead to the reconstruction of current industrial processes that will implement the sustainable utilisation

of renewable resources for the production of biofuels, biopolymers and chemicals. The development of integrated biorefinery concepts that will be incorporated into existing industrial plants exhibits a feasible approach, considering that agri-industrial waste and by-product streams are currently under-exploited. On the other hand, production of commodity and specialty products from these renewable feedstocks will enhance sustainability and broaden the market outlets of traditional industrial sectors. In this viewpoint, the present study was directed towards the development of a novel biorefinery concept valorising the by-products from a sunflower-based biodiesel process to generate poly(hydroxyalkanoates) along with value-added products.

CHAPTER 3

OBJECTIVES

The biodiesel production process generates sunflower meal (SFM) and crude glycerol as the main by-products. The tremendous increase of biodiesel production entails a proportional increase in by-products formation. The eventual depletion of fossil resources raises significant environmental concerns, rendering inevitable the substitution of conventional petroleum-derived products with their corresponding counterparts deriving from biomass. Under this prospect, conventional plastics are envisaged to be replaced by biopolymers including polyhydroxyalkanoates (PHAs) synthesised from renewable resources. However, the high production cost of PHAs attributed mainly to the high cost of raw materials is the main reason that hinders the industrial production of PHAs as substitutes for conventional plastics (e.g. polyethylene).

In the future bio-economy era, biorefinery concepts using renewable resources for the production of various products should be developed and gradually evolve into mature processes in a similar manner as petroleum refineries evolved since the beginning of the 20th century. Under this viewpoint, the present study focused on the development of an advanced sunflower-based biorefinery concept targeting the production of numerous products including PHAs, and more specifically polyhydroxybutyrate (PHB), using SFM and crude glycerol as the sole feedstocks. The main objectives of the experimental work are presented below (Figure 3.1)

- Optimisation of solid state fermentation using the fungal strain *Aspergillus oryzae* for the production of crude enzyme consortia

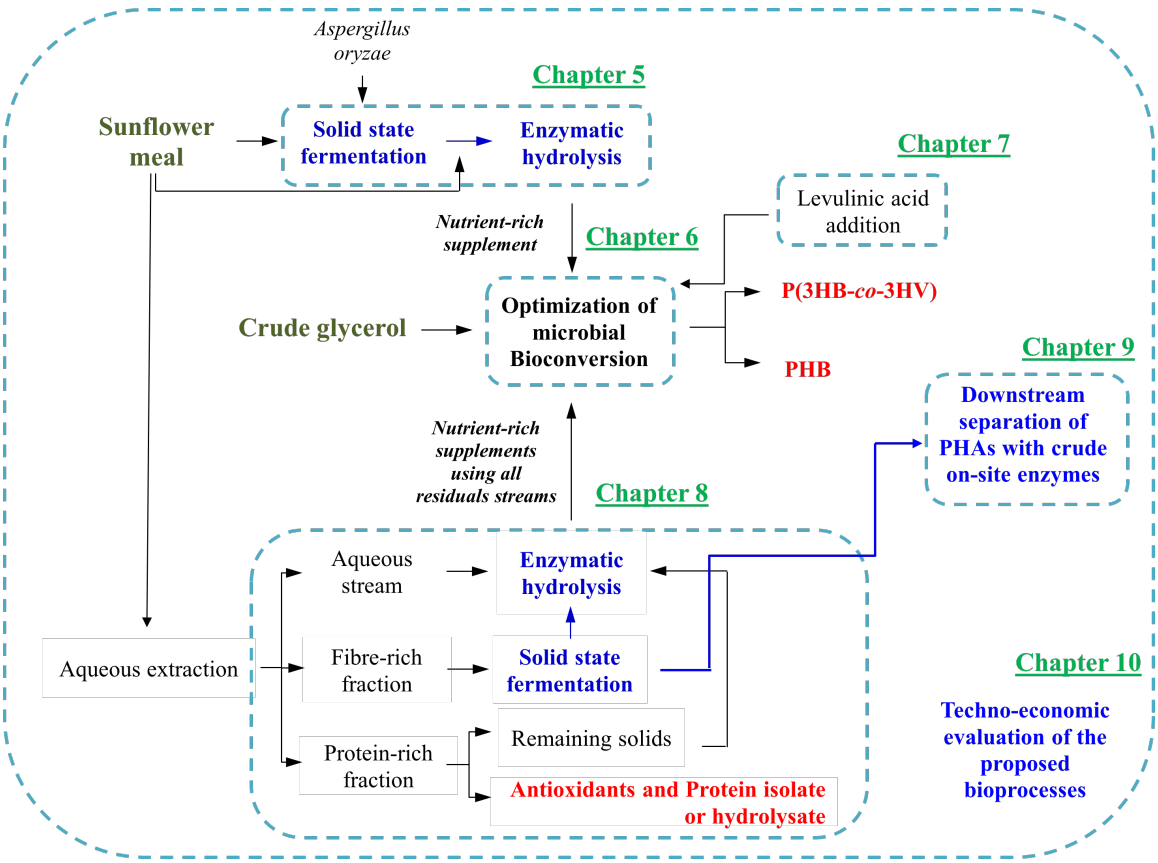

Figure 3.1 Overview of the experimental process designed during the present study for the valorisation of SFM and crude glycerol towards the development of an advanced biorefinery concept

- Optimisation of enzymatic hydrolysis of SFM to formulate a nutrient-rich fermentation feedstock for bacterial bioconversions
- Optimisation of bacterial fermentation for poly(3-hydroxybutyrate) production using *Cupriavidus necator* strains
- Evaluation of levulinic acid as a precursor for fermentative production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
- Development of a sunflower-based biorefinery process for the production of various value-added products including the extraction of an antioxidant-rich fraction and protein isolate
- Development of a novel downstream process for the separation of poly(hydroxyalkanoates) based on the utilisation of crude enzyme consortia produced via solid state fermentation for enzymatic lysis of non-PHA bacterial cell mass.
- Thermal characterisation of the produced biopolymers

- Techno-economic assessment of the proposed biorefinery concept

The experimental procedure was designed in order to fulfill each one of the above objectives. In some cases, a more detailed insight was required, thus various parameters and different fermentation strategies were implemented. More specifically, the optimisation of the two stage bioprocess that was developed for the production of the nutrient-rich supplement was divided into the optimisation of solid state fermentation (SSF) and subsequently in the optimisation of enzymatic hydrolysis (Chapter 5). SFM was initially used as substrate in SSF for the production of crude enzyme consortia, mainly protease. Different parameters affecting fungal growth and enzyme production (e.g. pH, temperature, initial moisture content) were studied targeting the maximisation of enzyme production. These enzymes were subsequently used for enzymatic hydrolysis of unprocessed SFM. The process parameters affecting the activity of enzymes during hydrolytic reaction were evaluated. The target was to yield maximum free amino nitrogen and inorganic phosphorus concentrations deriving from the hydrolysis of protein and phytic acid, respectively. The study was focused on these two nutrients since they are the most important ones, besides the carbon source, in fermentation processes.

The nutrient-rich supplement derived from SFM was combined with crude glycerol and the mixture was evaluated as the sole fermentation media for the production of PHB with different *C. necator* strains. The best performing strain, *C. necator* DSM 7237, was selected via shake flask cultures. Subsequently, bench-scale bioreactor fermentations were conducted under fed-batch mode to assess the capability of this strain to produce PHB (Chapter 6). The approach followed was to initially verify the utilisation of crude glycerol and whole SFM hydrolysates as sole renewable resources for the production of either PHB or P(3HB-*co*-3HV). A simple decanting step employed for crude glycerol (91 % purity) pretreatment proved to be adequate for further utilisation of crude glycerol (92.4 % purity) in bacterial bioconversions. Optimisation of fermentation media included the evaluation of the effect of carbon to free amino nitrogen ratio (C/FAN) to further elucidate bacterial growth and PHB accumulation. After the optimisation offermentation conditions, different bio-processing strategies were implemented aiming to improve the bioconversion process. The effect of levulinic acid as precursor for P(3HB-*co*-3HV) production was also investigated (Chapter 7) taking into consideration that incorporation of 3-hydroxyvalerate units in the bio-based polymer improves its thermo-mechanical properties.

SFM fractionation led to the production of an antioxidant-rich stream, protein isolate and nutrient-complete fermentation media for PHB production (Figure 3.1). Fermentation media were produced from remaining fractions that resulted during SFM fractionation. The recommended approach is based on the principle of optimised resource utilisation preventing the use of commercial nutrient supplements.

Another objective of this study was to demonstrate the ability to recover the P(3HB-*co*-3HV) or PHB produced during fermentation of crude glycerol, SFM hydrolysates and levulinic acid through enzymatic cell lysis using the crude enzyme consortia produced during solid state fermentation by *A. oryzae*. In this way, the enzymes produced via SSF could be employed for downstream separation of P(3HB-*co*-3HV), besides the production of nutrient-rich hydrolysates for the fermentation stage. A central composite design was applied in order to optimise the temperature and pH during enzymatic cell lysis. After the separation of P(3HB-*co*-3HV), the cell lysate was recycled as nutrient-rich supplement together with crude glycerol for the production of PHB, enhancing the sustainability of the proposed biorefinery.

CHAPTER 4

MATERIALS AND METHODS

4.1 Introduction

In this chapter, the applied processes and methods involved in the proposed biorefinery concept are described. Specifically, cultivation conditions for the microorganisms are presented, together with additional and more detailed information concerning all the analytical methods implemented. The study initiated with the evaluation of SFM to produce a nutrient rich supplement that could substitute yeast extract and expensive inorganic chemicals that are mainly employed in fermentation processes. This feedstock was combined with crude glycerol and was further employed in bacterial bioconversions for PHA production. The following step concerned the development of an advanced sunflower based biorefinery scenario that would exploit all streams occurring from SFM fractionation to generate a nutrient rich supplement and other high value products. Similarly, the nutrient supplements produced were implemented in PHA biosynthesis.

4.2 Crude renewable materials

4.2.1 Sunflower meal

The sunflower meal (SFM) used in this study was a by-product of the biodiesel production process and it was kindly supplied by P. N. Pettas S.A. industry (Patras, Greece).

4.2.2 Crude Glycerol

Crude glycerol is also a by-product of the biodiesel production process and was kindly supplied by P.N. Pettas S.A. industry. Crude glycerol is obtained after the oil transesterification process with methanol. The original crude glycerol was left overnight in separation funnels to produce a pretreated crude glycerol stream with slightly higher glycerol purity (92.4 %). In some cases pure glycerol (99 %) purchased from Sigma-Aldrich was employed as substrate.

4.3 The fungal strain Aspergillus oryzae

An industrial strain of *Aspergillus oryzae* isolated from a soy sauce starter produced from the company Amoy Foot Ltd. (Hong kong), kindly provided by Professor Colin Webb (Satake Centre for Grain Process Engineering, Schooll of Chemical Engineering and Analytical Science, University of Manchester, UK) was used for the production of crude enzyme consortia essentials for all the hydrolytic reactions performed during this study. The isolation and purification protocols of this fungal strain were reported by Wang et al. (2005). Spores were preserved in silica sand and were rehydrated regularly to preserve cells viability. Reconditioning of the spores was performed with PBS solution containing 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4. Prior to the experimental setup, *A. oryzae* was sporulated and maintained at 4 °C in slopes containing 30 g/L SFM, 20 g/L wheat bran and 20 g/L agar (Sigma-Aldrich). The slopes were used to inoculate flasks composed of the same solid substrate aiming to increase the spore concentration used as inoculum in solid state fermentations.

4.4 The bacterial strain *Cupriavidus necator*

Three different bacterial strains of the *Cupriavidus* sp. were initially investigated concerning their ability to metabolise crude glycerol towards PHA biosynthesis. The bacterial strains evaluated were *C. necator* DSM 545, *C. necator* DSM 11348 and *C. necator* DSM 7237. Preliminary experiments were performed in shake flask fermentations to assess glycerol utilisation. All bacterial strains were purchased from DSM culture collection and were initially in lyophilised form. Rehydration and cultivation of bacterial cells were performed using the appropriate substrate medium as it was specified by DSMZ (Medium 1), containing 5 g/L peptone (Fluka) and 3 g/L meat extract (Himedia). Bacterial stock cultures were stored at 4 °C in petri dishes and were used during this study for inoculum preparation used subsequently in shake flask and bioreactor fermentations. The strains *C. necator* DSM 545 and *C. necator* DSM 11348 were cultivated in liquid media composed of 10 g/L pure glycerol (99 %, Sigma-Aldrich), 10 g/L yeast extract (Himedia) and 5 g/L peptone (Fluka). For the strain *C. necator* DSM 7237 the liquid media for pre-cultures consisted of 10 g/L pure glycerol (99 %, Sigma-Aldrich), 10 g/L meat extract (Himedia) and 5 g/L peptone (Fluka). The above mentioned media were supplemented with 20 g/L agar and were also used as solid substrate in petri dishes. Pre-cultures were

incubated at 30 °C, 180 rpm in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) in 250 mL Erlenmeyer flasks with 50 mL broth volume for 12 h using the strains *C. necator* DSM 545 and *C. necator* DSM 11348 and 14 hours when the *C. necator* DSM 7237 was employed. In shake flask bioconversions, 1 mL of the pre-culture was aseptically transferred in the main culture broth. In the case of bioreactor fermentations, inoculum volume corresponded to 10 % v/v of the bioreactor working volume. Microorganisms were also preserved in cryovials at -80 °C with pure glycerol (1:1).

4.5 Solid state fermentation

Solid state fermentations (SSF) of sunflower meal or lignocellulose-rich fraction were conducted for the production of crude enzyme consortia, in 250 mL Erlenmeyer flasks at 30 °C. A quantity of 5 g of solid substrate was added in each flask that was autoclaved at 121 °C for 20 min. Subsequently, the solids in each flask were inoculated with a fungal spore suspension that was also used to adjust the moisture content of the substrate at 65 % (w/w, on a dry basis), unless otherwise stated. To increase the fungal spore suspension concentration of the inoculum to 2×10^6 spores mL^{-1}, *A. oryzae* spores were freshly prepared in Erlenmeyer flasks (250 mL) on solid medium identical to the one used in slopes. Specific volume (10 mL) of sterilised tap water and Tween 80 (0.01 %, v/v) (Sigma-Aldrich) were aseptically added into each slope and the surface was scratched with a wire loop. Subsequently, 2 mL of this spore suspension were added on the surface of the solid medium in each flask. Incubation was performed at 30 °C in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China), for 4 days allowing the fungus to grow over the surface. Subsequently, sterilised tap water (50 mL), Tween 80 (0.01 %, v/v) and some glass beads (4 mm diameter) were added in each flask to obtain the fungal spore suspension, followed by vigorous shaking. All flasks with inoculated SFM or LF were incubated at 30 °C for 48 h except for the experiments performed for the optimisation of enzymatic activities. Remaining solids were subsequently mixed with unprocessed SFM, LF or solid residue from SFM fractionation in order to exploit the crude enzyme consortia for further hydrolysis of macromolecules, aiming to generate a nutrient-rich fermentation supplement.

4.6 Hydrolysis of sunflower meal and production of hydrolysates

The first set of experiments focused on the optimisation of SFM hydrolysis conditions. After the end of SSF, the fermented solids were suspended in 500 mL sterilised tap water and were subsequently macerated using a kitchen blender. The resulting suspension was added in 1 L Duran bottles that contained specific known quantity of unprocessed sterilised SFM. Hydrolysis temperature was regulated by placing Duran bottles in water baths. Mixing of the suspension was conducted using magnetic stirrers.

Five sets of experiments were carried out to evaluate the effect of temperature, pH, SSF time, initial solid concentration and initial proteolytic activity. In the first 4 cases, the remaining solids from SSF were mixed with unprocessed SFM at the beginning of each hydrolysis. In the set of experiments that focused on the evaluation of initial proteolytic activity, enzyme extracts were separated from SSF solids via vacuum filtration in order to evaluate only the hydrolysis of SFM protein and avoid the autolysis of remaining fungal cells.

The following experimental setup focused on the evaluation of all residual solid and liquid streams remaining after the fractionation of SFM. Similarly, after the end of SSF, fermented solids were macerated using a kitchen blender though in this case solids were suspended in either 500 mL of tap water or 500 mL of the residual soluble fraction (Figure 4.1). When tap water was used, the suspension was mixed with unprocessed LF for the production of hydrolysate I. When the residual soluble fraction was used, the suspension was mixed with the solid residue fraction generated after alkaline solubilisation of proteins (Figure 4.1, Figure 4.13) for the production of hydrolysate II. Enzymatic hydrolysis was carried out using either 50 g/L or 100 g/L initial solid concentration with the same initial proteolytic activity. Hydrolysis was performed in 1 L Duran bottles at 45°C and uncontrolled pH. Agitation was conducted using magnetic stirrers.

Samples were collected at random intervals and the solids were separated via centrifugation (10 min, 3000 g). The supernatant was used for the analysis of free amino nitrogen (FAN) and inorganic phosphorus (IP). Hydrolysis experiments, as well as subsequent analysis of their products, were carried out in triplicates.

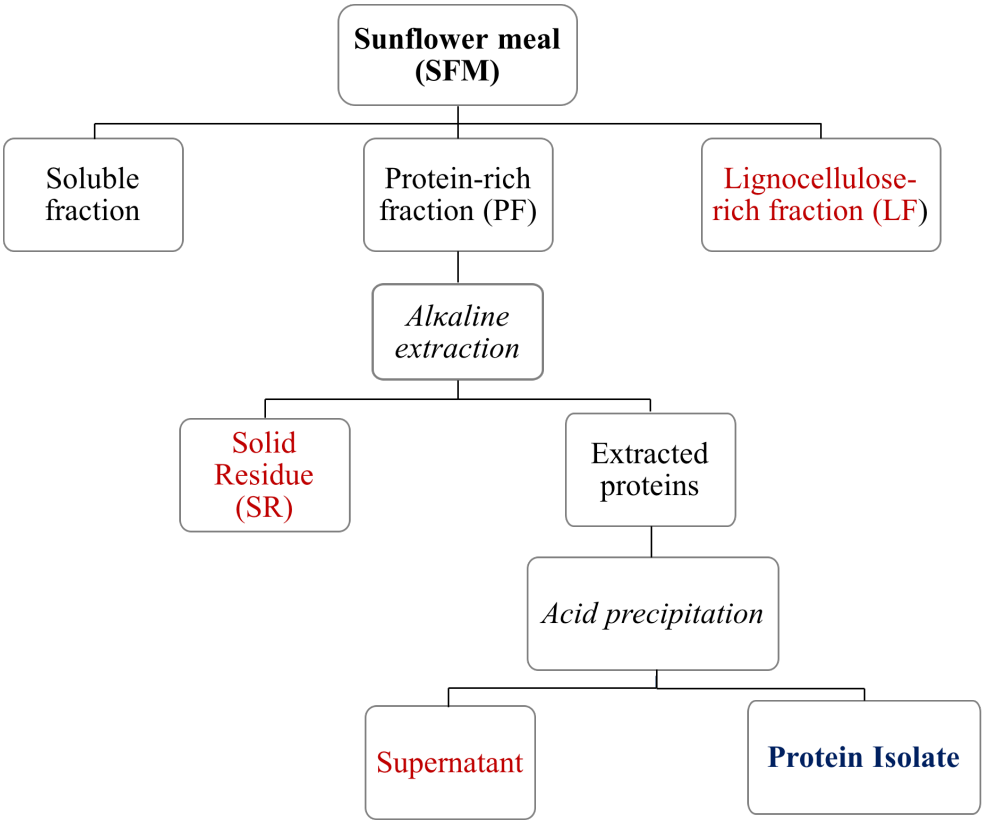

Figure 4.1 SFM fractionation protocol followed for the production of protein isolate (adapted by Villaneuva et al. 1999)

After the end of hydrolysis, remaining solids were removed by vacuum filtration and SFM hydrolysates were filter-sterilised using a 0.2 μm filter unit (Polycap ^{TM} AS, Whatman Ltd.). The pH of SFM hydrolysates was adjusted to the optimum pH range (6.7 – 6.9) for *C. necator* growth with 5 M NaOH.

4.7 Fractionation of sunflower meal

A slightly modified method originally reported by Parrado et al. (1991) was implemented for the fractionation of SFM and the production of protein isolate (Figure 4.1). More specifically, fractionation initiated with suspension of SFM in water (1:10, w/v) leading to the production of three distinct phases: 1) the lignocellulose-rich fraction (LF) that floated at the top of the aqueous suspension, 2) the protein-rich fraction (PF) that precipitated at the bottom and 3) the intermediate aqueous solution (soluble fraction) that contained soluble compounds from SFM (water soluble proteins and sugars).

Extraction of phenolic compounds was performed either from the SFM or the PF fraction by treatment with a solution of ethanol:water (1:1, v/v). Subsequently, alkaline treatment with 5 M NaOH until a pH value of 10.5 was carried out in order to solubilise the protein fraction. Solubilisation of protein was performed twice with reduced volume of alkali solution for 1 h under agitation with overhead stirrer. After separation of residual solids, the pH value of the liquid stream was reduced to the isoelectric region (around pH value of 4.3) leading to the precipitation of sunflower proteins. Protein isolate was collected by centrifugation and lyophilised for further use. The residual streams of this process are the soluble fraction, the LF, the solid residue (SR) generated after alkaline solubilisation of proteins and the supernatant that remains after precipitation of the protein isolate stream. All these residual streams were evaluated in enzymatic hydrolysis to generate nutrient supplements as noted in the previous section. Subsequently, the obtained hydrolysates were employed in bioreactor fermentations to assess their capability for PHB synthesis, as will be described in following section.

4.8 Shake flask fermentations for PHA production

Initial preliminary experiments to evaluate SFM hydrolysates and crude glycerol as fermentation supplements were performed in 250 mL Erlenmeyer shake flasks with 50 mL of broth volume. Incubation was carried out in a rotary shaker (ZHWY-211C Series Floor Model incubator, PR China) at 180 rpm, 30 °C and initial pH in the range of 6.7 – 6.9. The pH was manually adjusted during the fermentation by adding 5 M NaOH or 10 % (v/v) H_2SO_4. The pH was regulated by running simultaneously two identical shake flask fermentations. One was used to aseptically measure the pH value and identify the volume of NaOH or H_2SO_4 necessary to re-adjust pH. The same volume of base or acid was aseptically added in the other shake flask that was used to collect samples. Preliminary experiments have proved that results from the two flasks were identical and therefore reproducible. Bacterial bioconversions were carried out in duplicate and data presented are the mean values of two independent experiments.

Crude glycerol (25 g/L) was used as a sole carbon source in the fermentation medium. Flasks were aseptically inoculated with 1 mL of the liquid pre-culture (after specified time of pre-culture for each strain) and were subsequently incubated at 180 rpm and 30 °C. Fermentations for the bacterial strain *C. necator* DSM 545 were performed

under fed-batch mode by adding crude glycerol at 58 h and 81 h. Concerning the strain *C. necator* DSM 7237, shake flask fermentations were carried out under fed-batch mode by supplementing culture broth with a concentrated solution of levulinic acid (LA) (75 %, v/v) as a precursor in random intervals, aiming to evaluate the production of the co-polymer P(3HB-*co*-3HV). Initial FAN concentration of SFM hydrolysates in these particular experiments was around 365 mg/L.

Fermentation samples were regularly withdrawn and centrifuged at 3000 g for 10 min (Hettich Universal 320-R, Germany). Supernatant was collected and used for the analysis of glycerol, FAN, IP and LA. The sediment was suspended in distilled water to wash the cells and centrifuged again. Acetone was added in the solid fraction and transferred in pre-weighed McCartney universal bottles (14 mL) for the determination of residual cell weight (RCW) and PHA concentration.

4.9 Bioreactor fermentations for PHA production

All of the fed-batch mode fermentations conducted during this study were performed in bioreactors. The majority of them was conducted in 1 L bioreactors (New Brunswick Scientific Co, New Jersey, USA) with 0.8 L working volume while in some experiments bioreactors of 2 L total volume, with 1.6 L working volume were employed. In each case inoculation was carried out with a 10 % (v/v) pre-culture medium as previously specified. The aforementioned bioreactors were fully equipped with pH probe (Mettler Toledo, USA), dissolved oxygen (DO) probe, temperature control with external thermal jacket and temperature detector, agitation, gas inlet and outlet, pumps and sampling system. The temperature was maintained at 30 °C and the pH was regulated in the range of 6.7 - 6.9 using NaOH 5 M and H_2SO_4 10 % (v/v) through automated pumping with sterilised tubes. The aeration was maintained at a flow rate of 1 vvm. Dissolved oxygen (DO) was set at 25 % of saturation, while the agitation speed was regulated in the range of 200-500 rpm in order to maintain the DO level in the bioreactor. The pH probe was calibrated before autoclaving while the calibration of the DO probe was carried out prior to inoculation. Foaming was prevented by manually addition of a sterilised dilute antifoam solution (1 %, v/v).

Figure 4.2 Bioreactor employed in bacterial bioconversions

Initial crude glycerol concentrationwas in the range of 20-25 g/L unless otherwise stated. The purity of crude glycerol was included in the respective calculations to determine the appropriate initial quantity of glycerol. A concentrated solution of crude glycerol (75 % v/v) was manually inserted through a butyl septum on the head plate of the bioreactor unless otherwise stated.

Different sets of fed-batch bioreactor experiments were performed. First the effect of crude glycerol pretreatment was assessed. Experiments were performed with pure glycerol, with and without supplementation of micro elements. The trace element solution employed in the fermentation carried out with pure glycerol contained: 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L $FeCl_3 \cdot 6H_20$, 0.02 g/L $ZnSO_4$, 0.06 g/L $MnSO_4$, 0.15 g/L $CaCl_2 \cdot 2H_20$. Subsequently, crude glycerol and pretreated crude glycerol via decanting were evaluated in microbial bioconversions. In all these experiments SFM hydrolysates were utilised as nutrient supplements with the same initial FAN concentration.

The next step employed the evaluation of the effect of carbon to nitrogen ratio (C/N) on PHA accumulation. A range of increasing initial free amino nitrogen concentrations was evaluated aiming to identify the optimum ratio that shifts bacterial metabolism towards biopolymer synthesis.

Subsequently, different bioprocessing strategies were implemented targeting to enhance the residual cell weight during the exponential phase or during PHA

accumulation. Firstly, the addition of phosphate salts was examined aiming to increase the initial inorganic phosphorus concentration. In a subsequent fermentation, continuous feeding of a mixture composed of concentrated sunflower meal hydrolysate and crude glycerol was implemented during the accumulation phase.

In the optimum fermentation conditions established, the addition of levulinic acid as a precursor to copolymer P(3HB-*co*-3HV) synthesis was examined. A known volume of concentrated solution of levulinic acid was manually added in the culture broth in order to reach the specific concentrations under investigation. Moreover, the frequency of levulinic acid pulses was also studied. A concentrated solution of glycerol was added as previously described.

Finally, the last set of experiments concerned the evaluation of different hydrolysates obtained by combining and hydrolysing all the residual streams after fractionation of SFM to assess their potential as fermentation feedstocks. Three sets of experiments with different hydrolysates were performed as described in previous section (see 4.6 *Hydrolysis of SFM and production of hydrolysates*).

Fermentation samples were collected at frequent intervals (specifically every 1-3 h during the exponential phase) and centrifuged at 3000 × g for 10 min (Hettich Universal 320-R, Germany). The solid fraction was washed with distilled water and centrifuged again. The sediment was re-suspended in acetone and transferred in pre-weighed McCartney universal bottles (14 mL) for the determination of residual cell weight (RCW) and PHA concentration. The supernatant was used for the analysis of glycerol, free amino nitrogen, inorganic phosphorus and levulinic acid.

4.10 Extraction of enzymes and assay of enzymatic activities

Optimisation of SSF was performed based on the maximum protease activity since nitrogen was the main macromolecule of SFM to be hydrolysed. In a following step, phytase, xylanase and cellulase production was also studied in the optimum culture conditions of protease production. In order to determine the enzymes secreted during SSF of SFM with *A. oryzae,* the whole content of each flask was collected at different intervals. A known quantity of the fermented mass (~2.5 g) was mixed with 50 mL of buffer solution and macerated using a kitchen blender. The mixture was subsequently centrifuged (5886 ×

g, 15 min) and 5 mL of the enzymatic extract were utilised in enzymatic assay after mixing with 5 mL of the corresponding substrate. In each case, the reaction was terminated by adding trichloroacetic acid (5 %, w/v) and samples were stored until further analysis.

Protease activity was quantified by the formation of free amino nitrogen that resulted during hydrolysis of 15 g/L of casein (Merck) at 55 °C in 200 mM, pH 6 phosphate buffer. One unit of protease activity (U) was defined as the protease required for the production of 1 μg FAN in one minute.

Phytase activity was assayed by the quantification of inorganic phosphorus (IP) released from the hydrolysis of sodium phytate (1 %, w/v) at 50 °C in 5mM acetate buffer (pH 5.5). One unit of phytase activity (U) was defined as the phytase required for the production of 1 μg of IP in one minute.

Xylanase activity was measured by the production of xylose (as reducing sugar) from the hydrolysis of Beechwood xylan (Sigma-Aldrich) (1 %, w/v) at 50 °C in 0.05 M citrate buffer (pH 5.4). One unit of xylanase activity (U) was defined as the xylanase required for the production of 1 μmol of xylose in one minute.

Cellulase activity was determined by the quantification of glucose released from the hydrolysis of carboxymethyl cellulose (Sigma-Aldrich) (1 %, w/v) at 40 °C in 0.1 M citrate-phosphate buffer (pH 6). One unit of cellulase activity (U) was defined as the cellulase required for the production of 1 μmol of glucose in one minute.

4.11 Determination of free amino nitrogen concentration

The free amino nitrogen (FAN) concentration in hydrolysis and fermentation samples was determined according to the ninhydrin colorimetric method (Lie, 1973) promulgated in the European Brewery Convention. This method is based on the oxidative decarboxylation of α-amino acids by ninhydrin under heating in pH value of 6.7. A blue color is produced by the reaction of reduced ninhydrin with unreduced ninhydrin and the generated NH_3.

The concentration of FAN refers to the nitrogen contained in free amino groups of amino acids and peptides. For quantification of FAN the following reagents were employed:

1) *Color reagent*: This reagent was prepared by mixing 49.71 g di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), 5 g ninhydrin, 3 g fructose and approximately 60 g mono-potassium phosphate (KH_2PO_4) until the pH of the reagent was in the range of 6.6 – 6.8. The final volume of the mixture was adjusted to 1 L.
2) *Dilution reagent*: This reagent was prepared by diluting 2 g of potassium iodate (KIO_3) in 616 mL of deionised water and subsequently adding 384 mL of absolute ethanol (99% vol/vol Sigma-Aldrich) to reach final volume of 1 L.
3) *Glycine stock solution*: 0.1072 g of glycine was dissolved in deionised water in a volumetric bottle of 100 mL.
4) *Glycine standard solution*: 1 mL of the glycine stock solution was transferred and diluted in deionized water until 100 mL final volume in order to obtain a final concentration of FAN 2 mg/L.

All the aforementioned reagents were stored at 4 ^{o}C and were regularly prepared.

After the centrifugation of the samples, supernatant was collected and properly diluted prior to analysis. 1 mL of the diluted sample was added into glass test tubes (16x150 mm), followed by the addition of 0.5 mL of color reagent. A blank sample was also prepared with water instead of diluted sample. All tubes were sealed with alufolie and placed in boiling water for exactly 16 min. Subsequently, the samples were cooled down for 20 min, at 20 ^{o}C and then 2.5 mL of dilution reagent were added. Samples were vigorously shaken with vortex and then absorbance was read in spectrophotometer at 570 nm (U-2000, Spectrophotometer, Hitachi) against blank sample. All the samples were carried out in duplicate.

Calibration curve was obtained by diluting glycine standard solution to achieve different concentrations of FAN (0.5, 1, 1.5 and 2 mg/L). A representative equation employed to calculate FAN concentration in samples is presented in Figure 4.3.

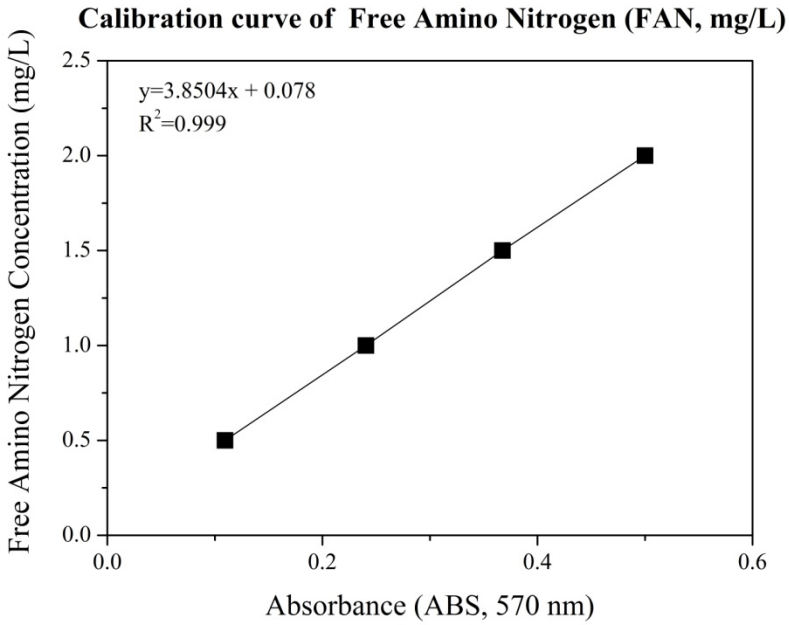

Figure 4.3 Calibration curve of free amino nitrogen (FAN)

4.12 Determination of inorganic phosphorus concentration

Inorganic phosphorus (IP) was assayed by the ammonium molybdate spectrophotometric method described by Harland and Harland (1990). This method is based on the oxidation of organically combined phosphorus with perchloric acid to produce orthophosphate. Under acidic conditions ammonium molybdate reacts with orthophosphate to form phosphomolybdate that is further reduced to molybdenum blue. The blue colour is measured spectrophotometrically at 730 nm.

To determine IP concentration, samples were appropriately diluted to 5 mL final volume into glass test tubes (16x150 mm). In each tube, 0.4 mL of perchloric acid (60 %, v/v) was added under the fuming cabinet and the samples were vortexed for 10 sec. Then, 0.3 mL of freshly prepared ascorbic acid solution (1 %, w/v) was introduced and the samples were vortexed again for 10 sec. Finally, 0.4 mL of ammonium molybdate (4 %, w/v) were added and the samples were vigorously shaken. The tubes were left for 10 min, allowing for the colour to develop and then absorbance was read at 730 nm using a spectrophotometer (U-2000, Spectrophotometer, Hitachi) against water as a blank sample. All the samples were carried out in 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. This reagent was employed to prepare the calibration curve after the respective dilutions to obtain different IP concentrations (2, 4, 6, 8 and 10 mg/L). An indicative equation employed to assay IP concentration in hydrolysis and fermentation samples is presented in Figure 4.4.

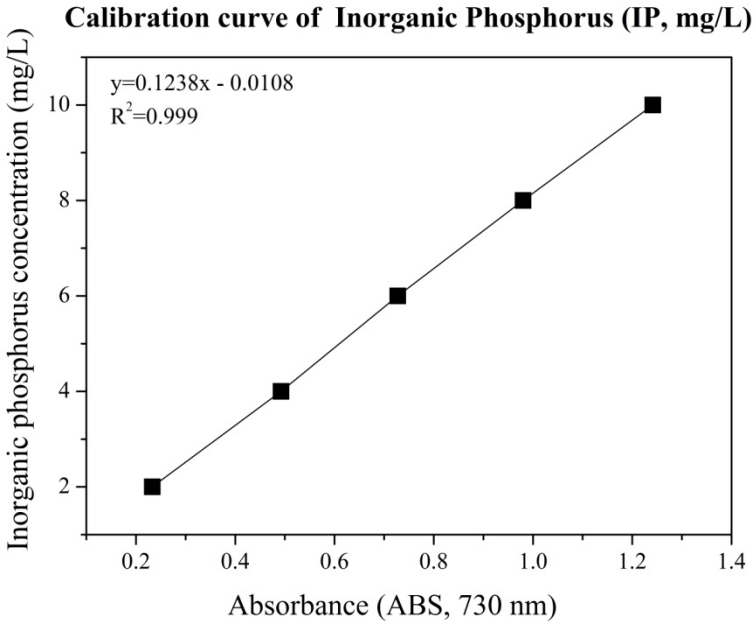

Figure 4.4 Calibration curve of Inorganic Phosphorus (IP)

4.13 Determination of reducing sugars

Reducing sugars and specifically glucose and xylose during the enzymatic assays were determined with the dinitrosalicylic colorimetric method reported by Miller et al (1959). The principle of the method is the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid under alkaline environment and heating, which develops orange colour that absorbs at 540 nm.

For the experimental procedure, 0.5 mL of properly diluted sample and 0.5 mL of DNS reagent were introduced in glass test tubes (16x150 mm) and heated (100 °C) exactly for 5 min. After cooling down to ambient temperature, 5 mL of distilled water were added in each tube and absorbance was measured in the photometer at 540 nm (U-2000, Spectrophotometer, Hitachi) against blank sample. Blank sample preparation was performed by substituting sample with water. Calibration curves were carried out with

glucose (0.5, 1, 1.5, 2 g/L) and xylose solutions (0.5, 1, 1.5, 2 g/L), as demonstrated in Figure 4.5 and Figure 4.6 respectively.

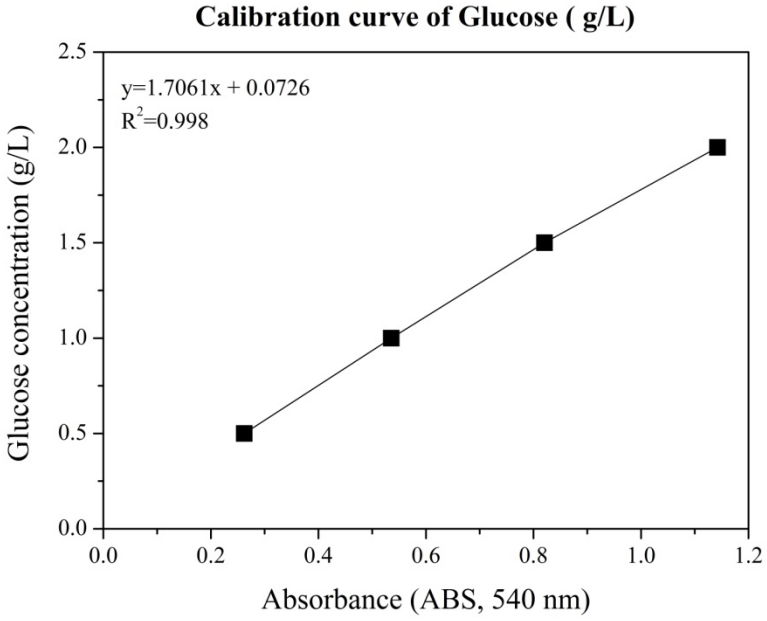

Figure 4.5 Calibration curve of glucose

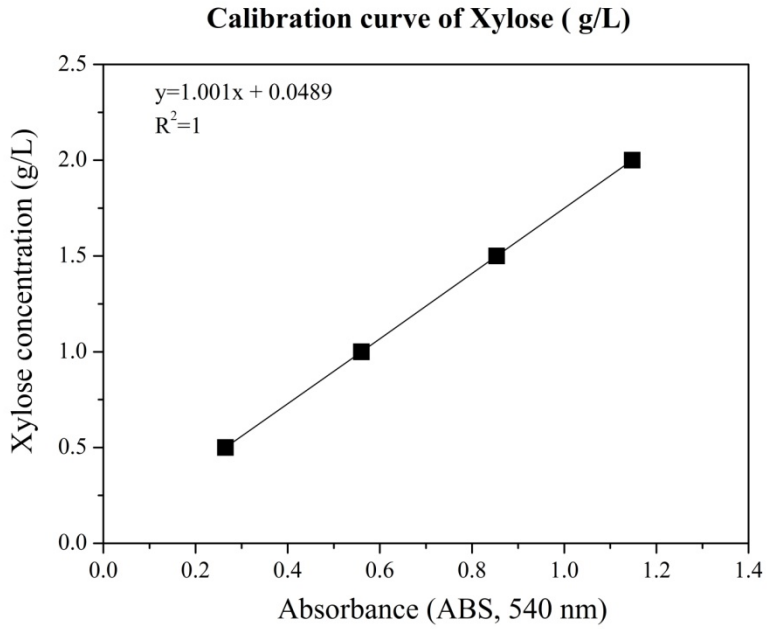

Figure 4.6 Calibration curve of xylose

4.14 Determination of glycerol and levulinic acid concentration

Glycerol and levulinic acid (LA) concentration in fermentation samples were determined by High Performance Liquid Chromatography (HPLC, Waters 600E) equipped with an Aminex HPX-87H (300 mm x 7.8 mm, Bio Rad, CA) column, coupled to a differential refractometer (RI Waters 410). Operating conditions were as further described: mobile phase was an aqueous solution of 0.005 M H_2SO_4 and the flow rate was set to 0.6 mL/min. Injection volume was 40 μL and the column temperature was 65 °C. Prior to injection, the samples were diluted to appropriate concentration with deionised water and filtered through a 0.2 μm membrane filter. Calibration curves of glycerol and LA are illustrated in Figure 4.7 and Figure 4.8 respectively.

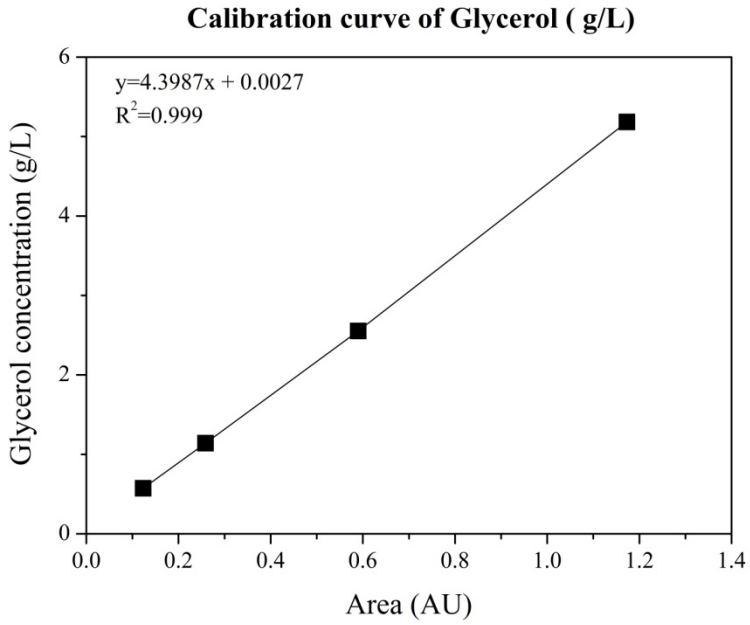

Figure 4.7 Calibration curve of Glycerol

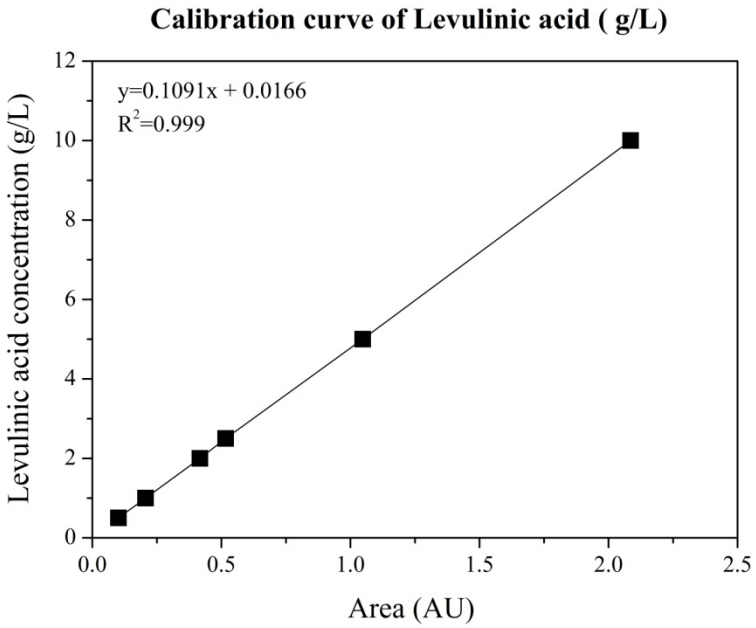

Figure 4.8 Calibration curve of Levulinic acid

4.15 Determination of total dry weight (TDW) and residual cell weight (RCW)

Throughout the fermentation and specifically during the exponential phase, samples were frequently withdrawn (1-3 h). Cell growth was estimated indirectly by reading optical density (OD) at 650 nm using a spectrophotometer (U-2000, Spectrophotometer, Hitachi). An approximate calculation of dry weight was performed by employing the following equation (Figure 4.9) which correlated OD with total dry weight.

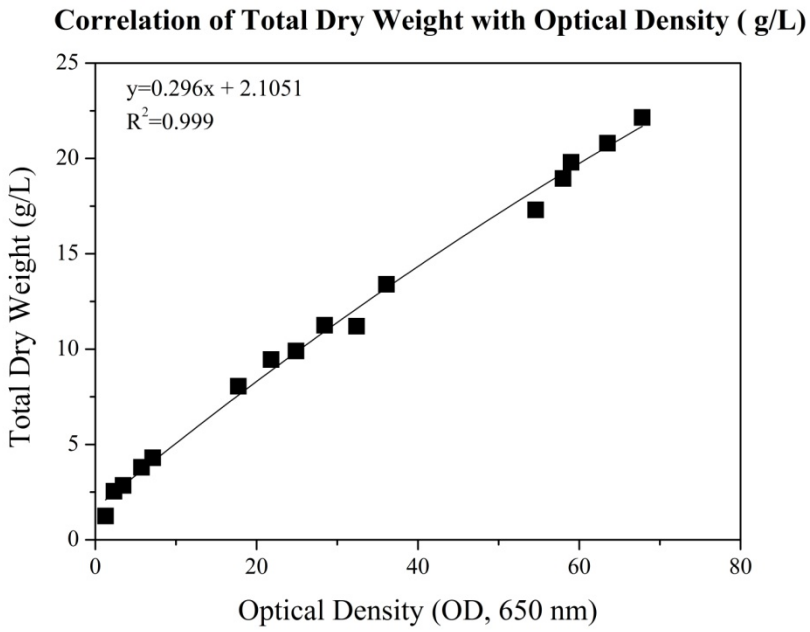

Figure 4.9 Correlation curve of Total Dry Weight (TDW) with Optical Density (OD)

Subsequently, samples were centrifuged as previously described (see 4.*9 Bioreactor fermentations for PHA production*). The sediment was washed with distilled water, re-suspended in acetone and transferred in pre-weighed McCartney bottles (14 mL). Total dry weight (TDW) was determined by drying the sediment from each fermentation sample at 50 °C until constant weight in an analytical balance. The residual cell weight (RCW) was determined by subtracting the PHA concentration measured by GC from the TDW.

4.16 Quantification of PHA concentration

PHA concentration was determined by the method described by Riis and Mai (1988) based on the derivatization of 3-hydroxybutyric acid to propyl ester. PHA samples were hydrolysed and propylated with a mixture of 1,2-dichloroethane (DCE) and acidified propanol. Benzoic acid (99.6%, Acrōr Organics) was used as internal standard at a concentration of 200 mg/L.

More specifically, in the McCartney universal bottles containing dry biomass cells, 2 mL of 1,2-dichloroethane and 2 mL of acidified propanol (a mixture of HCl : n-propanol 1:4) were introduced. The samples were placed in a water bath at 100 °C for 2 hours. Subsequently, the samples were removed and left to cool down. Phase separation was

achieved by the addition of 4 mL deionized water. Samples were vigorously vortexed and left to settle. Propylated 3-hydroxybutyric acid was extracted in 1,2-dichloroethane and 1 μL was injected in GC:

A gas chromatograph (Fisons 8060) equipped with a Flame Ionization Detector (FID) and a Chrompack column CP-WAX 52 CB (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific) was used for PHA concentration measurements. Helium was used as carrier gas at a flow rate of 2 mL/min. Oven initial temperature was set at 120 ^{o}C for 1 min, followed by a temperature ramp of 10 ^{o}C/min to 200 ^{o}C, held constant for 5 min, and then increased to 220 ^{o}C at 10 ^{o}C/min with a final isothermal period of 3 min. The injector and detector temperatures were set at 230 °C. The peak of each PHA monomer (propyl esters of 3HB and 3HV) in the chromatogram was confirmed based on the retention time of respective monomers from commercial PHB and P(3HB-*co*-3HV) standards (Sigma-Aldrich). The equation employed to calculate PHB and P(3HB-*co*-3HV) concentration is presented in Figure 4.10 and Figure 4.11.

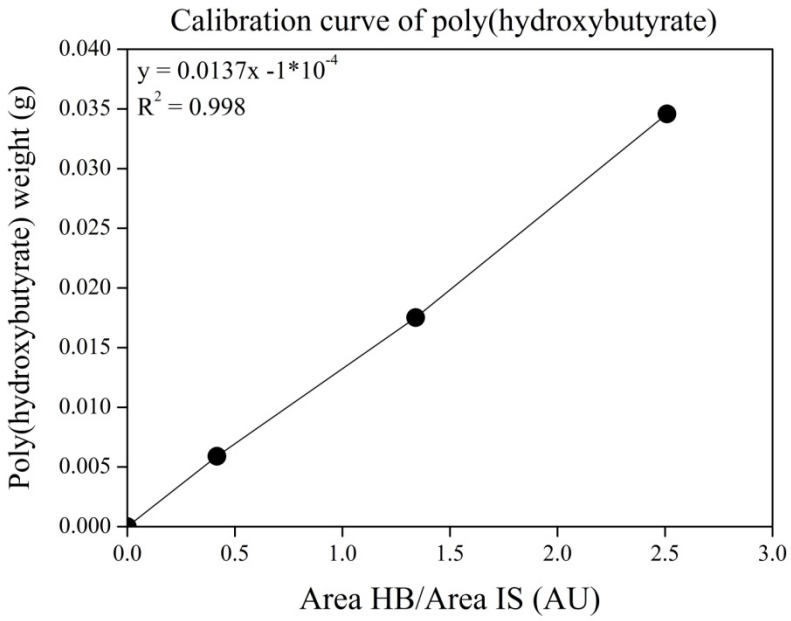

Figure 4.10 Calibration curve of P(3-hydroxybutyrate) with internal standard (benzoic acid)

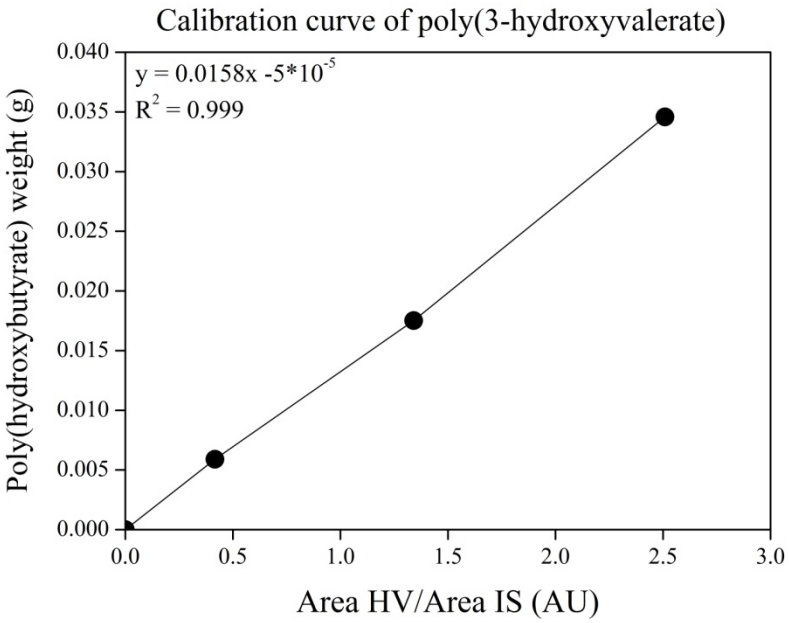

Figure 4.11 Calibration curve of P(3-hydroxyvalerate) with internal standard (benzoic acid)

4.17 Total phenolic content

The total phenolic content (TPC) was estimated by the Folin-Ciocalteu colorimetric method according to the procedure reported by Wojdyło et al. (2007) using chlorogenic acid (CGA) as standard phenolic compound according to the standard curve of Figure 4.11. Briefly, 0.2 mL of Folin-Ciocalteu reagent and 2 mL of distilled water were added in methanol and the ethanol extracts and allowed to stand for 3 minutes. Subsequently, 1 mL of 20 % sodium carbonate (Na_2CO_3) was added and the samples were incubated for 1 h. Absorbance of the developed blue colour was measured at 765 nm. The TPC was expressed as CGA equivalents (CGAE) (mg per 100 g of dry mass).

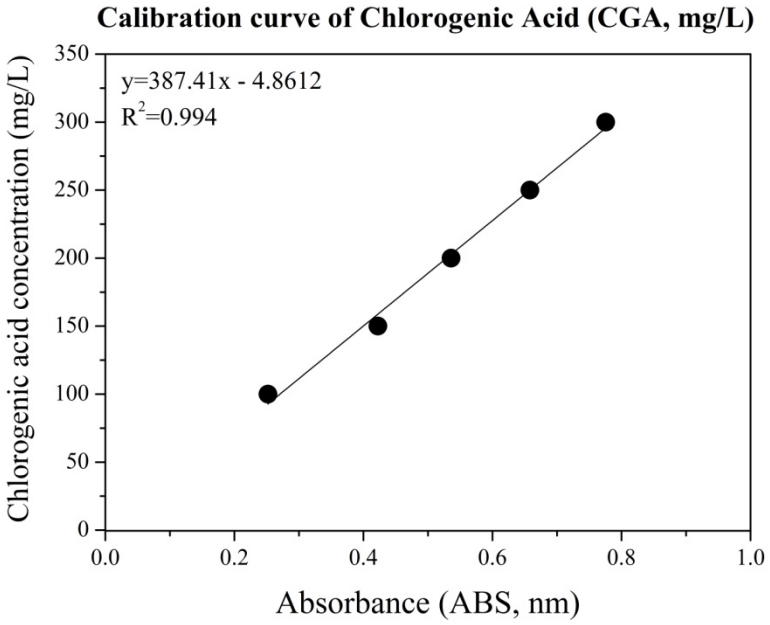

Figure 4.12 Calibration curve of chlorogenic acid for the determination of total phenolic content

4.18 DPPH assay

Free radical-scavenging activity of the sample extracts was evaluated with the modified DPPH$^{\bullet}$ (1,1-diphenyl-2picrylhydrazil radical) assay and evaluated using the IC_{50} method (Gardeli et al., 2008). In particular, 1 mL of sample extract was added in 3 mL of freshly prepared DPPH solution (10^{-4} M in aqueous methanol, 70:30 v/v). The diluted sample was vigorously shaken and incubated at room temperature in dark for 30 min. The decrease in absorbance was determined at 517 nm against a blank solution (aqueous methanol). A sample containing 1 mL of aqueous methanol an 3 mL DPPH was employed as the control. The IC_{50} value stands for the minimum concentration of extract that is required to scavenge 50% of the DPPH free radical.

4.19 Extraction of intracellular PHA

Extraction of intracellular PHA was carried out following a slightly modified method to the one reported by Hahn et al. (1999). Bacterial cells were separated from the fermentation broth via centrifugation at 3000 g for 15 min, washed with water and lyophilised. Each gram of lyophilised bacterial mass was subsequently mixed with 30 mL

of chloroform and 30 mL of sodium hypochlorite solution (20%, v/v in water). The suspension was agitated at 150 rpm using an orbital shaker for 120 min at ambient temperature. Three distinct phases were formed after centrifugation of the mixture at 3000 g for 10 min. The upper phase was sodium hypochlorite solution, middle phase composed of cell debris and PHA was dissolved in chloroform in the bottom phase. After the removal of the upper phase using a pipette, the remaining two phases were left to settle in a separating funnel. The lower phase was collected and filtered (Whatman N° 1 filter paper, VWR Scientific, USA) in order to recover the chloroform. PHA recovery was achieved by precipitation with ice-cold methanol.

4.20 Analysis of micronutrients

Fe^{+2}, Ca^{+2} and Mg^{+2} were measured using an atomic absorption spectrophotometer (Varian SpectrAA-300). Ca^{+2} analysis was performed under N_2O/Acetylene flow while for Fe^{+2} and Mg^{+2} a mixture of Air/Acetylene was employed. Na^{+} quantification was performed with a PGI 2000 Flame Photometer.

4.21 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was carried out to determine thermal stability of the biopolymer using a Netzsch STA 409 at a rate of 10 °C/min under a constant flow of nitrogen (100 mL/min). In each case 50 mg of sample was employed. Thermogravimetric analysis (TGA) was performed during a short term scientific mission (STSM) in the Green Chemistry Centre of Excellence in the University of York included in Cost Action TD1203 named “Food waste valorization for sustainable chemicals, materials & fuels (EUBis)”.

4.22 Nuclear magnetic spectrometry

^{1}H and ^{13}C NMR spectra were recorded on a Jeol ECX-400 spectrometer at 400 MHZ (^{1}H) and 100 MHz (^{13}C). The central resonances of $CDCl_3$ (δ_H = 7.24 ppm; δ_C = 77.23 ppm) were used as the internal references. Typically 10 mg sample was dissolved in 1 mL $CDCl_3$. The low concentration necessitated the use of a higher number of scans i.e. 512 for the ^{1}H NMR spectra and 2048 for the ^{13}C NMR spectra.

Nuclear magnetic spectrometry was performed during a short term scientific mission (STSM) in the Green Chemistry Centre of Excellence in the University of York included in Cost Action TD1203 named "Food waste valorization for sustainable chemicals, materials & fuels (EUBis)".

4.23 Neutral Detergent Fiber, Acid Detergent Fiber, Acid Detergent Lignin

Cellulose, hemicellulose and lignin analysis was performed by determination of acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) according to AOAC Official Method 973.18 and Fiber (Acid Detergent) and Lignin (H_2SO_4) in Animal Feed and was based to Gerhardt application fiber bag-system. Composition of cellulose was obtained by subtracting ADL from ADF while hemicellulose is represented by the difference between NDF and ADF.

4.24 Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) was implemented to evaluate the thermal properties of the produced polymers by using a Differential Scanning Calorimeter DIAMOND DSC of Perkin Elmer calibrated with indium, tin and zinc. Samples (approximately 5-10 mg) were weighed and enclosed in aluminum pans. First heating cycle was performed from -20 ºC to 200 ºC at a rate of 20 ºC/min and kept for 5 min to eliminate polymer history. Subsequently samples were cooled from 200 ºC to -20 ºC at a rate of 20 ºC/min and then heated again from -20 ºC to 200 ºC at a rate of 20 ºC/min. Melting temperature (T_m) and glass transition temperature (T_g) were determined based on the obtained thermograms. Crystallisation temperature (T_c) was obtained from the cooling cycle while cold crystallisation temperature (T_{cc}) was calculated from the second heating cycle. Percentage of crystallinity was calculated by implementing Equation 3 where ΔH_m is the thermodynamic enthalpy of fusion obtained from the first heating cycle and ΔH^0_{PHB} is the enthalpy of fusion for PHB (146 J/g). (Li et al. 2003)

$$\%X = \frac{\Delta H_m}{\Delta H^0_{PHB}} * 100 \qquad \text{Eq. 4.1}$$

4.25 Gel permeation chromatography (GPC)

Molecular weights of the extracted PHAs were determined by employing gel permeation chromatography (GPC) coupled with a Waters HPLC 1525 equipped with a Waters 996 photodiode array detector and a TOSOH Bioscience TSKGEL GMHHR column (300 mm x 7.8 mm). Chloroform was used as elution solvent at a flow rate of 1 mL/min. Briefly, samples were dissolved in chloroform at a final concentration of 0.5 % (w/v) and the obtained solution was filtered (PTFE membrane, 0.22 μm) prior to analysis. Calibration curves were performed with polymethyl metacrylate (PPMA) standards at a range from 31,110 to 2,136,000 g/mol (Agilent technologies). Integration and molecular weight calculations were carried out using Empower GPC software (Waters).

Characterisation of the produced polymers employing DSC and GPC were conducted in AIMPLAS, Instituto Technológico del Plástico, in Valencia during a four month placement carried out with ERASMUS+ program.

4.26 Response surface methodology

The temperature and pH during enzymatic P(3HB-*co*-3HV) extraction from bacterial cell mass was optimised via response surface methodology using a central composite design (CCD). The lysis of non-PHA bacterial mass is accomplished via synergistic action of various enzymes produced by *A. oryzae* during SSF. Therefore, identifying the optimum temperature and pH is essential in order to maximise bacterial cell lysis. The ranges of temperature and pH values used in the CCD were selected by conducting preliminary experiments for bacterial cell lysis. CCD was developed so as to introduce quadratic terms, and hence, allow the estimation of curvature for the prediction of the free amino nitrogen (FAN) concentration produced via bacterial cell protein hydrolysis and the percentage of cell lysis achieved at the end of enzymatic P(3HB-*co*-3HV) extraction. Table 4.1 presents the design of the experiments and the corresponding ranges of the two factors.

Table 4.1 The examined value ranges of the two factors for the CCD experiment

Level	Temperature:X_1	pH:X_2
+1.414	65.2	7.6
+1	60.0	7.0
0	47.5	5.5
-1	35.0	4.0
-1.414	29.8	3.4

Twelve runs were implemented for the CCD, four star points for the extreme values, four at the corner of the design box and four runs (replicates) at the central point for statistical purposes. Equation 4.2 was used in order to code the examined variables.

$$x_i = \frac{X_i - X_i^{CP}}{\Delta X_i} \qquad \text{Eq. 4.2}$$

where i is the independent variables (T or pH), x_i is the coded value, X_i is the real value, X_i^{CP} is the real value at the central point and ΔX_i is the step change value.

The response of the system was predicted by using a 2nd order polynomial equation (Equation 4.3).

$$Y = b_1 + b_2 x_1 + b_3 x_2 + b_4 x_1 x_2 + b_5 x_1^2 + b_6 x_2^2 \qquad \text{Eq. 4.3}$$

where Y is the simulated response which in this case is the *FAN* concentration measured in mg/L, or percentage of cell lysis, $b_{(j)}$ is the parameter vector, b_1 is the intercept element, b_2 *and* b_3 are the linear effects of the two parameters T and pH, respectively, b_4 is the interaction parameter and b_5 and b_6 are the quadratic parameters for T and pH, respectively. The parameters were obtained by linear regression minimising the difference between predicted and experimental values.

4.27 Techno-economic evaluation

A techno-economic assessment was performed for the sunflower-based biorefinery concept (Figure 4.13) developed in this study generating protein isolate, crude

phenolic extract and poly(3-hydroxybutyrate). Costing studies were based on preliminary economic assessment (accuracy up to 30 %) that was conducted to estimate the total capital investment and the cost of manufacture for the process flow sheets of the abvanced biorefinery concept. The industrial plant was projected to operate 7920 h/y. The material and energy balances were performed using experimental results presented in Chapters 5-9, while equipment sizing was carried out using well known procedures in chemical engineering process design and rules of thumb (Ulrich, 1984; Peters et al., 2003; Turton et al., 2003; Humbird et al., 2011; Koutinas et al., 2014d)

The techno-economic evaluation that will be presented in Chapter 10 was carried out for the extraction of antioxidant-rich fraction (AREA 100) and protein isolate (AREA 200) as well as the production of PHB (AREA 300) using entirely crude glycerol and sunflower meal hydrolysates. The PHB was extracted using crude enzymes produced via solid state fermentation of *A. oryzae* (AREA 400). The annual biodiesel production of 50,000 t is derived from the reaction of 50,789.1 t/yr of oil that was assumed to contain only triolein as triglyceride. Considering that the oil content of sunflower seed is approximately 40 % (w/w), 126,982.8 t/y of sunflower seeds are processed generating 76,183.7 t/y of sunflower meal as side stream. The annual capacity of PHB production was calculated based on the crude glycerol generated for the annual production of 50,000 t biodiesel and the optimal glycerol to PHB conversion yield achieved in fermentation studies. The highest TDW and PHB concentration achieved were 66.1 g/L and 57.02 g/L, respectively, corresponding to a PHB content of 86.26 %. Fermentation time was 142 h thus productivity was 0.4 g/L/h. Overall, the conversion yield of glycerol to PHB was found to be 0.475 g/g.

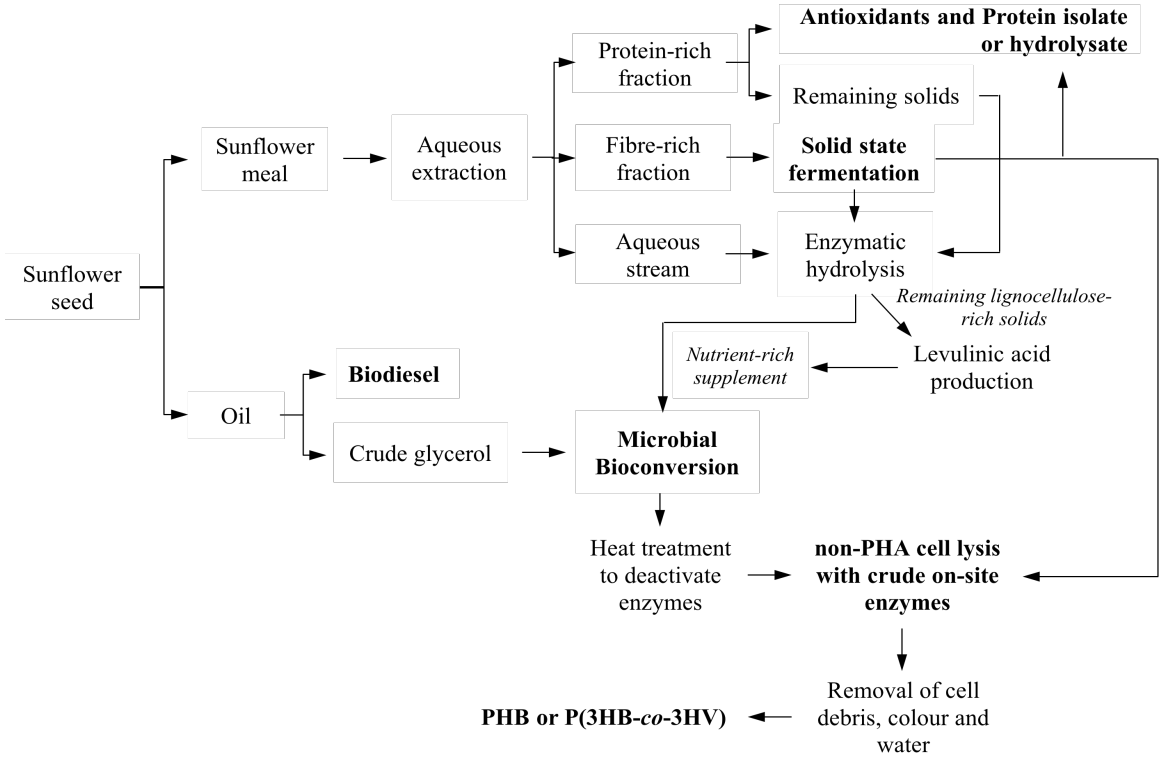

Figure 4.13 Sunflower-based biorefinery concepts for PHA production. Utilisation of SFM for the production of antioxidants, protein isolate and PHB or P(3HB-*co*-3HV)

CHAPTER 5

OPTIMISATION OF SOLID STATE FERMENTATION AND ENZYMATIC HYDROLYSIS OF SUNFLOWER MEAL FOR THE PRODUCTION OF FERMENTATION FEEDSTOCK

5.1 Introduction

The implementation of biotechnological processes for chemical and biopolymer production is currently hindered by the high cost of manufacture deriving mainly from the cost of raw material employed in microbial bioconversions. Consequently, research is directed towards exploiting renewable resources in order to substitute for the expensive conventional carbon and nitrogen sources with nutrients supplements occurring from renewable resources. Under this prospect, one of the main objectives in this study was to generate a nutrient rich fermentation feedstock from sunflower meal (SFM) and crude glycerol that could substitute yeast extract and conventional carbon sources utilised in traditional fermentation processes. Composition analysis and characterisation of these streams was performed at the beginning of the experimental set up. Optimisation of solid state fermentations (SSF) with the fungal strain *Aspergillus oryzae* using SFM as substrate was carried out aiming to maximise the production of crude enzyme consortia. Subsequently, optimisation of enzymatic hydrolysis of SFM was carried out under the viewpoint of producing a nutrient rich fermentation supplement for PHA production.

5.2 Characterization and analysis of by-products

5.2.1 Sunflower meal

The composition of oilseed cakes including sunflower meal can significantly vary depending on extrinsic factors during cultivation, the sunflower variety or the application of dehulling and/or oil separation through solvent extraction processes implemented in industrial scale. The composition of SFM is presented in Table 5.1. The ash content of the SFM used in this study is similar to literature-cited publications (Lomascolo et al., 2012; Parrado et al., 1993; Bautista et al., 1990). The lipid content found in this study is lower than 1% because a solvent extraction process was employed in the removal of sunflower oil that was used in biodiesel production. Phenolic compounds are also present in SFM

either in soluble form or connected to proteins (Dominguez et al., 1995), thus affecting the nutritional value of SFM as animal feed.

Table 5.1 also demonstrates the composition of SFM in lignin, hemicellulose and cellulose represented by the ADF, ADL and NDF contents. It is easily observed that the reported values comply with literature-cited publications (Lomascolo et al., 2012; Koutinas et al., 2014c). More specifically, ADL content corresponding to lignin content in SFM was 9.3 %. Cellulose content (28.3 %) was calculated by subtracting ADL from ADF. Finally the subtraction between ADF and NDF led to the calculation of hemicellulose (8.8 %).

The major component in SFM is protein. Salunkhe et al. (1992) reported that the crude protein content in dehulled seeds is approximately 20 – 40 % depending on the sunflower variety. Removal of the hull results in an increase in the protein content of SFM. The crude protein is constituted of true protein (87 – 99 %) and peptides, amino acids and other nitrogenous substances (1 – 13 %) (Gassmann, 1983; Bhatty et al., 1973). Lomascolo et al. (2012) and Villanueva et al. (1999) reported similar protein contents in SFM in the range of 29 – 34 %.

Table 5.1 Composition of sunflower meal

Composition	Content	Content from literature	References
Moisture (%, wet basis)	3.8 ± 0.15	7.73-9.68	Bautista et al., 1990; Villanueva et al., 1999
Total Nitrogen (TN) (mg/g, dry basis)	42.6 ± 0.47		
Protein (%, 6.25 × TN, db)	26.6 ± 0.29	20-40	Salunkhe et al., 1992; Villanueva et al., 1999
Oil (%, db)	0.9 ± 0.08	1.3-3.2	Geneau-Sbartai et al., 2008
Ash (%, db)	6.8 ± 0.35	5.75-7.47	Lomascolo et al., 2012; Parrado et al., 1993; Bautista et al., 1990
Dietary Fibre (%, db)	19.5 ± 0.63	14-25	Bautista et al., 1990, Gassmann, 1983
ADF (%, db)	37.6	30.9-32	Lomascolo et al., 2012; Koutinas et al., 2014c
ADL (%, db)	9.3	8.4-10.5	
NDF (%, db)	46.4	43.8-45.1	
Phenolic compounds (g/100 g SFM)	3.8	3-4	Dominguez et al., 1995

5.2.2 Crude glycerol

The vast production of crude glycerol as a by-product from the transesterification reaction to yield fatty acid alkyl esters has impelled the valorisation of this stream in biotechnological processes to replace conventional expensive carbon sources (e.g. glucose). The raw material used in biodiesel production process affects the final composition of crude glycerol consisting of miscellaneous impurities. The final composition is accordingly dependent on the type of catalyst and alcohol utilised as well as the biodiesel separation process employed. In general, the glycerol-rich stream that remains after separation of FAME contains around 50% glycerol (w/w), methanol, most of the catalyst, fatty acid soaps, remaining FAME, moisture and various impurities depending on the feedstock (Van Gerpen et al., 2004). Current biodiesel producers process this stream initially through acidification therewith neutralising the catalyst and splitting the fatty acid soaps into salts and free fatty acids, which are not soluble in glycerol and separate naturally from the glycerol-rich stream. After acidification, methanol is separated via a vacuum flash process, an evaporator or vacuum distillation (the latter is used when the processing capacity is more than 25 t per day) (Van Gerpen et al., 2004). Methanol is removed completely as it is recycled in the transesterification process to reduce the processing costs. The final crude glycerol stream contains 77 – 90% (w/w) glycerol 5.3 – 14.2% (w/w) water, 4.2 – 7% NaCl or 0.8 – 6.6% K_2SO_4 depending on the catalyst used in the transesterification stage, less than 1% methanol and low quantities of free fatty acids, FAME, mono- and di-acylglycerols (Chatzifragkou and Papanikolaou, 2012; Mothes et al., 2007). Table 5.2 presents the composition of crude glycerol and pretreated crude glycerol, obtained by a simple decanting step.

During this study, a crude glycerol with a purity of 91% was used, deriving from the transesterification process of sunflower oil as starting material with NaOH as catalyst and methanol. The presence of impurities is strongly associated with the microbial metabolism of crude glycerol by influencing the fermentative bioconversions demonstrating an inhibition effect on cell growth and product formation. Consequently, pretreatment steps constitute a prerequisite prior to implementation of crude glycerol as fermentation feedstock. A simple decanting step in separation funnels led to the formation of an upper phase consisting of non-polar compounds, not miscible with glycerol (Figure 5.1).

Table 5.2 Composition of crude glycerol before and after treatment via decanting

Component	Crude Glycerol	Crude Glycerol after decanting	Upper phase after decanting
Glycerol (%, w/w)	91	92.4	-
Na^+ (%, w/w)	1.33	1.3	-
Ca (mg/g)	3.4	3.4	-
Mg (mg/g)	0.37	0.37	-
Fe (mg/g)	0.61	0.61	-
Total non-polar compounds extracted with petroleum ether (%, w/w)	0.424	0.099	0.325
Total FAME (%, w/w)[1]	0.361	0.084	0.268
FAME produced via MeONa treatment (%, w/w)			0.024
FAME produced via treatment with HCl with methanol (%, w/w)			0.244

[1] produced via esterification or transesterification reactions using sodium methoxide and hydrochloric acid with methanol, respectively

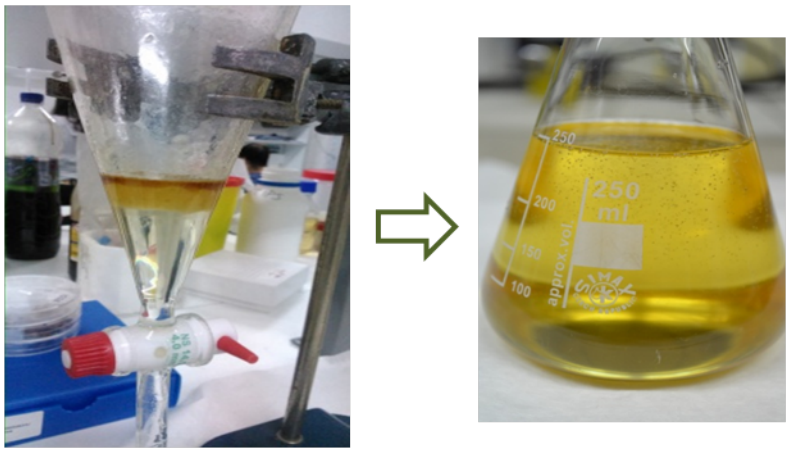

Figure 5.1 Crude glycerol decanting in separation funnels

Both the initial and pre-treated crude glycerol streams were treated with petroleum ether in order to verify the removal of non-polar compounds during decanting (Table 5.2). It is obvious that the top fraction produced via decanting contained most (77%) of the non-polar compounds (mainly free fatty acids as well as various glycerides) present in crude glycerol. This was further validated by analyzing the FAME produced from all fractions

after combined esterification and transesterification reactions with MeONa and HCl with methanol, respectively, in order to convert both free fatty acids and various glycerides into the respective esters. The results presented in Table 5.2 indicate that the FAME content of the crude glycerol fraction produced after decanting contained a 4-fold lower content on non-polar compounds compared to the initial crude glycerol.

It should be also noted that numerous chemical components, such as calcium, magnesium, iron, phosphorus etc., are present in crude glycerol at varying concentrations. Elemental analysis was also performed (Table 5.2) indicating that the concentration of Ca, Mg and Fe could provide substantial supplementation of micronutrients critical for microbial proliferation.

5.3 Optimisation of solid state fermentation

The main objective was the production of a nutrient rich supplement that could substitute for yeast extract and inorganic chemicals that are usually employed in fermentation processes. Since nitrogen source is the second most important nutrient (after carbon source) in fermentation processes and protein is the main component in SFM, the main criterion used for the evaluation of SFM hydrolysis was the production of free amino nitrogen (FAN) generated during protein hydrolysis, which is dependent on the activity of protease produced via SSF. The fungal strain of *A. oryzae* used in this study is a prolific producer of protease (Wang et al., 2005; Kiran et al., 2012). It can also produce other enzymes, such as phytase, xylanase and cellulase that enhance the hydrolysis of crude oilseed meals (Kiran et al., 2012; Wang et al., 2010).

The optimisation of solid state fermentation focused on the maximisation of enzyme production that would be subsequently implemented in the hydrolysis of macromolecules contained in sunflower meal. The effect of different processing parameters on fungal growth and enzyme production was investigated. In particular, initial moisture content, pH value in the substrate and incubation temperature were investigated in the experimental set up.

5.3.1 Effect of initial moisture content

The first step of the experimental setup employed the investigation of the effect of the initial moisture content of the substrate. Based on previously cited studies on the maximisation of proteolytic activity, different moisture contents were evaluated in the range of 55 – 85 % and the results are presented in Figure 5.2. Limited fungal growth and significantly low protease activities were observed when initial moisture contents of 55% and 85% were employed. Inadequate quantity of water results in deficient dispersion of nutrients thus hindering fungal proliferation (Gervais and Molin, 2003). On the other hand, excess water present in the substrate leads to decreased porosity thus restricting oxygen transfer (Shingania et al., 2009). The optimum moisture content appeared to be in the range of 60 – 75%.

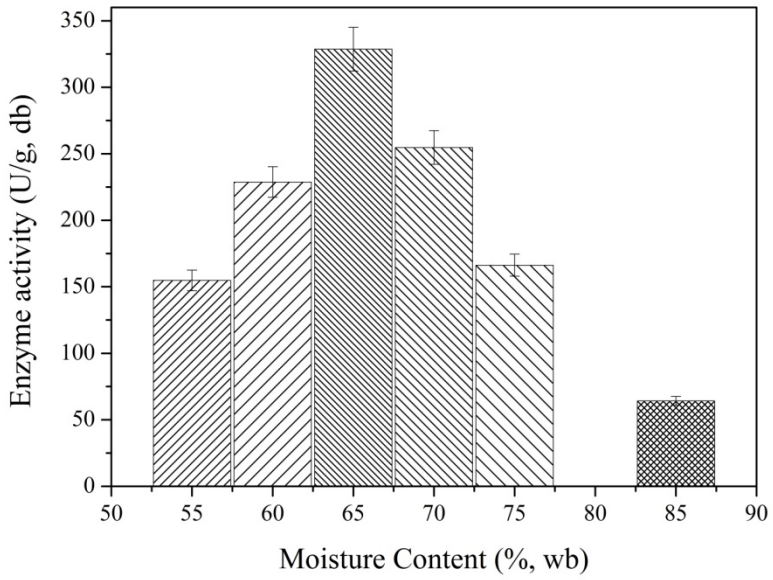

Figure 5.2 Enzymatic activities in different initial moisture contents at 48 h of SSF

Figure 5.3 presents the profile change of protease activity during four SSF performed at different initial moisture contents namely 60, 65, 70 and 75 %. Protease activity in all different cases examined was increased up to 48 h followed by a decreasing trend. The highest protease activity was observed at approximately 48 h when an initial moisture content of 65 % was employed, corresponding to 400 U/g (db). Initial moisture content of 70 % resulted in notable protease production (380 U/g at 60 h), while in the other two examined cases the proteolytic activity was reduced approximately by 25 % (less than 300 U/g).

Several agro-industrial residues have been widely implemented in SSF bioconversions to yield enzymes. The moisture content of the substrate is strongly correlated with water retention capacity, thus affecting the initial moisture content of SSF. Moreover, protein content of the substrate constitutes a substantial factor in the production of protease. Wang et al. (2009) performed SSF with A. *oryzae* on wheat pieces with 10.9 % (db) nitrogen content at initial moisture content of 60 %. The maximum protease activity reached 172.8 U/g at 97 h, while similarly a declining trend after achieving the maximum value was observed. The utilisation of rapeseed meal containing 38.87 % protein in SSF with *A. oryzae*, resulted in the production of 728 U/g of protease activity after 67 h (Wang et al., 2010). The optimum initial moisture content for rapeseed meal was 65 % (Wang et al., 2010; Kiran et al., 2012). The optimisation of initial moisture content was also carried out in wheat milling by-products with the fungal strain of *Aspergillus awamori* leading to 65 % as the optimum moisture content (Tsakona et al. 2014).

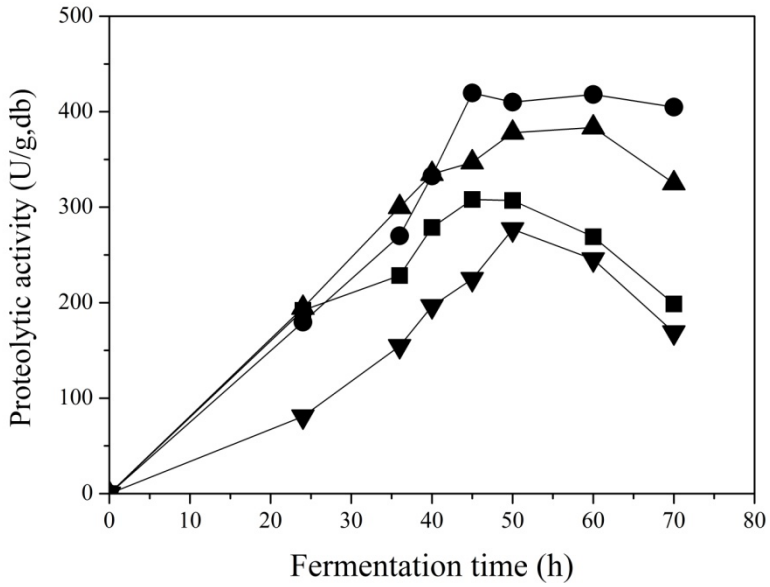

Figure 5.3 Proteolytic activity during SSF with *A. oryzae* carried out at four different initial moisture contents (■: 60 %, ●: 65 %, ▲: 70 %, ▼: 75 %)

5.3.2 Effect of pH

The protease produced by *Aspergillus oryzae* comprise of neutral, alkaline and acidic protease. Furthermore, the optimum pH for fungal growth varies significantly (3.8-6.0) among filamentous fungi (Gowthaman et al., 2001). The synthesis of protease is

strongly associated with the pH of the substrate, affected mainly by the modifications of cell membrane allowing for the exchange of various compounds. (Battaglino et al., 1991; Sandhya et al., 2005). In order to optimise the pH value during fermentation leading to optimum protease production, various pH values were evaluated using the optimum initial moisture content of 65 %. Phosphate buffer solutions were employed to maintain the pH value during SSF at 4, 4.5, 5.5, 6.5 and 7. In addition, one experiment was carried out at natural (uncontrolled) pH. As presented in Figure 5.4, the protease activity produced during SSF carried out at uncontrolled pH was significantly higher as compared to SSF where the pH was controlled. In all cases, the proteolytic activity was increased up to 70 h followed by a slight decreasing trend.

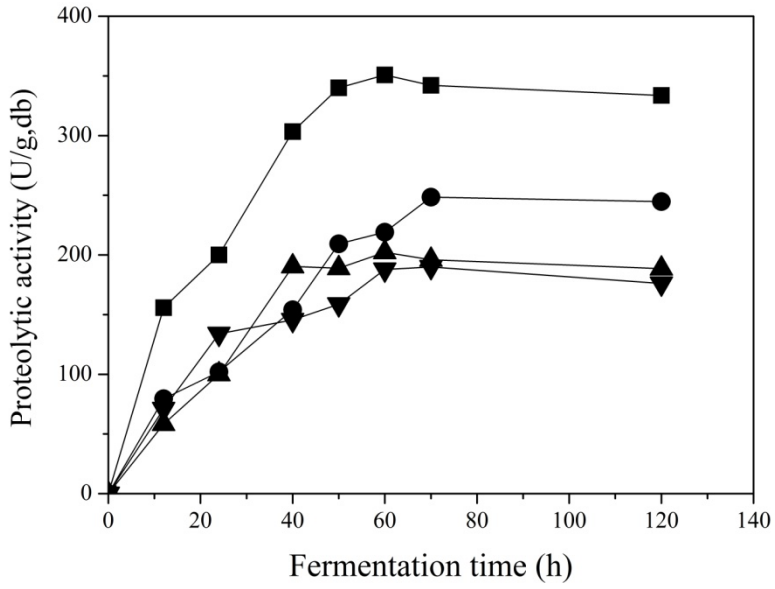

Figure 5.4 Proteolytic activity during SSF with *A. oryzae* carried out at four different pH values (■: natural, ●: 6.5, ▲: 5.5, ▼: 4.5) with 65 % initial moisture content

It is generally acknowledged that the pH value does not remain constant during SSF leading to pH variations (Gowthaman et al., 2001). In the case of SSF carried out at controlled pH, the buffer capacity was maintained throughout fermentation contrary to the SSF carried out under uncontrolled pH condition. In the latter case, it is speculated that fungal growth allowed pH regulation thus enabling crude protease consortium to reach maximum activity by synergistic actions of neutral, alkaline and acid proteases. Similar observations were presented in the production of polygalacturonases by *Aspergillus oryzae*, where maximum activities were obtained in the case that the pH value in the

medium was not controlled (Malvessi et al., 2004). Finally, pH control during SSF would constitute an additional expenditure increasing the cost of the whole process.

5.3.3 Effect of temperature

In order to conclude with the optimisation of the processing parameters affecting the production of proteolytic enzymes in SSF, the effect of incubation temperature was also evaluated. The fungi *Aspergillus oryzae* proliferates promptly at 30 °C (Chutmanop et al., 2008). Protease production is reported to significantly decrease at 25 °C and above 35 °C (Vishwanatha et al., 2010; Franscis et al., 2003). Therefore, the evaluation of temperature was restricted at 30 °C and 35 °C. Two different initial moisture contents were also employed in the experimental set-up in order to better correlate the effect of the initial moisture content of the substrate and the temperature (Figure 5.5).

From the results presented in Figure 5.5, it is easily noticed that increasing incubation temperature to 35 °C resulted in more than 50 % decrease in the obtained proteolytic activity for both initial moisture contents. More specifically, when 65 % initial moisture content was implemented, the proteolytic activity decreased from 350 U/g to 177 U/g at 50 h. The same trend dominated in all the examined cases leading to the selection of 30 °C as temperature during SSF.

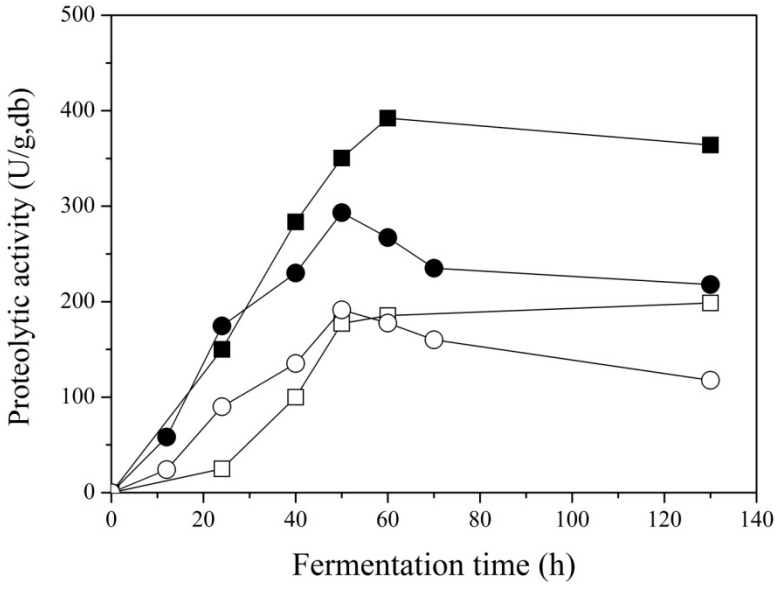

Figure 5.5 Proteolytic activity during SSF with *A. oryzae* carried out at two different incubation temperatures with two different initial moisture contents (■: 65 % moisture

content and 30 °C, ●: 70 % moisture content and 30 °C, □: 65 % moisture content and 35 °C, ○: 70 % moisture content and 35 °C)

5.3.4 Production of enzymes in optimum SSF conditions

The prolific ability of *A. oryzae* to produce various enzymes during solid state fermentation has been widely demonstrated employing an ample variety of substrates, especially agricultural waste and by-products (Wang et al., 2005; Kiran et al., 2012). The capability of SFM as substrate in SSF to generate proteolytic enzymes has been demonstrated in the previous sections. However, considering that a crude consortium of enzymes is secreted, it was important to elucidate the different enzymatic activities that affect hydrolytic reactions. The study was performed in the optimum conditions for the production of proteases, i.e. 65 % (w/w) initial moisture content, uncontrolled pH and 30 °C. Figure 5.6 presents the profile change of phytase, xylanase and cellulase during SSF carried out on SFM with *A. oryzae*.

SFM contains significant quantities of phytic acid that contains inorganic phosphorus, which is necessary in microbial growth and product formation. The hydrolysis of phytic acid is essential in order to release inorganic phosphorus. Consequently, the phytase activity was studied yielding the release of inorganic phosphorus from phytic acid. As demonstrated in Figure 5.6, the phytase activity follows an increasing trend until 48 h reaching a maximum value of 318 U/g, coinciding also with the optimum solid state fermentation time for protease production (400 U/g at 48 h).

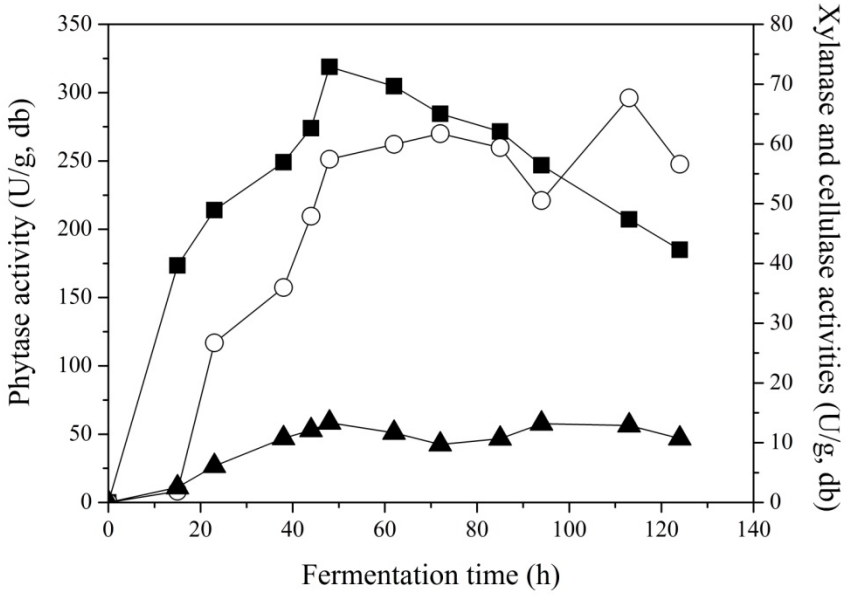

Figure 5.6 Profile changes of phytase (■), xylanase (○) and cellulase (▲) during SSF carried out at 65 % (w/w) initial moisture content, uncontrolled pH and 30 °C

The production of xylanase and cellulase were also investigated (Figure 5.6). A comparable trend is noted hereof indicating an increase in xylanase activity up to 48 h reaching a value of 57.5 U/g followed by a plateau until 94 h. Finally, cellulase activity and more specifically endo-1,4-β-glucanase assay was performed, though the results obtained were significantly lower compared to the other enzymes produced by *A. oryzae* and remained approximately constant after 48 h of fermentation.

Phytase, xylanase and cellulase have been widely studied in solid state fermentations employing various substrates and microorganisms, particularly filamentous fungi. Botella et al. (2005) reported on xylanase, cellulase and pectinase production by *Aspergillus awamori* using grape pomace. Maximum xylanase activity obtained reached approximately 40.4 ± 15.6 U/g at 24 h. Enzyme production by *A. oryzae* was also studied by Yamane et al. (2002) evaluating different solid feedstocks, such as wheat bran, where xylanase production reached a maximum activity at 96 h (60 U/g). Rani and Ghosh (2011) studied phytase production in SSF by *Rhizopus oryzae* utilising various agro-industrial streams including sunflower oil cake. Furthermore, Chen et al. (2014) evaluated phytase production by *A. oryzae* cultivated on soy meal, reporting a maximum phytase activity of 58.7 U/g when the initial moisture content was 41 % (w/w). Generally, the term enzymatic activity is characterised by a wide range of definitions and for this reason direct

comparison between different studies is difficult. Hence the results of this study on phytase production (318 U/g) were converted in μmol of inorganic phosphate per 45 minutes of reaction yielding 99.45 U/g, which is significantly higher than the reported phytase activity reported by Chen et al. (2013).

5.4 Optimisation of enzymatic hydrolysis

Following the successful optimisation of SSF for the production of crude enzyme consortia, subsequent experiments focused on the optimisation of the hydrolysis of sunflower meal. During the experimental set up one parameter at a time was evaluated. The production of nutrient-rich fermentation supplements from SFM was mainly focused on efficient hydrolysis of protein, as well as on the release of phosphorus as the most important micro-nutrient that is necessary in fermentation media formulation. It should be stressed that this is a complex hydrolytic reaction that involves hydrolysis of SFM macromolecules, hydrolysis of macromolecules from macerated fungal cells and possibly autolysis of remaining fungal cells due to the absence of oxygen during hydrolysis performed in Duran bottles. The effect of temperature, pH, initial SFM concentration, initial enzyme activity and SSF time course on the production of FAN and IP during hydrolysis was evaluated.

5.4.1 Effect of pH

Enzymes exhibit an optimum pH value at which they present maximum reaction rates due to the maintenance of its three-dimensional structure in the active site and the ionisation of groups in the active site of enzymes or the substrate (Whitaker, 2000). Figure 5.7 presents hydrolysis experiments carried out at pH values of 4.8, 5.3, 5.8, 6.3, 6.8, 7.3 and natural (uncontrolled) pH to identify the optimum pH value. The pH values were set with phosphate buffers. FAN production was initiated immediately after the addition of the SSF aqueous suspension.

It was observed that even though the initial FAN concentration was similar in all cases (approximately 2.3 mg/g), FAN production was considerably increased by approximately 45 % when the pH of hydrolysis was not controlled. In the hydrolysis carried out at uncontrolled pH, the pH value at the beginning of the hydrolysis was 6.8 and

it was gradually reduced to 5.2 towards the end of hydrolysis when FAN production reached a plateau. The synergistic action of the different enzymes in conjunction with the ample range of pH values that proteases act (acidic, neutral, alkaline) could provide a possible interpretation for this observation. Inorganic phosphorus quantification was not carried out due to the phosphate buffers employed to regulate the pH value in hydrolysis.

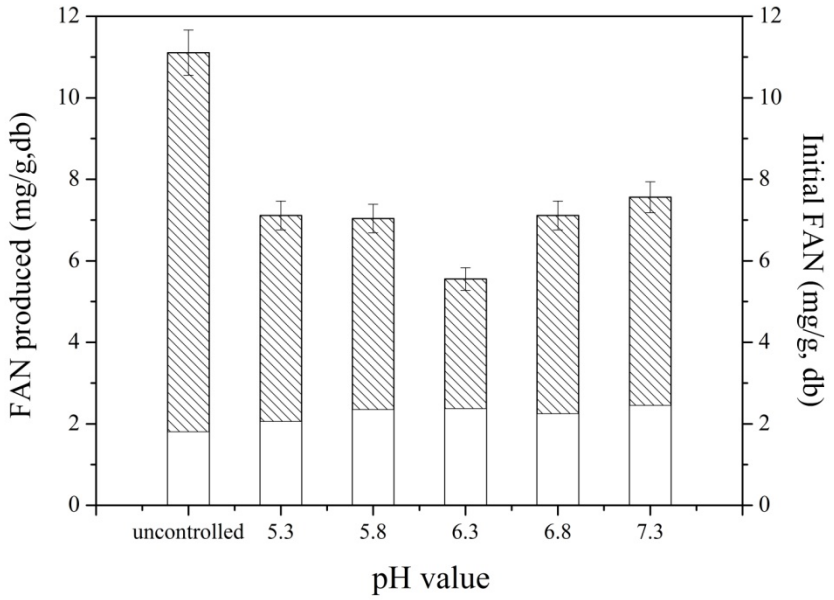

Figure 5.7 Effect of pH value on FAN production during hydrolysis of 45 g/L initial solid (SFM and remaining SSF solids) concentration using different pH values

5.4.2 Effect of temperature

The temperature influences the stability of enzymes, the enzyme activity and the efficiency of hydrolysis including the reaction rate and the substrate to product conversion rate (Klibanov, 1983). Increase in reaction temperature induces denaturation of enzymes, thus reducing the efficiency of hydrolysis.

The effect of temperature on FAN and IP production was studied by conducting experiments at 35, 40, 45, 50, 55, 60 and 65 °C. The optimum temperature for protease produced by this strain of *A. oryzae* is 55 °C during wheat flour hydrolysis (Wang et al. 2009). However, hydrolysis of complex renewable resources is a concerted action among different enzymes and therefore a wide temperature range was studied. Figure 5.8 presents the final FAN and IP concentrations produced at different temperatures employed during hydrolysis. Hydrolytic reactions at 45 °C presented the highest FAN production (16.1

mg/g) which was proved to be optimum for IP production as well (3 mg/g). Temperatures higher than 45 °C showed a steadily declining trend for FAN and IP production. Although the optimum temperature for the protease produced by this strain of *A. oryzae* is 55 °C when casein was used as substrate, the optimum temperature for hydrolysis of SFM-based substrate was 45 °C indicating that hydrolysis of crude renewable resources is strongly dependent on the concerted action of various enzymes.

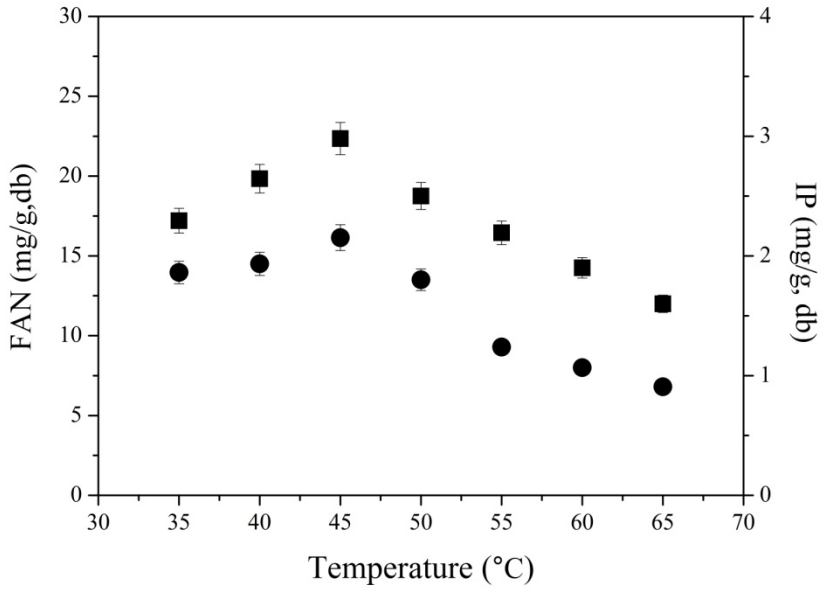

Figure 5.8 Effect of temperature on FAN (●) and IP (■) production during hydrolysis of 45 g/L initial solid (SFM and remaining SSF solids) concentration using an initial enzyme activity of 6.4 U/mL

The fungal strain *A. oryzae* used in this study has been employed for the production of enzymes that were subsequently used for the hydrolysis of various renewable resources. Hydrolytic reactions carried out in yeast cells derived via fractionation of wine lees using crude enzymes produced by the same strain of *A. oryzae* showed the highest FAN production at 40 °C, thus achieving 50 % conversion yield of TKN to FAN (Dimou et al., 2015). In the case of protein hydrolysis using rapeseed meal as raw material, the optimum temperature was 40 °C (Wang et al., 2010). Pleissner et al. (2014) employed the fungal strains of *A. oryzae* and *A. awamori* for the enzymatic hydrolysis of food waste conducted at 55 °C. Similarly, hydrolysis performed at 45-55 °C proved to be optimum in the case of flour-rich waste streams hydrolytic reactions by *A. awamori* achieving conversion yield of

starch to glucose 86.6 % and TKN to FAN 13.8 % (at 55 °C) respectively (Tsakona et al. 2014).

Subsequent experiments focusing on SFM hydrolysis were carried out at 45 °C under uncontrolled pH as these operating conditions led to the highest production of fermentation nutrients (i.e. FAN and IP).

5.4.3 Effect of SSF duration on SFM hydrolysis

This set of experiments studied the effect of SSF duration on SFM hydrolysis. During the experiments performed in SSF optimisation, it was clearly stated that the optimum protease production was accomplished at 48 h. Nevertheless, the analytical method implemented to determine the proteolytic activity used casein as substrate, thus it was crucial to elucidate the effect of fermentation duration on SFM hydrolysis. Consequently, hydrolysis experiments were conducted at 45 °C, with 45 g/L initial solid concentration, employing crude enzymes produced at 36, 42, 48, 53 and 60 h of SSF, respectively. Figure 5.9 illustrates the effect of SSF duration on FAN production during hydrolysis of sunflower meal. Initial FAN concentration was subtracted from the corresponding final measurement to evaluate FAN production.

It can be easily noticed that hydrolysis of protein to amino acids and peptides, as expressed by the production of FAN, is distinctively higher in the case where hydrolysis was carried out with crude enzymes generated at 48 h of SSF. This observation is in accordance with the results obtained during the optimisation of SSF.

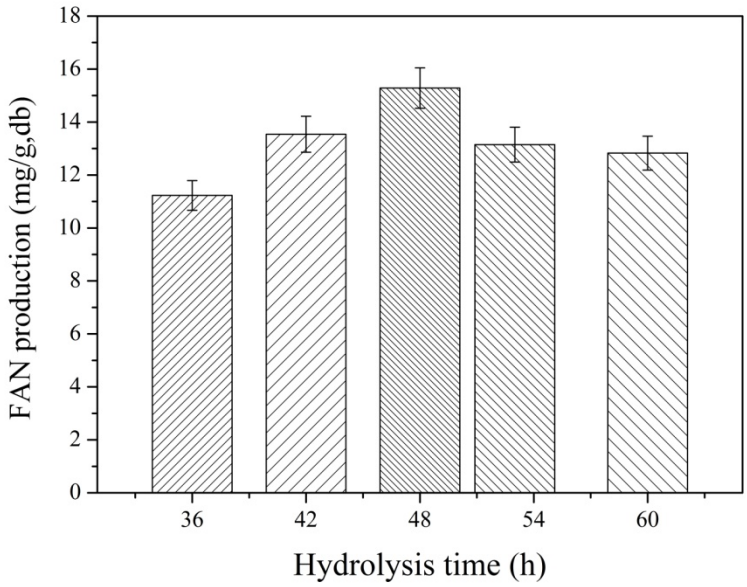

Figure 5.9 Effect of time course of SSF on FAN production during hydrolysis of 45 g/L initial solid concentration (SFM and remaining SSF solids)

5.4.4 Effect of initial SFM concentration

Industrial implementation of enzymatic hydrolysis would require operating conditions of high solid concentrations. Thereof, the effect of initial SFM concentration on FAN production was evaluated. Figure 5.10 presents the profile change of FAN concentration during hydrolysis at various initial solid concentrations, namely 45, 68 and 90 g/L. These concentrations represent both remaining SSF solids and unprocessed SFM (on a dry basis) mixed at the beginning of hydrolysis. However, TKN to FAN conversion yields were calculated based on the total SFM utilised for both SSF and hydrolysis reactions as illustrated in Figure 5.11.

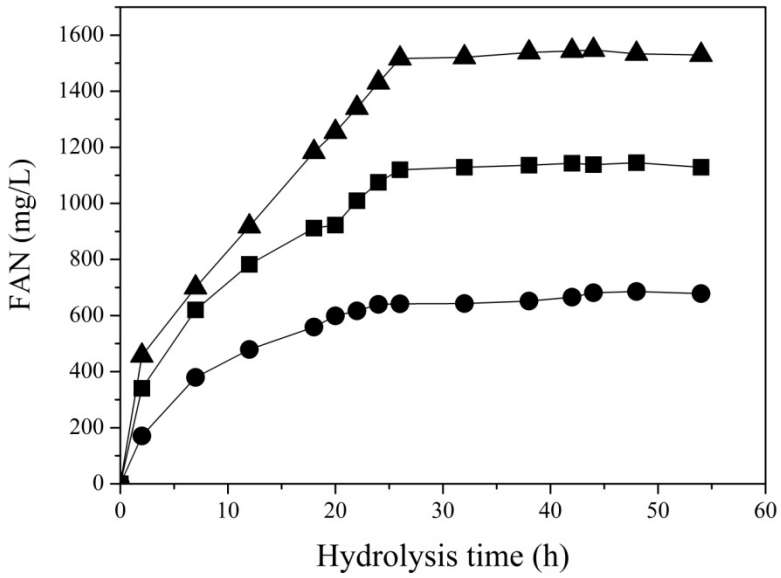

Figure 5.10 FAN production during solid (SFM and remaining SSF solids) hydrolysis at initial solid concentrations of 46.3 g/L (●), 70.4 g/L (■) and 92.2 g/L (▲) using an initial enzyme activity of 6.4 U/mL

Likewise, the SFM that was consumed during SSF for fungal proliferation has also been taken into consideration for the calculation of the overall TKN to FAN conversion yield. The initial FAN concentration at the beginning of each hydrolysis was not zero but it was subtracted from each FAN production curve in order to evaluate only FAN production during hydrolysis. The initial FAN concentration at the beginning of hydrolysis has been included in the calculation of the TKN to FAN conversion yield because in that case the total SFM employed for both SSF and hydrolysis reactions was used. It should be also stressed that FAN production is a result of both SFM enzymatic hydrolysis and fungal cell enzymatic degradation. Fungal cells were macerated with a blender and the protein was hydrolysed by proteolytic enzymes. Fungal cell autolysis may have also occurred due to the absence of dissolved oxygen during hydrolysis. The occurrence of autolysis enhances the production of nutrients such as FAN and IP due to secretion of autolysins by fungal cells (Koutinas et al., 2005).

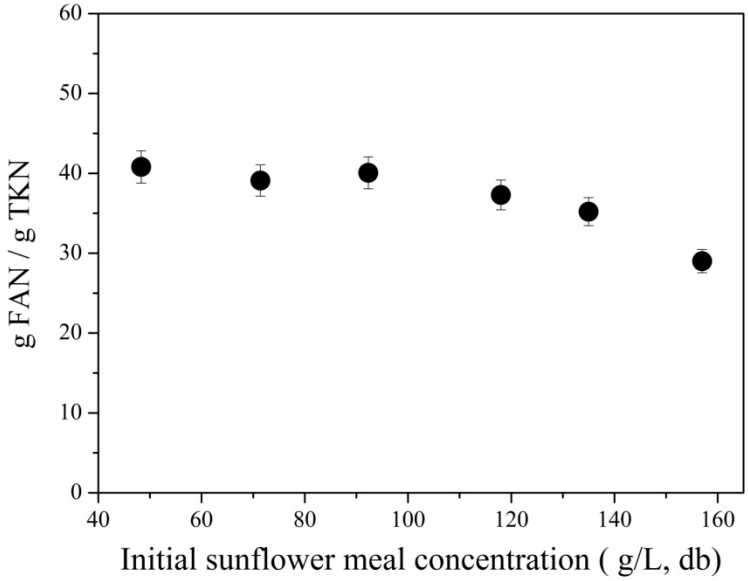

Figure 5.11 Overall TKN to FAN conversion yield at different total SFM concentrations used for both SSF and hydrolysis stages

Figure 5.11 shows that the final FAN concentration in the reaction mixture was increased with increasing initial solid concentration. The maximum FAN concentration (1.5 g/L) was achieved when an initial solid concentration of 90 g/L was used. This initial solid concentration corresponds to 92.3 g/L when the total SFM used for both SSF and hydrolysis is taken into consideration. The achieved concentration of FAN corresponds approximately to 30 g/L of yeast extract.

Slightly higher FAN concentrations were achieved with slightly higher initial SFM concentration, but the TKN to FAN conversion yield is gradually reduced at higher initial SFM concentrations (Figure 5.11). The TKN to FAN conversion yield achieved was 42% when the total SFM employed for both SSF and hydrolysis is taken into consideration. The yield is reduced at overall SFM concentrations higher than 100 g/L, whereas at concentrations higher than 120 g/L the high viscosity of the suspension prevented agitation of the mixture. Wang et al. (2010) reported that hydrolysis of rapeseed meal resulted in the production of 2087.2 mg/L FAN concentration and 449 mg/L IP concentration, while the TKN to FAN conversion yield was 54 %.

Figure 5.12 presents the profile change of IP concentration during SFM hydrolysis at various initial solid concentrations (45, 68 and 90 g/L). As in the case of FAN production,

final IP concentration was increased with increasing initial solid concentration. The IP concentrations achieved in this study are lower than previous reports conducted for the generation of a nutrient rich supplement by fungal autolysis (Koutinas et al., 2005) that reached an IP concentration of 0.5 g/L. The highest IP concentration (246 mg/L) was achieved when an initial solid concentration of 90 g/L was used at the beginning of hydrolysis.

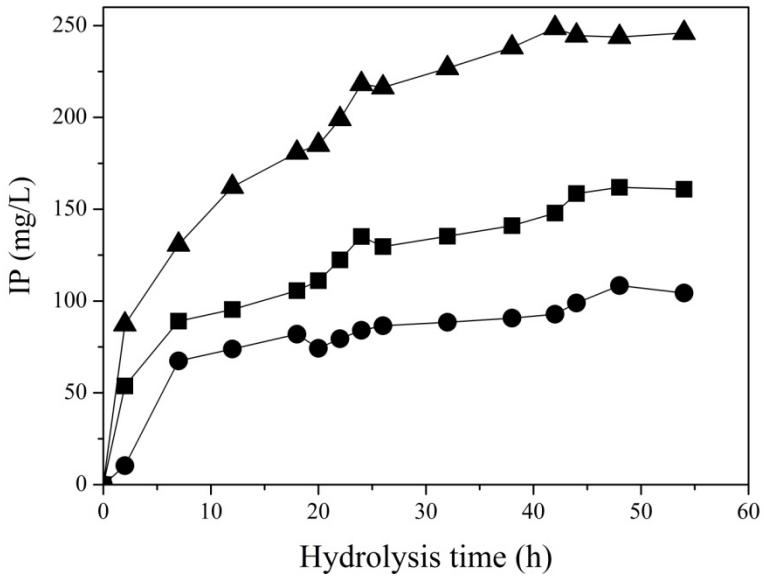

Figure 5.12 IP production during solid (SFM and remaining SSF solids) hydrolysis at initial solid concentrations of 46.3 g/L (●), 70.4 g/L (■) and 92.2 g/L (▲) using an initial enzyme activity of 6.4 U/mL

Under the rationale of developing a bioprocess that would evaluate a low cost substrate for the production of a generic fermentation feedstock, noticeable FAN and IP production were attained, capable to support microbial growth and substitute yeast extract and expensive inorganic chemicals.

5.4.5 Effect of initial enzymatic activity

The initial enzymatic activity relates to the total amount of enzymes employed in hydrolysis reaction thus affecting the catalysis rate. During these experiments, the fermented solids from SSF were removed by vacuum filtration and only the liquid phase containing the crude enzyme consortia was utilised. Therefore, FAN production in these

experiments can be correlated only to protein hydrolysis from SFM. Figure 5.13 presents the FAN production during hydrolysis of 102 g/L (db) of unprocessed SFM at four different initial proteolytic activities (3.2, 6.4, 9.6 and 16 U/mL).

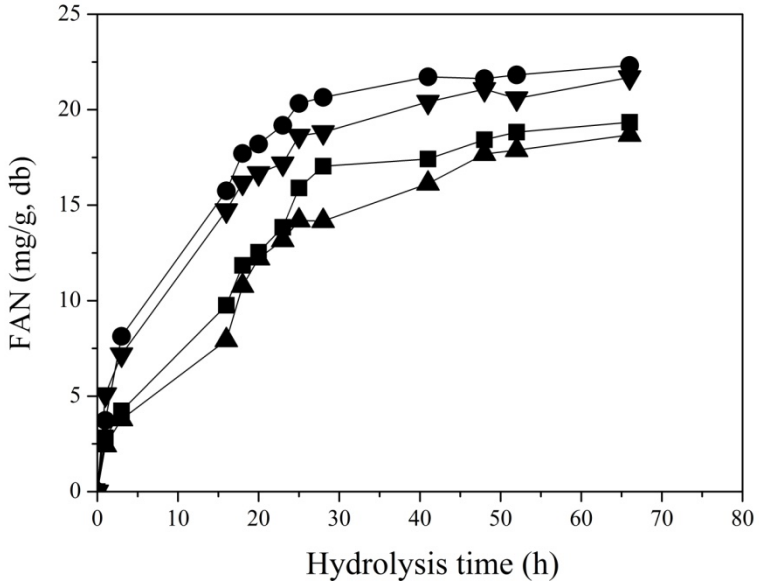

Figure 5.13 FAN production during SFM hydrolysis at different initial proteolytic activities (▲: 3.2 U/mL, ■: 6.4 U/mL, ▼: 9.6 U/mL, ●: 16.4 U/mL) using the same initial SFM concentration of 102 g/L (db)

Figure 5.13 clearly demonstrates that FAN production increases with increasing initial proteolytic activity resulting to a higher TKN to FAN conversion yield. Specifically, when an initial proteolytic activity of 3.2 U/mL was employed, the TKN to FAN conversion yield was 42 %, which was increased up to 52 % when the initial proteolytic activity was increased to 16 U/mL. The TKN to FAN conversion yield was 45 % and 48.5 % when initial proteolytic activities of 6.4 and 9.6 U/mL were used, respectively. In the case of wine lees hydrolysis, implementing the same strain of *A. oryzae*, an analogous increase in hydrolysis yield was obtained with an initial enzymatic of 24 U/mL (Dimou et al., 2015). A comparable trend was demonstrated by Tsakona et al. (2014) where a 4-fold increase in the amount of initial enzymatic activity significantly enhanced flour-rich waste hydrolysis achieving conversion yield 84 % (w/w) of starch to glucose.

5.5 Conclusion

The results of this chapter demonstrate that SFM occurring as a by-product from the biodiesel production process can be successfully implemented in a two-stage bioprocess for the production of a nutrient-rich fermentation feedstock. Crude enzyme consortia including mainly proteases and phytases, produced by *Aspergillus oryzae* during SSF presented high capability to hydrolyse macromolecules of SFM into micro-nutrients providing the possibility to substitute conventional commercial nutrient supplements. The highest free amino nitrogen and inorganic phosphorus concentrations achieved were 1.5 g/L and 246 mg/L, respectively, when an initial proteolytic activity of 6.4 U/mL was used. The FAN concentration was increased to 2.3 g/L when the initial proteolytic activity was increased to 16 U/mL. In the following chapters SFM hydrolysates will be further evaluated combined with crude glycerol in the optimisation of bacterial bioconversions for PHB and P(3HB-*co*-3HV) production.

The results presented in Chapter 5 have been published in the Waste and Biomass Valorisation journal:

Kachrimanidou, V., Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Yanniotis, S., Kookos, I., Koutinas, A.A. Utilization of by-products from sunflower-based biodiesel production processes for the production of fermentation feedstock. Waste Biomass Valor (2013) 4:529-537.

CHAPTER 6

OPTIMISATION OF BACTERIAL BIOCONVERSIONS USING SUNFLOWER MEAL HYDROLYSATES AND CRUDE GLYCEROL

6.1 Introduction

The predominant aim of this chapter was to study and optimise the production of PHB and P(3HB-*co*-3HV) using crude glycerol and SFM, generated by a biodiesel industry, as the sole nutrient sources. The production of a nutrient rich supplement through enzymatic hydrolysis of SFM has been demonstrated in the previous chapters following a two-stage bioprocess. Apparently, SFM hydrolysates contain significant quantities of FAN derived from protein hydrolysis, IP produced from the hydrolysis of phytic acid and other micronutrients such as Mg, Ca and Fe.

Initially, a literature review was performed to select bacterial strains specifically strains belonging in the genus *Cupriavidus* that are known to metabolise glycerol and potentially produce intracellular poly(3-hydroxybutyrate). For this purpose, shake flask fermentations were conducted to evaluate the ability of *C. necator* DSM 545, *C. necator* DSM 11348 and *C. necator* DSM 7237 to proliferate on SFM hydrolysates and synthesise PHB. SFM hydrolysates served as the sole nutrient source of nitrogen, phosphorus and micronutrients unless otherwise stated. Subsequently, fed-batch experiments were performed in bioreactors to assess the capability to ferment crude glycerol. After selecting the best performing bacterial strain, evaluation of glycerol bioconversion was carried out by testing glycerol of different purities. Furthermore, the effect of carbon to nitrogen ratio (C/N) was studied in fed-batch experiments with the addition of glycerol. Finally, different bioprocessing strategies were implemented aiming to further improve production of PHB through the increase of residual cell weight.

6.2 Shake flask fermentations for bacterial strain selection

As mentioned above, the strains *C. necator* DSM 11348, *C. necator* DSM 7237 and *C. necator* DSM 545 were evaluated for PHB production using SFM hydrolysates and crude glycerol as the sole fermentation substrates. Concerning the strains *C. necator* DSM 7237 and *C. necator* 11348, they are known to assimilate glycerol compared to *C. necator* DSM 545 that was observed to consume glycerol at a very low rate (approximately 0.2 g per L per h in shake flask fermentation) when the initial glycerol concentration was 10 g/L. At initial glycerol concentrations of 20 g/L or higher, glycerol was not consumed at the end of the fermentation. The low glycerol consumption rate resulted in a slow bacterial growth rate. To adapt the bacterial strain to glycerol and therefore increase its consumption rate, successive cultivations in shake flasks at gradually increasing glycerol concentrations (5 – 25 g/L) were carried out. Pure glycerol (99%, Sigma-Aldrich) was used as carbon source in these experiments, in order to avoid potential inhibition phenomena caused by crude glycerol impurities. After successive cultivations, the glycerol consumption rate by *C. necator* was improved (more than 0.3 g per L per h in shake flask fermentation). In addition, glycerol was entirely consumed even at initial glycerol concentrations higher than 20 g/L.

Preliminary shake flask experiments were performed at initial glycerol concentrations of 25 g/L and a wide range of initial FAN concentrations (250, 400 and 550 mg/L) employing the three *Cupriavidus necator* strains. Figure 6.1 shows the profile change of FAN, glycerol, total dry weight and PHB during cultivation of the strain *C. necator* DSM 11348 on SFM hydrolysate (550 mg/L initial concentration) and crude glycerol. Although significant amount of FAN was consumed during the first 24 h of cultivation and TDW reached approximately 4 g/L, PHB accumulation was rather low indicated by a low PHB content of 1% (w/w). Consequently, experiments were performed under fed-batch mode with the addition of glycerol to assess the capability to produce higher concentrations of PHB, still the results obtained were not favorable and for this reason the strain was not further evaluated.

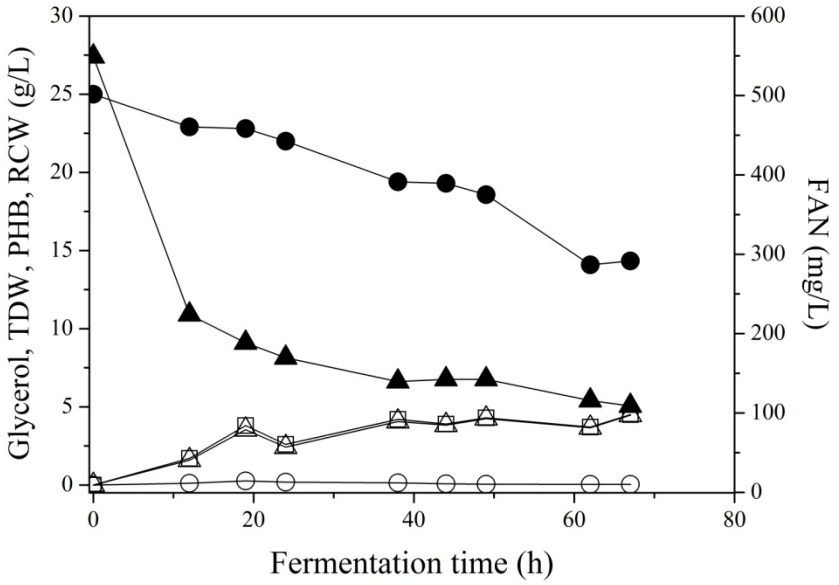

Figure 6.1 Profile change of FAN (▲), glycerol (●), TDW (□), PHB (○) and RCW (△) concentration during shake flask fermentation of *C. necator* DSM 11348 on SFM hydrolysate and crude glycerol.

The bacterial strain *C. necator* DSM 545 was cultivated in shake flasks with SFM hydrolysate and crude glycerol as nitrogen and carbon sources, respectively. Preliminary experiments carried out in batch mode showed that the SFM hydrolysate could promote bacterial growth and P(3HB-co-3HV) accumulation (results not presented). Subsequently, fermentations were carried out in fed-batch mode in shake flasks with glycerol addition at two interval steps (Figure 6.2). Initial FAN and glycerol concentrations in the medium were 540 mg/L and 25 g/L, respectively.

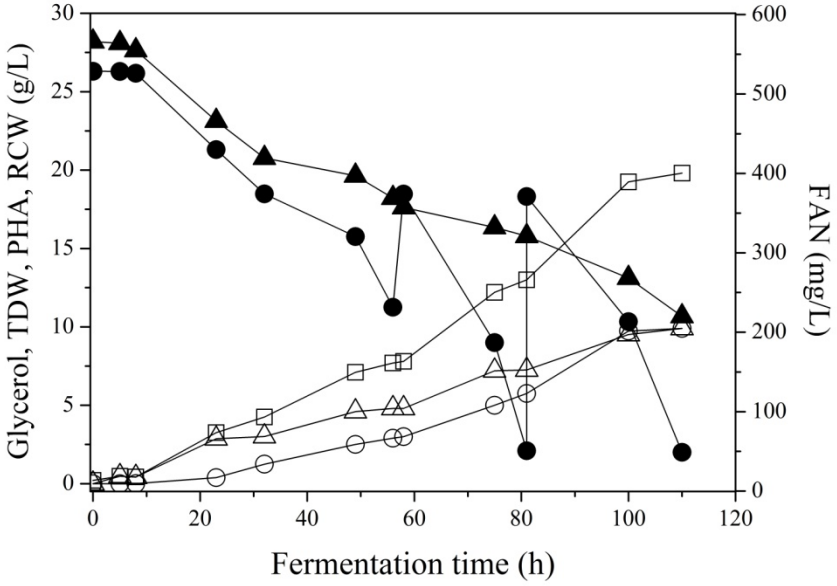

Figure 6.2 Profile change of FAN (▲), glycerol (●), TDW (□), PHA (○) and RCW (△) during shake flask fermentation of *C. necator* DSM 545 on SFM hydrolysate and crude glycerol.

It can be easily observed that microbial growth and PHA accumulation occurred simultaneously with glycerol and FAN consumption (Figure 6.2). This observation is in contrast to previous studies, where production of PHAs occurs under nitrogen limitation (Cavalheiro et al., 2009; Cavalheiro et al., 2012; Mothes et al., 2007). This could be attributed to the fact that in shake flask cultures, dissolved oxygen depletion could become the limiting factor for PHA accumulation. Cell growth was observed during the whole cultivation time. In previous studies carried out with *C. necator* NCIMB 11599 for the production of PHB from wheat-based fermentation media (Koutinas et al., 2007b; Xu et al., 2010), PHB accumulation occurred only after the exhaustion of FAN. The fermentation was extended by addition of crude glycerol at two different intervals (56 and 81 h). The addition of glycerol during fed-batch fermentation resulted in a significant increase of total dry weight (19.8 g/L) and PHA concentration (9.9 g/L). The PHA content was 50 % (w/w). The biopolymer produced contained both 3HB and 3HV monomers. The 3HV molar content was higher at the beginning of the fermentation (9 mol %) and it was gradually reduced towards the end of the fermentation (3 mol %). The same results have also been observed with the same microorganism when rapeseed meal hydrolysates were used as fermentation feedstock (Garcia et al., 2013). The incorporation of 3HV in the co-

polymer is advantageous because it improves the properties of the biopolymer. The production of PHA copolymers in the case of glycerol fermentations has been reported by Cavalheiro et al. (2012) by using 3HV precursors. In this study, 3HV monomers were produced without any precursors. This could be attributed to the consumption of specific amino acids contained in the SFM hydrolysate. Steinbüchel and Lütke-Eversloh (2003) reported that specific amino acids, such as valine, isoleucine, threonine and methionine, are precursors for 3HV synthesis because they are catabolised via propionyl-CoA. Fermentation experiments were also conducted with the strain *C. necator* DSM 545 in bioreactors. However, high concentration of PHA was not accomplished, thus this strain was not utilised during further optimisation of bacterial bioconversions.

In the following stage, *C. necator* DSM 7237 was studied in shake flask experiments to evaluate the ability of this strain to consume crude glycerol combined with SFM hydrolysates in order to produce PHAs. Figure 6.3 presents the profile change of FAN, glycerol, IP, TDW and PHB in shake flask fermentation evaluating the original crude glycerol with 91 % purity. The initial FAN concentration was 338 mg/L and the initial glycerol concentration was 24 g/L. FAN consumption was initiated within the first hours post inoculation, while IP was depleted from the fermentation broth after 10 h triggering PHB synthesis. Although TDW production was satisfactory (more than 7 g/L after 49 h), the PHB concentration was lower than expected and the intracellular biopolymer content did not exceed 58 %. Therefore, a simple pretreatment step via decanting using separation funnels was employed to remove the non-polar compounds present in the crude glycerol stream. The obtained pretreated crude glycerol demonstrated a slightly higher purity (92.4 %) and was further evaluated in bioconversions with three different initial FAN concentrations (250, 365, 550 mg/L).

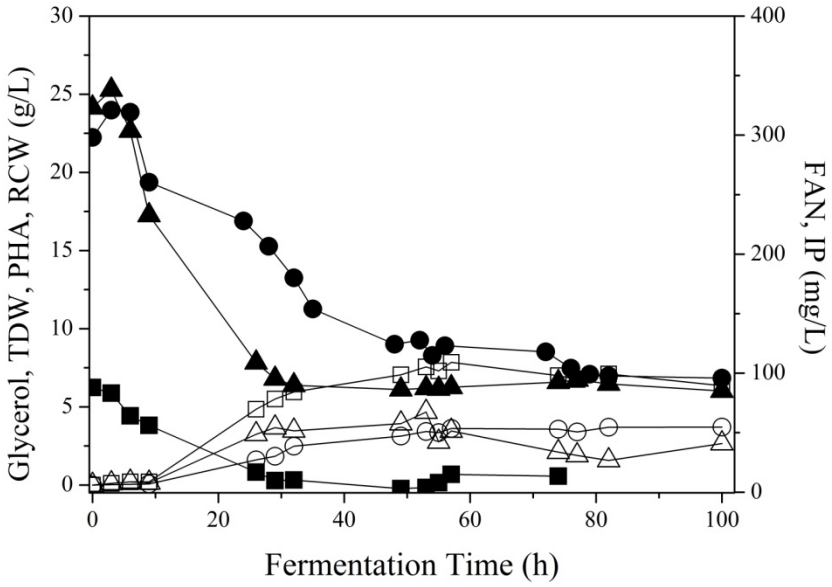

Figure 6.3 Profile change of FAN (▲), IP (■) glycerol (●), TDW (□), PHB (○) and RCW (△) during shake flask fermentation of *C. necator* DSM 7237 on SFM hydrolysate and original crude glycerol with a purity of 91 % (w/w).

Figure 6.4 presents the consumption of FAN, glycerol and IP together with the production of TDW and PHB during shake flask fermentation using pre-treated crude glycerol. More specifically, the initial FAN and glycerol concentrations were 365 mg/L and 25 g/L, respectively. Glycerol consumption was initiated at the point where FAN was almost depleted from the fermentation broth leading to the production of 11.3 g/L of TDW and 7.2 g/L of PHB corresponding to an intracellular content of more than 65 % (w/w). Similar yet lower results were achieved when the initial concentration of nitrogen was increased (550 mg/L FAN) reaching a PHB content of 52 % (w/w). Consequently, the *C. necator* DSM 7237 was selected for the optimisation of bioreactor cultures for the production of PHAs. It is worth noting that the aforementioned strain could only produce PHB compared to *C. necator* DSM 545 that synthesised P(3HB-*co*-3HV) without the addition of precursors. For this reason, the effect of levulinic acid as a precursor on copolymer synthesis was evaluated and will be described in Chapter 7.

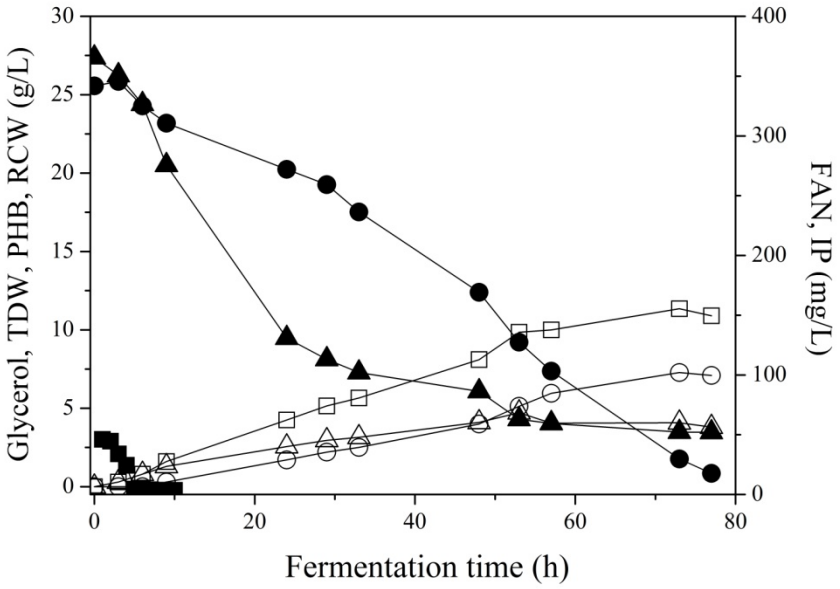

Figure 6.4 Profile change of FAN (▲), IP (■) glycerol (●), TDW (□), PHB (○) and RCW (△) during shake flask fermentation of *C. necator* DSM 7237 on SFM hydrolysate and pre-treated crude glycerol with a purity of 92.4 % (w/w).

6.3 Optimization of crude glycerol utilisation

After selecting the best performing bacterial strain in shake flask fermentations, the following experiments were designated to optimise PHB or P(3HB-*co*-3HV) production in bioreactor cultures utilising SFM hydrolysates and crude glycerol. As previously mentioned in section 5.2.2, the glycerol–rich stream after separation of FAMEs contains approximately 50 % glycerol (w/w). Sequential treatment processes to neutralise the catalyst and recover methanol entail the production of a crude glycerol stream composed of 77-90 % (w/w) glycerol as well as 4.2 – 7 % NaCl or 0.8 – 6.6 % K_2SO_4 depending on the catalyst employed during the transesterification reaction.

Sodium salts have been identified as significantly more inhibitory compounds than potassium salts regarding *C. necator* growth and PHB production (Garcia et al., 2013; Mothes et al., 2007). A sodium chloride concentration of 6 g/L causes inhibition of bacterial growth and PHB production. Mozumder et al. (2015) reported that PHB synthesis was completely terminated at 10.5 g/L of Na^+ concentration. The crude glycerol employed in this study contained a relatively low Na^+ concentration (1.33%, w/w) compared to other

crude glycerol streams. In all literature-cited studies presented so far, crude glycerol resulted in a lower PHA yield as compared to pure glycerol. This was also observed in this study (Figure 6.5) when the initial untreated crude glycerol was used. For this reason, the first set of bioreactor experiments focused on evaluating the potential of minimum crude glycerol pre-treatment in order to achieve PHB production at similar levels as achieved with pure glycerol.

Figure 6.5 presents the profile change of TDW and PHB accumulation during four fed-batch fermentations carried out with different types of glycerol at an initial concentration of around 25 g/L, apart from the fermentation carried out with crude glycerol in which the initial concentration was 15 g/L. The same SFM hydrolysate was used in all fermentations. Initial FAN and IP concentrations were around 670 mg/L and 115 mg/L, respectively, occurring from the dilution of a concentrated SFM hydrolysate to obtain the appropriate initial FAN concentration. The Mg, Ca and Fe concentrations were 157±13 mg/L, 23±2 mg/L and 915±79 mg/L respectively. Fermentations carried out with pure glycerol stopped prematurely after 43 h with relatively low TDW (12.8 g/L) and PHB (8 g/L) production. The same fermentation was carried out with supplementation of various inorganic chemicals as reported in literature-cited publications studying PHB production from glycerol with *C. necator* strains (Cavalheiro et al., 2009, 2012). The addition of inorganic chemicals improved TDW (31.9 g/L) and PHB (24.1 g/L) production corresponding to a PHB content of 75.5 % (w/w). Furthermore, the productivity (0.33 g/L/h) was increased almost two fold. Thus, SFM hydrolysate alone does not provide adequate quantities of micro-nutrients essential for bacterial growth and PHB production.

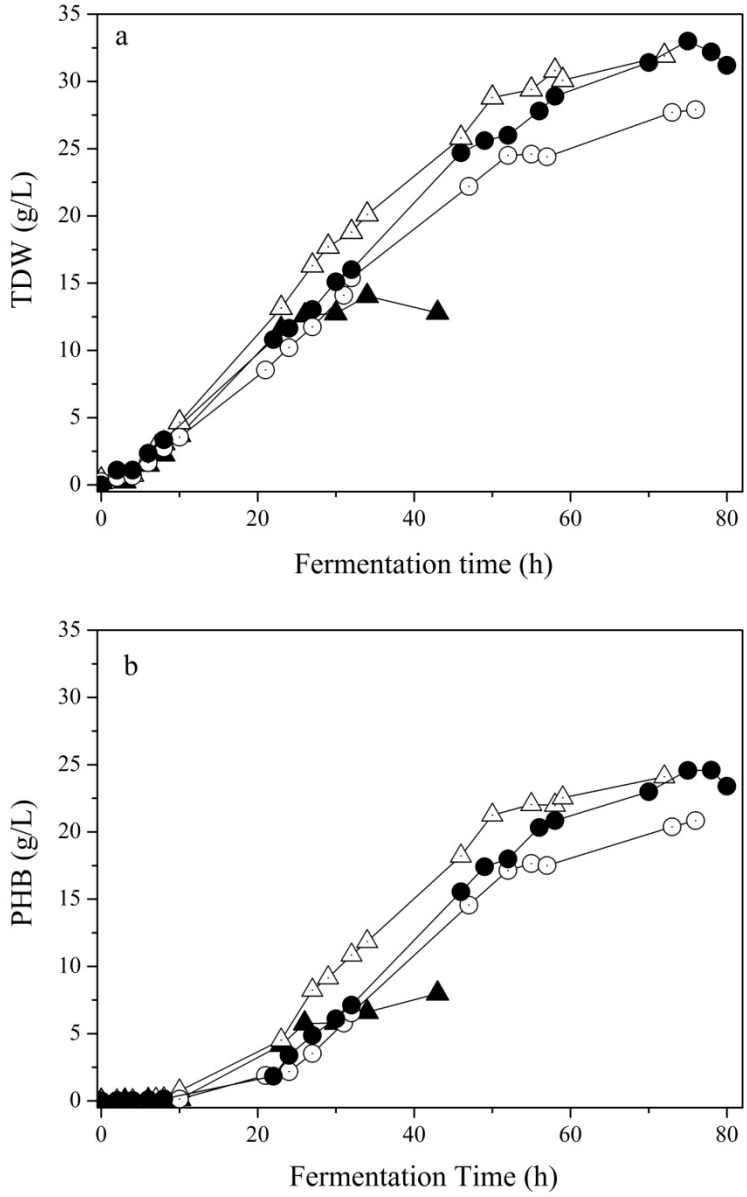

Figure 6.5 TDW (a) and PHB (b) production during *C. necator* fed-batch bioreactor cultivations using sunflower meal hydrolysates and four different crude glycerol solutions including (▲) pure glycerol, (△) pure glycerol supplemented with inorganic chemicals, (○) original crude glycerol and (●) pre-treated crude glycerol via decanting

Table 6.1 Effect of different crude glycerol and pure glycerol streams on *C. necator* fermentation during fed-batch bioreactor experiments using SFM hydrolysates as nutrient supplements

Glycerol type	T_f (h)	TDW (g/L)	RCW (g/L)	PHB (g/L)	PHB content (%)	Productivity (g/L/h)
PG	43	12.8 ± 0.2	4.8 ± 0.058	8 ± 0.21	62.5 ± 0.73	0.19 ± 0.005
PGS	72	31.9 ± 0.95	7.8 ± 0.096	24.1 ± 1.0	75.5 ± 0.91	0.33 ± 0.014
CG	76	27.9 ± 1.24	7.1 ± 0.36	20.8 ± 1.08	74.5 ± 1.17	0.27 ± 0.014
PCG	78	32.2 ± 1.48	7.6 ± 0.67	24.6 ± 0.83	76.4 ± 1.02	0.31 ± 0.011

PG: Pure glycerol; PGS: Pure glycerol supplemented with inorganic chemicals; CG: Original crude glycerol with 91% (w/w) purity; PCG: pre-treated crude glycerol via decanting

When crude glycerol with a purity of 91 % (w/w) was used, the initial glycerol concentration employed was 15 g/L. The initial glycerol concentration was lower than for other fermentations because preliminary shake flask cultures with crude glycerol (Figure 6.3) showed slight inhibition of microbial growth at higher initial glycerol concentrations. The maximum TDW concentration achieved was 27.9 g/L with a PHB content of 74.5 % (w/w) (Table 6.1). These results were promising as far as the whole bioprocess is concerned yet lower than that obtained in the fermentation with pure glycerol. The lower RCW (7.1 g/L) and PHB (20.8 g/L) concentration achieved could be attributed either to limiting concentrations of micro-nutrients or inhibiting compounds present in the crude glycerol. The first reason was excluded because a similar fermentation conducted with supplementation of inorganic chemicals resulted in similar results (data not shown). Potential inhibitory components in crude glycerol could be sodium salts or other compounds (e.g. free fatty acids, fatty acid soaps, mono- and di-acylglycerols, various impurities) that are present in low quantities. The former was not present in inhibitory concentrations even at the end of the fermentation as was calculated using the concentration of Na^+ in crude glycerol (Table 5.2) and the total volume of crude glycerol added in the bioreactor.

In an attempt to separate potential inhibitors using a simple method, crude glycerol was subjected to decanting leading to the production of pre-treated crude glycerol with a slightly higher purity of 92.4 % (w/w). This feedstock was employed in microbial

bioconversions that led to higher TDW (32.2 g/L) and PHB (24.6 g/L) concentration and a similar productivity (0.31 g/L/h) than the fermentation carried out with pure glycerol supplemented with inorganic chemicals (Table 6.1). Moreover, it is worth noting that the concentrations of RCW obtained with different types of glycerol-rich feedstocks are within the same range except for the first fermentation carried out with pure glycerol without supplementation with inorganic chemicals (Table 6.1). Another important observation is the fact that pre-treated crude glycerol, besides being free of inhibitors, also provided sufficient micro-nutrients that led to high microbial growth and PHB production (Figure 6.5). As it is shown in Table 5.2, crude glycerol contains Fe, Mg and Ca. Crude glycerol streams produced from various feedstocks and processes usually contain varying concentrations of micro-nutrients, such as calcium, magnesium, phosphorus and sulfur (Chatzifragkou and Papanikolaou, 2012; Thompson and He, 2006). Decanting is not considered an expensive unit operation that could be easily employed in order to increase PHB production. Posada et al. (2011) reported that glycerol purification to 98 % increases PHB production cost by only 6% influencing slightly the process economics.

Sodium ions (Na^+) contained in crude glycerol streams have been previously demonstrated to cause inhibition in PHB accumulation presumably by inducing osmotic pressure. Mothes et al. (2007) reported a reduction of PHB content from 70% (achieved with pure glycerol) to 48% when a crude glycerol stream with a purity of 80% and a NaCl content of 5.5% was used in fermentations with *C. necator* JMP 134. In the same study, a crude glycerol stream with a purity of 90% that contained the less inhibitory salt K_2SO_4 (1.6%) resulted in an intermediate PHB content (around 55%) compared to the previous two crude glycerol streams. Likewise, a reduction of PHB content from 62% to 50%, PHB concentration from 51.2 g/L to 38.1 g/L and PHB productivity from 1.5 g/L/h to 1.1 g/L/h was reported when *C. necator* DSM 545 was cultivated on crude glycerol with glycerol content of 1174.5 g of glycerol per L (Cavalheiro et al., 2009). On the contrary, a recent study performed with *C. necator* DSM 545 on volatile fatty acids and more specifically acetic acid, stated that addition of 6.5 g/L NaCl during the fermentation induced PHB synthesis probably by causing osmo-regulation stress in the bacterial culture (Passanha et al., 2014).

The generic applicability of decanting as a pre-treatment stage for improved PHB production via fermentation should be further evaluated with crude glycerol streams produced by various biodiesel producers.

6.4 Optimisation of bioconversions: Effect of carbon to nitrogen (C/N) ratio

After the optimisation of crude glycerol pretreatment, it was necessary to optimise the addition of SFM hydrolysate at the beginning of fermentation. The complexity of this medium prevented optimisation of all components. For this reason, the optimisation focused only on the nitrogen content that is the second most important nutrient after the carbon source in any fermentation process. The optimum nitrogen content could be produced via appropriate enzymatic hydrolysis of SFM. It was decided to test the effect of initial C/FAN ratio and FAN concentration on both microbial growth and PHB accumulation. FAN represents the nitrogen content of free amino groups present in peptides and amino acids.

Other targets of this set of fermentations were to elucidate bacterial growth during assimilation of FAN and IP, maximize RCW that is directly associated to maximum PHB production, assuming that the highest PHB content of 70 – 75% (w/w) will be accomplished in the accumulation phase, and achieve high glycerol to PHB production yield. The latter is an important parameter as high yields will reduce the quantity of crude glycerol required to achieve the highest PHB concentration avoiding inhibition due to critical Na^+ concentrations. High yields could be achieved via utilisation of amino acids and peptides contained in SFM hydrolysates not only as nitrogen sources but also as carbon sources reducing glycerol requirements for bacterial growth. This was observed in PHB production using wheat-based hydrolysates (Xu et al., 2010).

Based on the results of shake flask fermentations the study on the effect of initial FAN concentration initiated at around 412 mg/L (Figure 6.6). The TDW production achieved was 21.6 g/L with a PHB content of 69.9 % at 78 h. The glycerol consumption rate was significantly increased after the depletion of nitrogen in the fermentation broth. The exponential growth phase of the bacteria lasted until the depletion of IP, while the specific growth rate (μ) was 0.198 (1/h).

Table 6.2 presents the results from seven bioreactor fermentations, operated in fed-batch mode under varying initial FAN concentration and C/FAN ratios. It should be noted that only the carbon content from glycerol has been considered in the calculation of C/FAN ratios. The initial glycerol concentration was around 25 g/L (±10%) in all fermentations. Fermentation time (T_f) corresponds to the time that maximum TDW and PHB were obtained. The designated initial FAN concentrations were achieved by the appropriate conditions of enzymatic hydrolysis and dilution with tap water where

necessary. The highest TDW production (37 g/L) with a PHB content of 72.9 % (w/w) were achieved at 98 h when an initial C/FAN ratio of 17.05 g/g was employed (corresponding to 585 mg/L FAN). The TDW and PHB concentrations achieved at 77 h (Figure 6.7 and Figure 6.8) were similar to the results obtained in the fermentation where the initial C/FAN ratio was 14.05 g/g (Table 6.2). Therefore, PHB productivities were also similar in these two fermentations at the same culture time of 78 h.

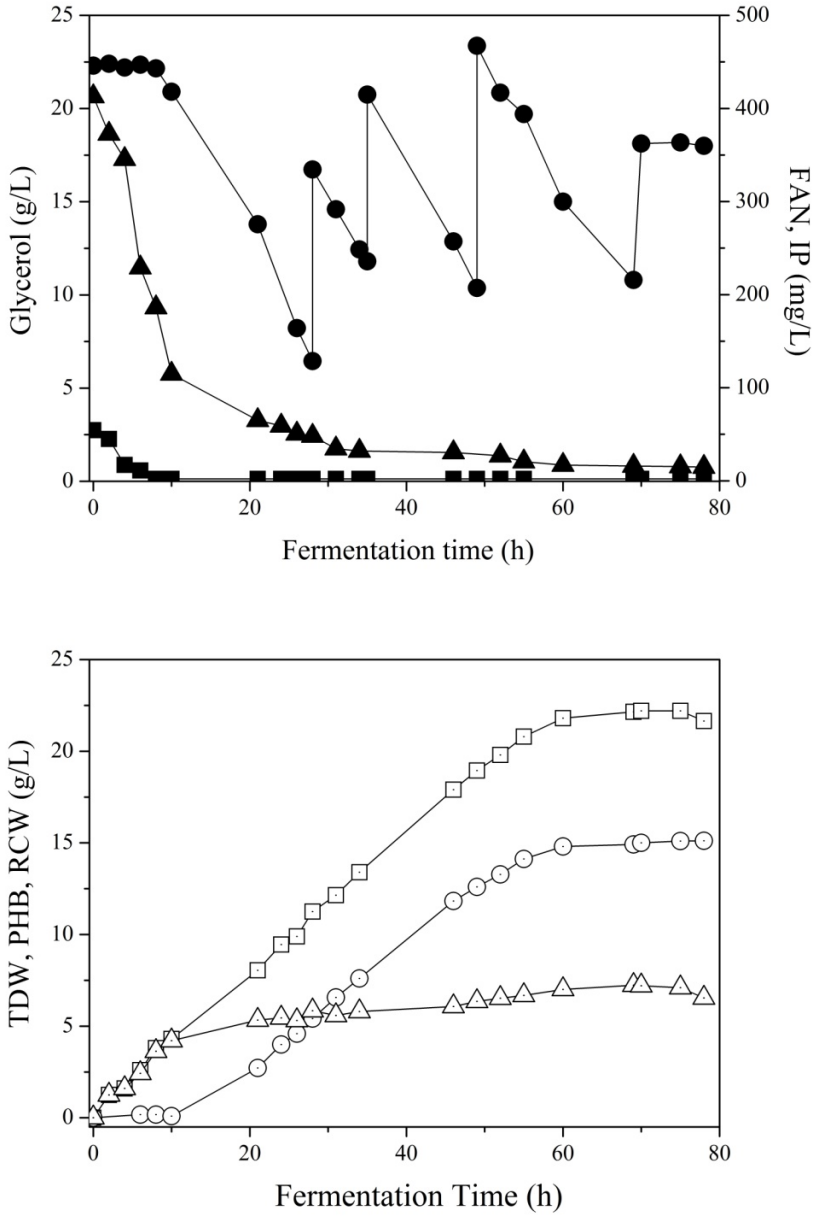

Figure 6.6 Profile change of glycerol (●), FAN (▲), IP (■), TDW (□), PHB (○) and RCW (△) during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with an initial FAN concentration of 412 mg/L (C/FAN ratio of 21.2 g/g)

Table 6.2 Effect of different C/FAN ratios on *C. necator* DSM 7237 fed-batch bioreactor fermentation using SFM hydrolysates and crude glycerol as the sole media

C/FAN (g/g)	T_f (h)	TDW (g/L)	RCW (g/L)	PHB (g/L)	PHB content (%)	Productivity (g/L/h)	Yield (g/g)
30	61	13 ± 0.62	5 ± 0.24	8 ± 0.38	61.5 ± 0.15	0.13 ± 0.01	0.24 ± 0.009
21.2	78	21.6 ± 0.81	6.5 ± 0.31	15.1 ± 0.5	69.9 ± 0.35	0.19 ± 0.006	0.34 ± 0.012
17.05	98	37 ± 0.73	10 ± 0.45	27 ± 0.36	72.9 ± 0.76	0.28 ± 0.004	0.32 ± 0.015
14.05	78	32.2 ±0.86	7.6 ± 0.24	24.6 ± 0.62	76.3 ± 0.13	0.31 ± 0.008	0.30 ± 0.013
12	76	29.8 ± 0.53	9.3 ± 0.18	20.5 ± 0.58	68.9 ± 0.85	0.27 ± 0.007	0.28 ± 0.011
11.08	79	22.5 ± 0.95	11 ± 0.41	11.5 ± 0.55	51.3 ± 0.33	0.15 ± 0.007	0.19 ± 0.008
9.97	82	19.8 ± 0.71	13.9 ± 0.50	5.9 ± 0.21	29.9 ± 0.015	0.07 ± 0.003	0.12 ± 0.004

This set of fed-batch fermentations showed that decreasing C/FAN ratio (corresponding to increasing FAN concentrations) results in increasing RCW production. The highest RCW (13.9 g/L) and the lowest PHB concentration (5.9 g/L) were achieved when the lowest C/FAN ratio was used because the highest volume of SFM hydrolysate was used for fermentation media formulation increasing the availability of all nutrients that are necessary for bacterial growth. This is logical because higher availability of nutrients will divert carbon flux towards microbial growth rather than PHB synthesis resulting in reduced PHB production. The metabolic pathway leading to PHB synthesis begins with condensation of two acetyl-CoA molecules for the production of acetoacetyl-CoA. Utilisation of acetyl-CoA for growth via the TCA cycle occurs when there is no nutrient limitation. This observation indicates that SFM hydrolysates in combination with crude glycerol provide adequate amount of nutrients to support microbial growth (Table 6.2). This was further verified by conducting shake flask fermentations with SFM hydrolysates as the sole nutrient source leading to the production of 3.65 g/L TDW after 32 h that was entirely RCW as PHB was not accumulated.

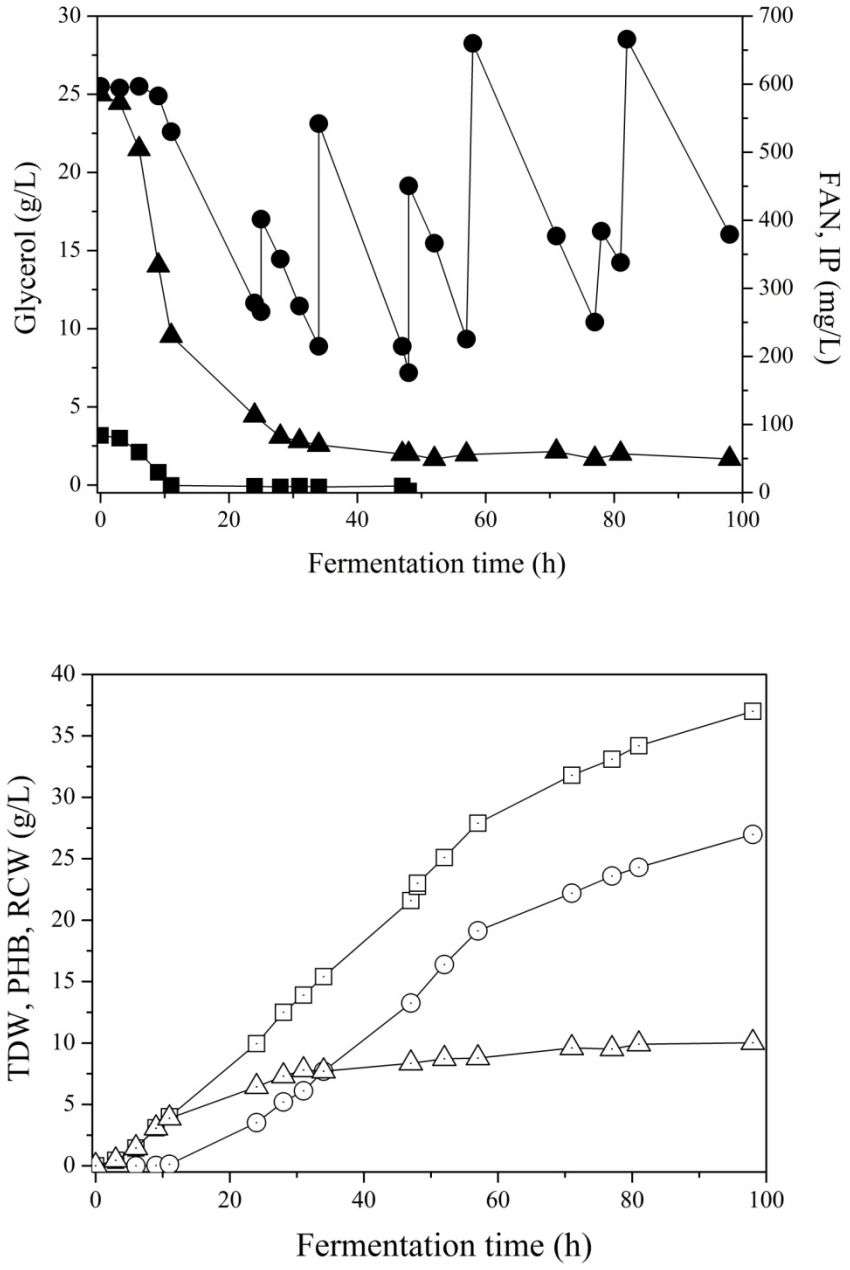

Figure 6.7 Profile change of glycerol (●), FAN (▲), IP (■), TDW (□), PHB (○) and RCW (△) during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with an initial FAN concentration of 585 mg/L (C/FAN ratio of 17.05 g/g)

Decreasing the C/FAN ratio results in an optimum range (14.05 – 17.05 g/g) that promotes maximum PHB concentration, content, productivity and yield, as presented in Table 6.2. These fermentation parameters followed a decreasing trend at initial FAN concentrations higher than 809 mg/L. Fed-batch fermentations at the optimum C/FAN ratio were carried out in duplicate to verify the obtained results. The lag phase was

increased significantly at fermentations carried out with an initial FAN concentration higher than 809 mg/L. The specific growth rate (μ) was in the range of 0.269-0.291 1/h for the optimum range of initial FAN concentrations examined.

Figure 6.7 presents the profile change of FAN, IP, glycerol, TDW, PHB and RCW during fed-batch fermentation carried out with an initial C/FAN value of 17.05 g/g. Two different phases can be observed. In the first phase that lasts up to complete IP consumption (around 10 h), microbial growth occurs simultaneously with FAN and IP consumption. The second phase is initiated when IP is completely consumed that triggers PHB synthesis. FAN consumption is completed at approximately 34 h. After IP depletion from the fermentation medium, the FAN is consumed at a slower rate. Therefore, in this fermentation, bacterial growth stops and PHB synthesis is triggered due to phosphorus limitation. Crude glycerol was fed periodically in the fermentation broth in order to enhance PHB formation, which reached a maximum concentration of 27 g/L at 98 h. The productivity of this fermentation at 98 h was 0.28 g/L/h, while at 77 h the productivity (0.31 g/L/h) was similar as in the fed-batch fermentation carried out with an initial C/FAN ratio of 14.05 g/g (Figure 6.7). The productivity achieved in this study is lower than productivities (up to 1.1 g/L/h) achieved in literature-cited publications using crude glycerol for PHB production (Cavalheiro et al., 2009; Ibrahim and Steinbuchel, 2009).

Figure 6.8 presents the consumption of glycerol, FAN and IP in accordance with the production of TDW and PHB. Likewise, the exponential phase lasted until complete phosphorus depletion in the medium leading to the intracellular accumulation of biopolymer. The maximum PHB production achieved was 24.6 g/L at 78 h after several pulses of crude glycerol to prevent carbon source exhaustion.

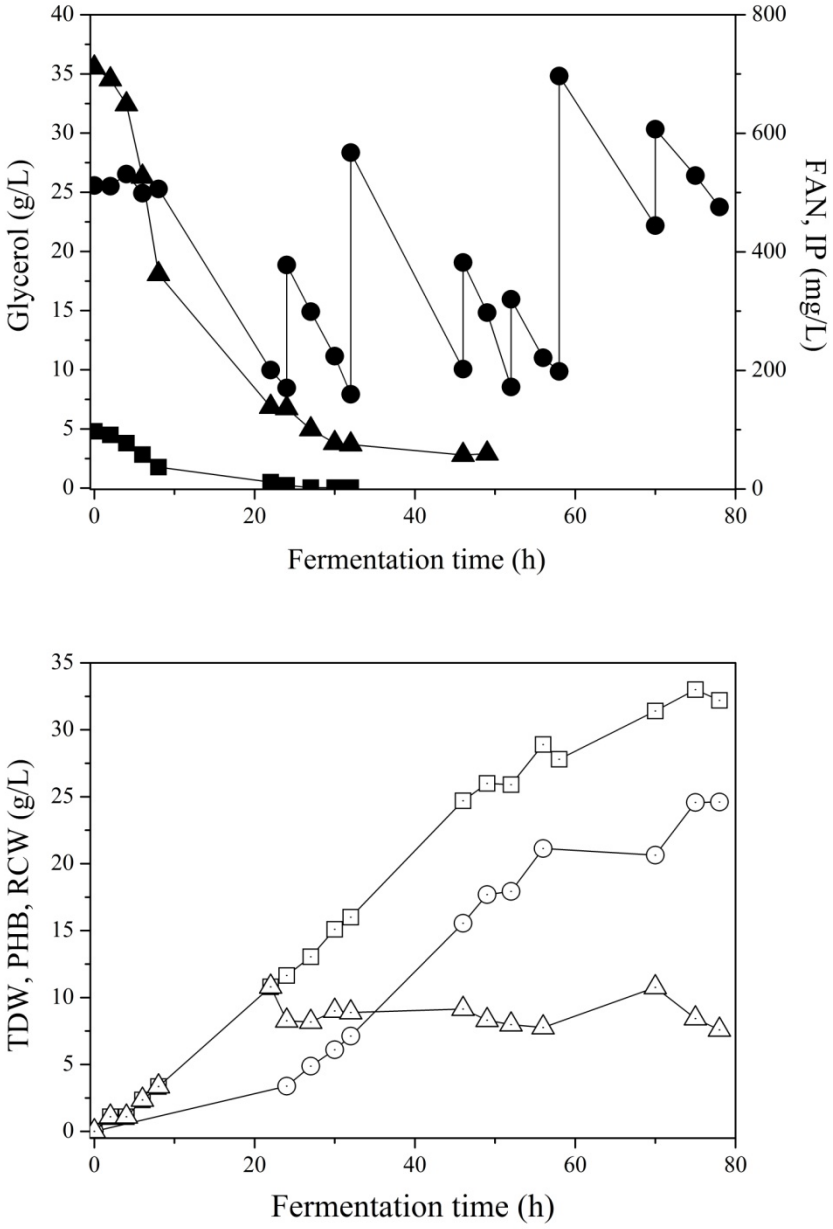

Figure 6.8 Profile change of glycerol (●), FAN (▲), IP (■), TDW (□), PHB (○) and RCW (△) during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with an initial FAN concentration of 710 mg/L (C/FAN ratio of 14.05 g/g)

The highest practical yield of PHB achieved in this study was 0.34 g/g, which is significantly higher than other studies (Cavalheiro et al., 2009; Ibrahim and Steinbuchel, 2009). This was achieved because bacterial growth takes place using amino acids and peptides as both carbon and nitrogen sources. This can be observed in Figure 6.7 and Figure 6.8 where glycerol is not consumed until complete IP depletion. The low glycerol

requirements for microbial growth lead to a higher overall glycerol to PHB conversion yield.

6.5 Evaluation of different bioprocessing strategies

Fed-batch bioconversions can lead to high conversion yields and productivities of the final product by modifying the feeding strategies (Lee et al., 1999). Implementation of diverse feeding strategies includes continuous feeding (Hafuk et al., 2011), pH-stat (Huschner et al., 2015), DO-stat (Cesario et al., 2014) and adjustment of feeding after on-line monitoring of carbon source (Kim et al., 1993). Moreover, high cell culture densities can be accomplished by maintaining nutrient and carbon source concentration within a specified range.

During the study on the evaluation of different initial FAN concentrations it was observed that for the optimum C/N ratio (14.05-17.05 g/g) the residual cell weight (RCW) ranged from 7.6 – 10 g/L. Thereof, it was decided that in order to enhance PHB production higher RCW should be achieved. The implementation of different bioprocessing strategies aimed at increasing the RCW during the exponential growth phase or during PHB accumulation. The first case was tested by the addition of excess phosphorus source in the fermentation feedstock aiming to prolong biomass production during the exponential growth phase. The glycerol feeding solution was added at random intervals in order to maintain the glycerol concentration in the bioreactor in the range of 5 – 10 g/L.

To assess the potential to increase the RCW during the PHB accumulation phase, the feeding solution of crude glycerol was substituted with a mixture of crude glycerol and SFM hydrolysate. The nutrient content in this feeding medium was low enough to allow cell maintenance or minimal cell growth since a higher nutrient content could stop PHB production and induce bacterial growth. Xu et al. (2010) reported that RCW and PHB production were improved when wheat hydrolysates were used as feeding media.

6.5.1 Fed-batch fermentation with increased IP concentration

The first set of experiments focused on the increase of the initial IP concentration in the fermentation medium by adding phosphate salts into the SFM hydrolysate. This experiment was carried out due to the fact that phosphorus depletion acts as the limiting

factor that switches cell metabolism to PHB accumulation. Thus, two different initial IP concentrations were evaluated (i.e. 2 g/L and 1 g/L). The total IP concentration included the IP present in the SFM hydrolysate. The phosphorus concentration was regulated using KH_2PO_4.

Figure 6.9 presents the profile change of glycerol, FAN, IP, TDW, PHB and RCW during fed-batch fermentation with an initial IP concentration of 2 g/L. Initial FAN and glycerol concentration were 716 mg/L and 25 g/L, respectively. FAN consumption ceased at 30 h indicating the onset of PHB accumulation. The total IP consumption from the beginning of the fermentation was approximately 200 mg/L and was terminated after 32 h. Considering that phosphorus exists in surplus quantity in the fermentation medium, it was deducted that nitrogen exhibited the limiting factor to trigger PHB synthesis. After FAN exhaustion, glycerol feeding with a concentrated solution (750 g/L) was initiated aiming to maintain glycerol concentration in the range of 5.1 – 15.8 g/L. The maximum TDW and PHB production reached were 37 g/L and 23.3 g/L, respectively, at 74 h of fermentation. However, at 68 h where the maximum RCW was achieved (16 g/L), the maximum PHB content was also observed (66.5 %, w/w). Eventually, although the residual cell weight was increased, the PHB concentration and content were not increased.

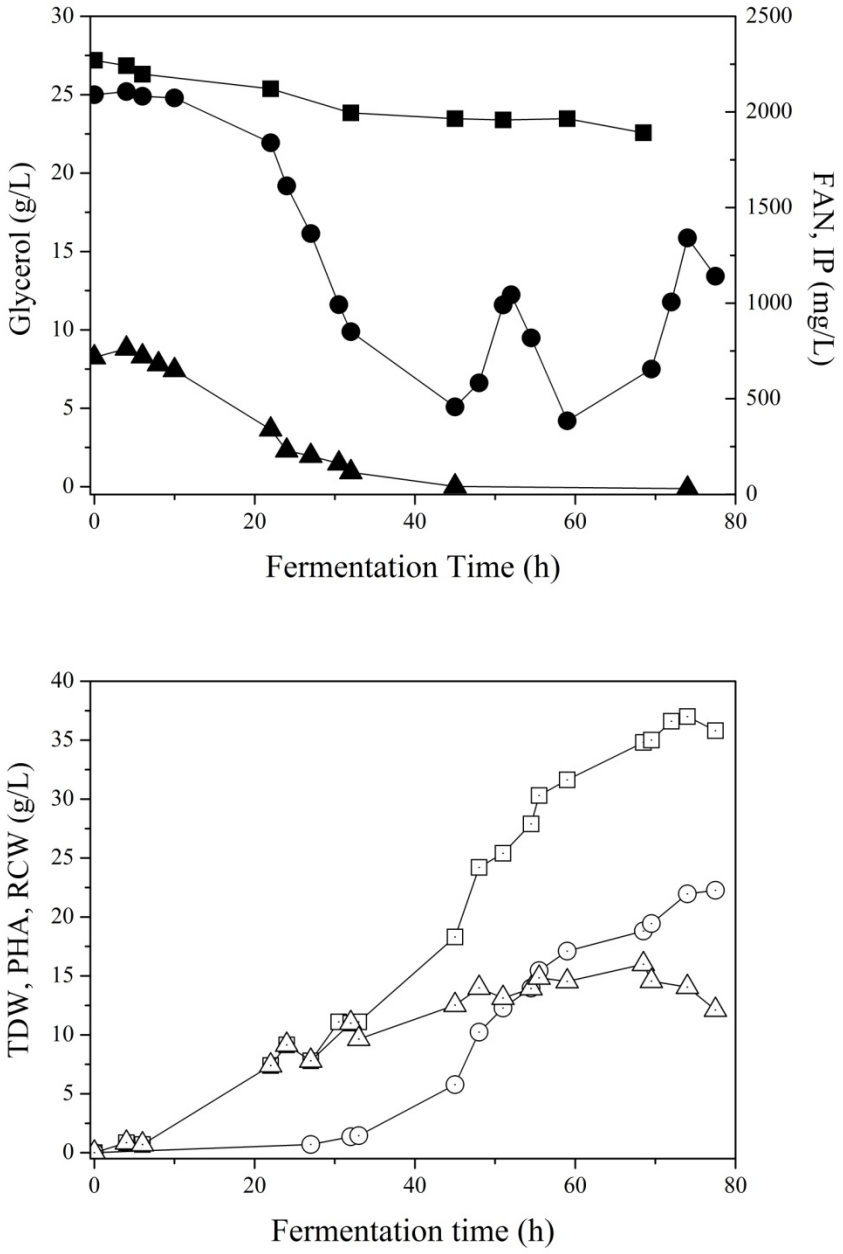

Figure 6.9 Profile change of glycerol (●), FAN (▲), IP (■), TDW (□), PHB (○) and RCW (△) during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with an initial IP concentration of 2 g/L

The consumption of glycerol, FAN and IP as well as the production of TDW, PHB and RCW is presented in Figure 6.10. During this experiment, the initial IP concentration was adjusted to 1 g/L, while the FAN concentration remained in the same range as in the fermentation presented in Figure 6.9 (711 mg/L). Glycerol addition was performed manually in various intervals. Likewise, the depletion of nitrogen from the fermentation

medium induced PHB accumulation at approximately 40 h. The maximum TDW produced was obtained at 124 h corresponding to 40.3 g/L with an intracellular content of 63.4 % (w/w). Similar to the previous case studied, the RCW was increased approximately by 20 % (up to 14.6 g/L). However, the PHB content did not exceed 64 % that was lower than previous fermentations presented in this chapter where the PHB content was higher than 70 % (Table 6.2). The volumetric productivity was 0.21 g/L/h when the maximum TDW and PHB concentration were attained (124 h).

In the fermentations presented in Figures 6.9 and 6.10, the inorganic phosphorus was not depleted from the fermentation media. Grousseau et al. (2014) demonstrated that phosphorus feeding led to higher productivity than phosphorus depletion conditions that was caused by maintaining residual cell growth. Different phosphorus feeding limitations had a pronounced effect on 3HB and 3HV molar ratios. The effect of initial phosphorus concentrations was also evaluated by Ryu et al (1999) using phosphate concentration in the range of 2.2 – 5.5 g/L. The latter case resulted in the highest cell dry weight and PHB concentration yielding 281 g/L and 232 g/L, respectively. at 74 h with a productivity of 3.14 g/L/h. In this study, an initial phosphate concentration of 1 g/L led to higher total dry weight and PHB concentration. However, the obtained PHB concentration and content were not higher than the ones achieved when only the SFM hydrolysate was used. As a conclusion, phosphorus depletion leads to higher PHB accumulation than nitrogen depletion. Similar observations were reported by Shang et al. (2003) during PHB production by *Ralstonia eutropha* in fed-batch cultures using phosphate limitation.

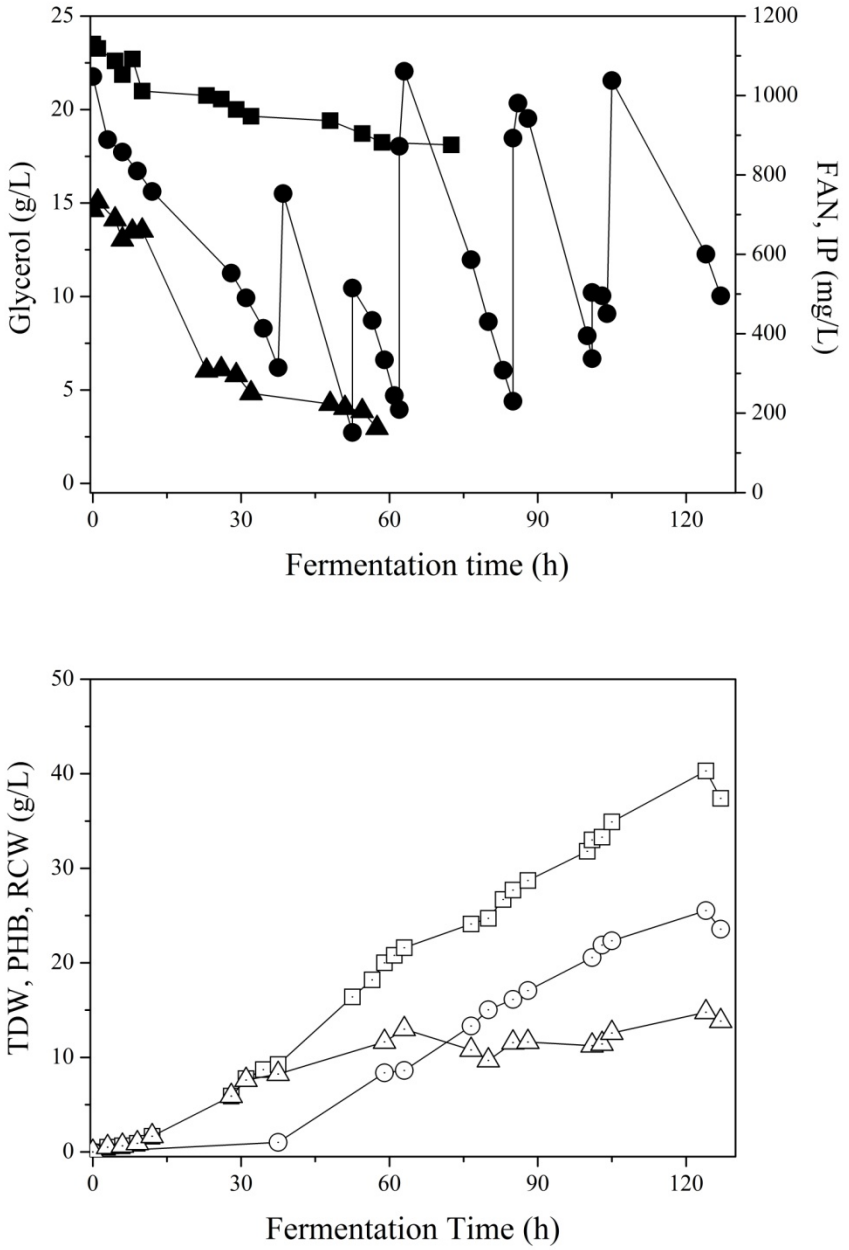

Figure 6.10 Profile change of glycerol (●), FAN (▲), IP (■), TDW (□), PHB (○) and RCW (△) during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with an initial IP of 1 g/L

6.5.2 Fed-batch fermentation with continuous feeding of glycerol and SFM hydrolysate

The following experiment focused on the effect of combined feeding of glycerol and SFM hydrolysate. The target was to extend bacterial proliferation and increase RCW through the optimisation of feeding solution to supply carbon source in conjunction with

other nutrients. Alongside the TCA cycle, acetyl-CoA is utilised to generate amino acids. Furthermore, NADPH is employed as a cofactor for acetoacetyl reductase, the second enzyme that is crucial for PHB synthesis. Thereof, external addition of amino acids will enable more acetyl-CoA for intracellular PHB synthesis since energy requirements of metabolism for protein synthesis are decreased (Lee et al., 1995).

The amount of FAN added in the bioreactor was relatively low targeting to sustain microbial maintenance without shifting the metabolism towards bacterial growth. The results obtained are illustrated in Figure 6.11. The initial FAN concentration was 611 mg/L, while the supply of feeding solution was initiated after 30 h of fermentation coinciding with PHB formation. The fermentation duration was prolonged up to 120 h yielding 42.2 g/L TDW and 32.6 g/L of PHB concentration corresponding to a PHB content of 77.2 % (w/w). The RCW was 9.6 g/L at 117 h, where the maximum TDW and PHB concentration were observed. Furthermore, during this experiment, the glycerol concentration remained within the range of 10 – 20 g/L by continuous feeding of the feeding solution. It was observed that when glycerol concentration was maintained within this range, the glycerol consumption rate was significantly enhanced (approximately 1.7 g/L/h) compared to experiments performed with external pulses of feeding solutions (around 0.8 g/L/h).

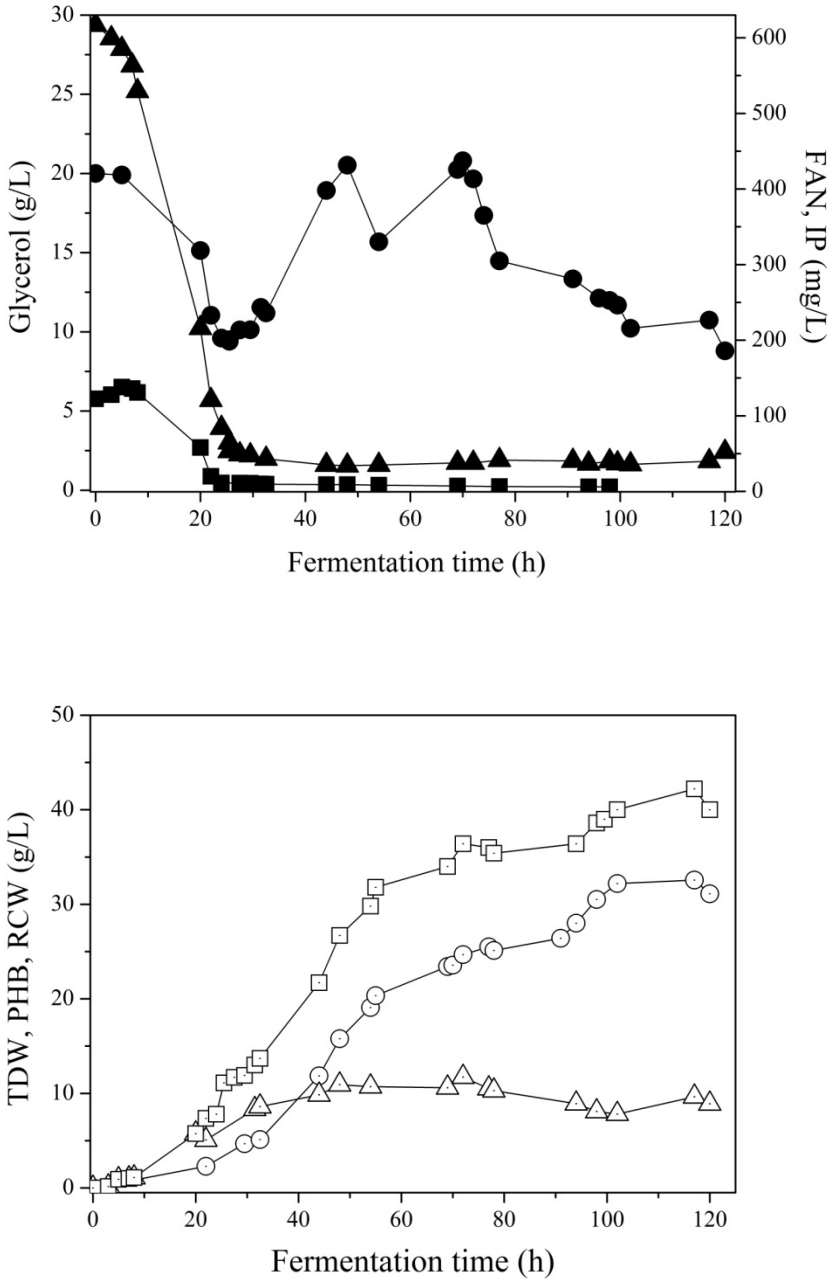

Figure 6.11 Profile change of glycerol (●), FAN (▲), IP (■), TDW (□), PHB (○) and RCW (△) during fed-batch bioreactor fermentation of *C. necator* with a combined continuous feeding of sunflower meal hydrolysate and crude glycerol

The fermentation presented in Figure 6.11 showed that low addition of nutrients during the PHB accumulation phase favours biopolymer production. Koutinas et al. (2007) implemented wheat-derived nutrient supplements for PHB production, observing that low nitrogen addition proved beneficial for the conversion yield of glucose to PHB. Similarly,

Xu et al. (2010) employed a feeding solution of wheat hydrolysates and pure glucose to demonstrate significant increase in PHB accumulation during bioreactor experiments performed under fed-batch mode. Literature-cited publications have well established the beneficial effect of amino acids contained in complex fermentation supplements deriving from renewable resources on PHB production (Fujita et al., 1993; Lee at al. 1995).

6.6 Conclusion

Consequently, based on the results of the Chapter 6, it can be stated that crude glycerol and SFM hydrolysates can successfully provide a feedstock for bacterial bioconversions, providing all essential nutrients to support microbial proliferation and PHB production. The substitution of conventional and expensive nitrogen sources paves the way towards the development of biorefinery concepts implementing renewable resources and re-structuring of current industrial processes. However, increased sustainability will only be feasible by employing all components of sunflower meal in order to generate diversified end-products targeting different market outlets.

The results presented in Chapter 6 have been published in the Bioresource Technology journal:

Kachrimanidou, V., Kopsahelis, N., Papanikolaou, S., Kookos, I., De Bruyn, A., Clark, J.H., Koutinas, A.A. Sunflower-based biorefinery: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production from crude glycerol, sunflower meal and levulinic acid. Bioresour. Technol. (2014) 172:121-130.

CHAPTER 7

PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYVALERATE) USING CRUDE RAW MATERIALS AND LEVULINIC ACID

7.1 Introduction

The optimisation of bacterial bioconversions using different glycerol streams and the C/N ratios demonstrated the optimum conditions for PHB production. This chapter focuses on the evaluation of poly(3-hydroxybutyrate-*co*-3-hyroxyvalerate), P(3HB-*co*-3HV), production using levulinic acid addition as pre-cursor during fermentation. Levulinic acid was selected because it is considered as a major platform chemical that can be produced from lignocellulosic biomass in future biorefineries (Bozell et al., 2000). The production of P(3HB-*co*-3HV) via levulinic acid supplementation was evaluated in shake flask fermentations and in fed-batch bioreactor experiments. In all cultures, crude glycerol and SFM hydrolysates were used as fermentation media. The biopolymers produced in this study were characterized using $_{1}H$ and $_{13}C$ Nuclear Magnetic Resonance spectroscopy (NMR) and thermogravimetric analysis (TGA).

7.2 Shake flask fermentations

The study described in Chapter 5 for the selection of the bacterial strain elucidated the capability of the strain *C. necator* DSM 545 to produce the copolymer P(3HB-*co*-3HV) when it was cultivated in shake flask fermentations with crude glycerol and SFM hydrolysates without the addition of any precursor. Similar results were obtained when crude glycerol and rapeseed meal hydrolysates were used as fermentation media in shake flask cultures with the same bacterial strain attaining a 3HV content of 12 mol% (Garcia et al., 2013). However, the strain *C. necator* DSM 7237 used in this study could only produce PHB when SFM hydrolysates and crude glycerol were used. The brittle and highly crystalline nature of PHB (55 – 80%) hinders industrial implementation. Furthermore, the degradation temperature of PHB does not differ significantly from the melting temperature causing problems during thermal processing (Wang et al., 2013). For this reason, various organic compounds like propionic acid, propanol, sodium propionate, pentanol, levulinic

acid and heptanoic acid (Aramvash et al., 2016) have been evaluated as precursors for copolymers synthesis, which show improved thermal and physical properties. The incorporation of 3-hydroxyvalerate units (3HV) in the backbone chain of PHB results in gradually decreasing crystallinity in parallel with a reduction of melting temperature, thus enabling the manufacture of biopolymers with adjustable properties (Ashby et al., 2012).

For this reason, levulinic acid (4-ketovaleric acid or 4-oxopentanoic acid) with a similar structure to valeric acid (pentanoic acid) was tested as a potential precursor for the synthesis of P(3HB-*co*-3HV). Levulinic acid was selected due to its future large scale production in various biorefineries from renewable lignocellulosic feedstocks using acid hydrolysis (Bozell et al., 2000). In particular, levulinic acid could be produced in the proposed biorefinery using the lignocelluloses-rich fraction remaining after enzymatic hydrolysis (Figure 4.12).

Table 7.1 presents the results obtained from four shake flask fermentations in which different cumulative quantities of levulinic acid (0, 2.56, 5.75 and 10.85 g/L) were added at random time intervals. The same SFM hydrolysate was used in all shake flask cultures corresponding to an initial FAN concentration of around 365 mg/L. The initial glycerol concentration was 25 g/L in all cases. A concentrated levulinic acid solution was added at random intervals in the fermentation broth 24 h post inoculation, when microbial growth had ceased and P(3HB-*co*-3HV) accumulation had started. Periodic addition of levulinic acid was chosen in order to prevent microbial growth inhibition caused by high levulinic acid concentrations at any stage during fermentation. The results presented in Table 7.1 demonstrate that consumption of increasing levulinic acid quantities led to increasing P(3HB-*co*-3HV) concentrations and 3HV content. The RCW remained constant as it was expected since the same SFM hydrolysate was used in all fermentations.

Table 7.1 Effect of different levulinic acid additions on *C. necator* growth and P(3HB-*co*-3HV) production during shake flask fermentations using crude glycerol and SFM hydrolysate

Initial FAN (mg/L)	T_f (h)	TDW (g/L)	RCW (g/L)	P(3HB-*co*-3HV) (g/L)	Levulinic acid* (g/L)	3HB (mol%)	3HV (mol%)	P(3HB-*co*-3HV) content (%)
365	73	11.35	4.08	7.27	0	99.14	0.86	64.1
353	77	13.45	4.77	8.68	2.56	89.4	10.6	64.5
382	73	14.55	4.98	9.57	5.75	86.2	13.8	65.8
364	79	15.5	4.12	11.38	10.85	78.5	21.5	73.4

* Cumulative consumption of levulinic acid

The TDW was increased by approximately 26 % when the highest addition of levulinic acid (10.85 g/L) was carried out. The P(3HB-*co*-3HV) content was increased only in the fermentation where the highest levulinic acid quantity was consumed. Based on the 3HB and 3HV quantities achieved in each fermentation, it can be observed that levulinic acid addition increased the production of both co-monomers (Figure 7.1). The increase of 3HB and 3HV concentrations with increasing cumulative levulinic acid consumption could be expressed by the following linear equations:

$$C_{3HB} = 0.156 \times W_{LA} + 0.08 \quad (R^2 = 0.99) \tag{7.1}$$

$$C_{3HV} = 0.21 \times W_{LA} + 0.12 \quad (R^2 = 0.98) \tag{7.2}$$

where C_{3HB} and C_{3HV} represent the 3HB and 3HV concentrations produced from increasing cumulative quantities of levulinic acid (W_{LA}). These equations were derived by subtracting the 3HB and 3HV concentrations produced in the shake flask fermentation conducted without levulinic acid addition from each one of the other three fermentations presented in Table 7.1 in which levulinic acid was added.

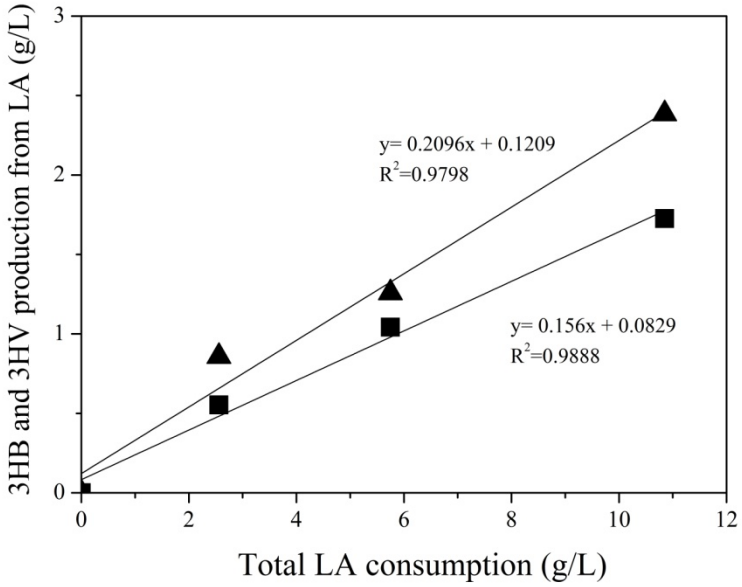

Figure 7.1 Production of 3HB and 3HV during shake flask fermentations of *C. necator* carried out on crude glycerol and sunflower meal hydrolysate at different supplementations of levulinic acid (▲) 3HV, (■) 3HB

Chung et al. (2001) evaluated the effect of levulinic acid, valerate and propionate on P(3HB-*co*-3HV) synthesis by *Ralstonia eutropha* KHB-8862 grown on fructose syrup. Levulinic acid proved to be superior than the other organic compounds used. Sustaining low concentrations of levulinic acid during fermentation by repeated additions promoted cell proliferation and copolymer synthesis leading to 3.4 g/L of TDW with 52 % (w/w) P(3HB-*co*-3HV) content. The stimulatory effect of levulinic acid on biomass and copolymer formation was also demonstrated by Ashby et al. (2012) in the cultivation of *Pseudomonas oleovorans* utilizing crude glycerol and levulinic acid. The levulinic acid enhances the synthesis of 3-ketovaleryl-CoA that is further reduced to 3-hydroxyvaleryl-CoA, the precursor of 3-hydroxyvalerate in the P(3HB-*co*-3HV) biosynthesis pathway (Chung et al., 2001; Jaremko and Yu, 2011).

The shake flask experiments presented above showed that the addition of levulinic acid can lead to the production of P(3HB-*co*-3HV). For this reason, subsequent bioreactor experiments were carried out in fed-batch mode with the combined addition of crude glycerol and levulinic acid to further evaluate the production of P(3HB-*co*-3HV) under controlled fermentation conditions.

7.3 Fed-batch fermentations for P(3HB-co-3HV) production

Two fed-batch fermentations in a bioreactor (0.8 L working volume) were conducted in order to evaluate the highest concentration of P(3HB-*co*-3HV) and the respective content of 3HV that could be achieved. Figure 7.2 presents the consumption of FAN, IP, glycerol and levulinic acid consumption as well as the production of TDW, P(3HB-*co*-3HV), 3HB and 3HV during a fed-batch fermentation carried out with an initial FAN concentration of 511 mg/L, an initial glycerol concentration of 27.3 g/L and levulinic acid addition every 3 h aiming to achieve a concentration of 1 g/L in each supplementation. It is easily observed that IP was consumed in less than 20 h, while FAN consumption ceased at 70 h. Levulinic acid periodic supplementation was initiated at 27 h. The maximum 3HV concentration achieved was 4.7 g/L at 60 h corresponding to a content of 27 % in the copolymer. Thereafter, the 3HV molar ratio reached a plateau and slightly decreased towards the end of the fermentation. Increased 3HV content at the beginning of the accumulation phase was also observed for *R. eutropha* KHB-8862 at 9 h of fermentation (Chung et al., 2001). Contrary to 3HV, 3HB production continued until 97 h reaching a concentration of 17 g/L. The P(3HB-*co*-3HV) concentration achieved at 78 h was 20.4 g/L that contained a 3HV content of 21.6 mol%.

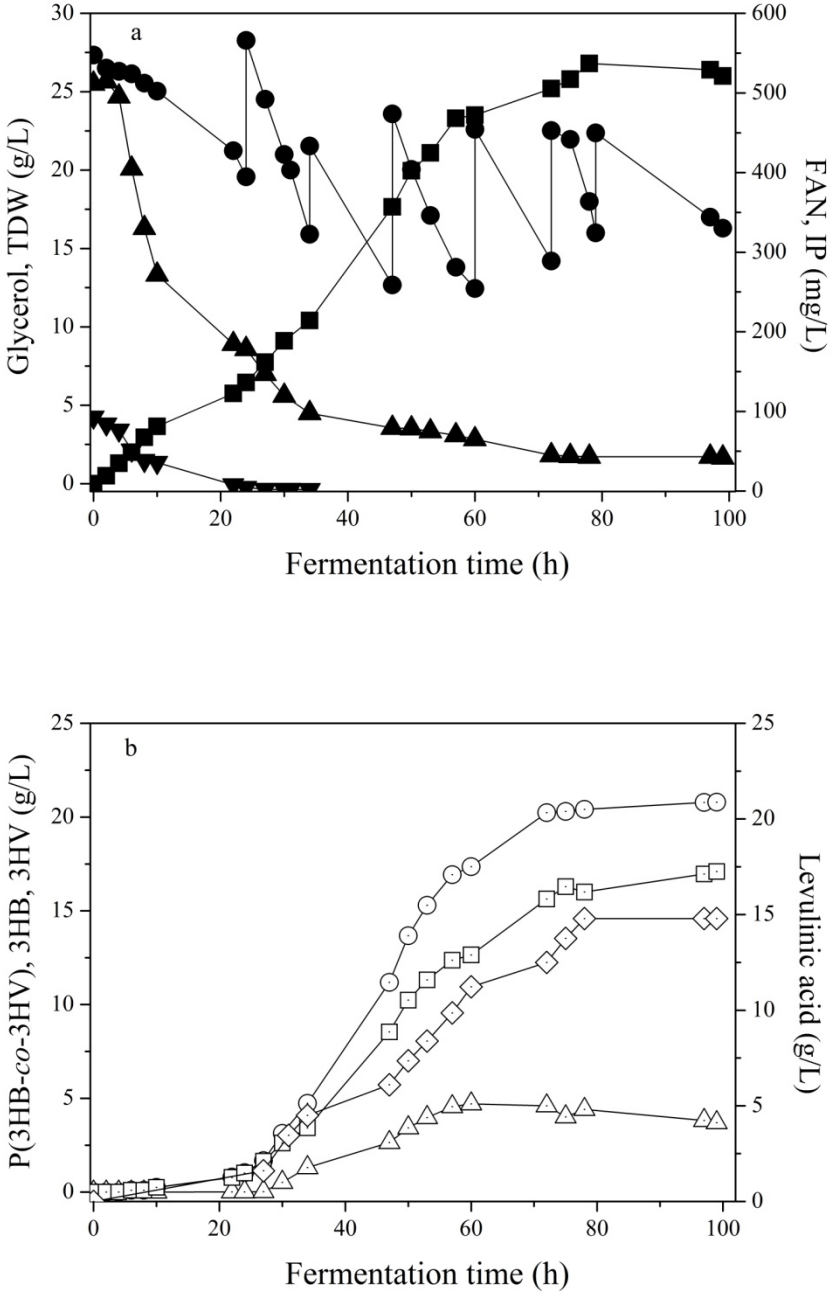

Figure 7.2 Consumption of (▲) FAN, (●) glycerol and (▼) IP as well as production of (■) TDW, (○) P(3HB-*co*-3HV), (△) 3HV, (□) 3HB and (◊) LA during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with feeding of crude glycerol and LA

The second fed-batch experiment was performed with higher initial FAN (797 mg/L) and IP (108 mg/L) concentrations, while initial glycerol concentration was maintained at approximately the same concentration (25.9 g/L). Levulinic acid

supplementation was carried out in higher quantities and shorter intervals to achieve a concentration of 1.5 g/L after each addition. As in the previous fermentation (Figure 7.2), P(3HB-*co*-3HV) accumulation was initiated after 20 h fermentation when IP was depleted. The maximum 3HV production was observed at 53 h corresponding to a 3HV content of 31 mol% (5.4 g/L). At this particular time, the total levulinic acid concentration consumed was 10.6 g/L compared to 7 g/L in the previous experiment (Figure 7.2), proving that more frequent additions of levulinic acid enhanced both P(3HB-*co*-3HV) and 3HV production. Although 3HV production ceased at 53 h, 3HB production continued up to 97 h reaching a final concentration of 18.1 g/L. The final P(3HB-*co*-3HV) concentration achieved was 23.4 g/L with a 3HV content of 22.5 mol%. The intracellular biopolymer content was around 66.4% (w/w).

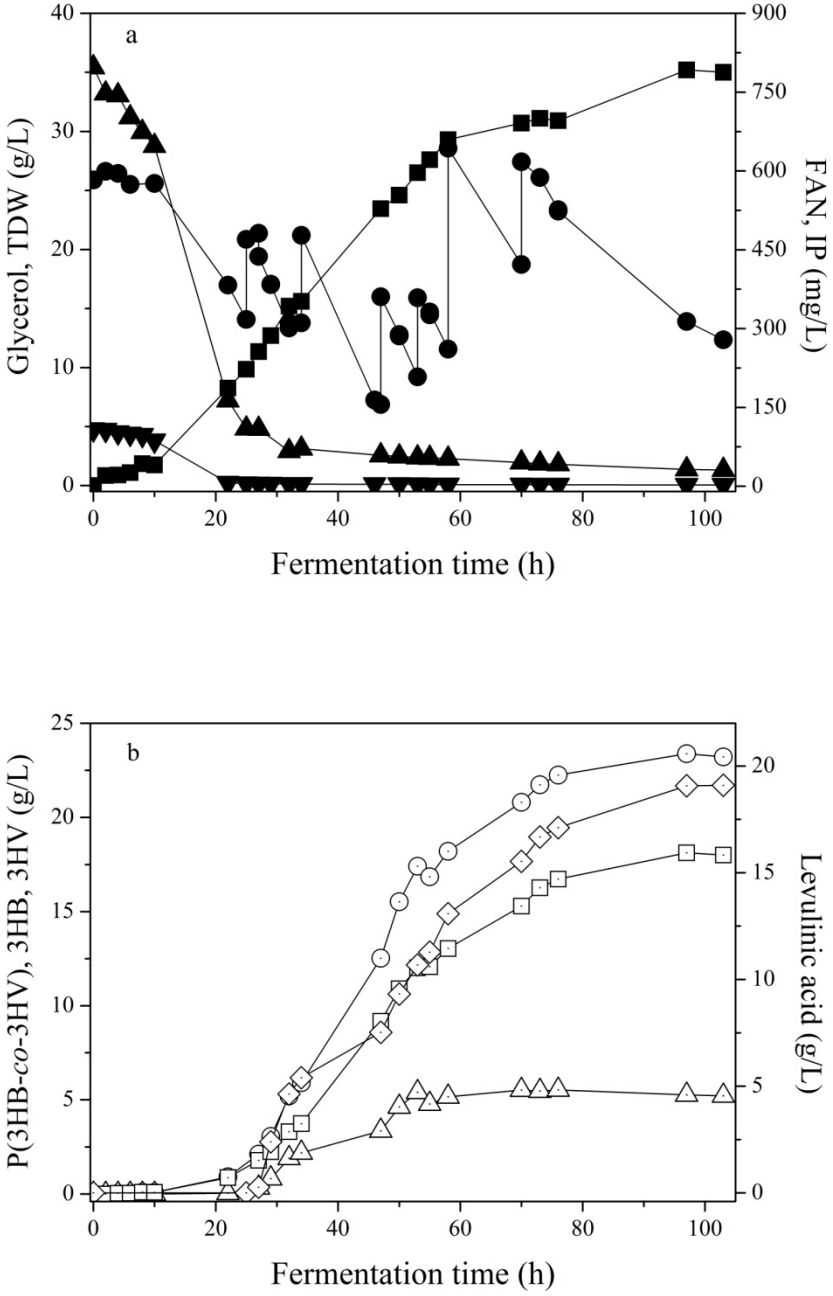

Figure 7.3 Consumption of (▲) FAN, (●) glycerol and (▼) IP as well as production of (■) TDW, (○)P(3HB-*co*-3HV), (△) 3HV, (□) 3HB and (◊) LA during fed-batch bioreactor fermentation of *C. necator* on sunflower meal hydrolysate with feeding of crude glycerol and LA

Jaremko and Yu (2011) reported that changing the carbon to nitrogen ratio may modify not only the P(3HB-*co*-3HV) concentration and intracellular content, but also the composition of 3HB and 3HV. Therefore, the increased concentration of P(3HB-*co*-3HV)

and 3HV content in the second fermentation could be attributed to both a higher levulinic acid supplementation rate and a different C/FAN ratio. Furthermore, Jaremko and Yu (2011) reported that levulinic acid consumption by *C. necator* occurred almost exclusively in the presence of glucose and fructose. However, contrary to this study, the consumption rate of glucose and fructose was significantly reduced in the presence of levulinic acid (Jaremko and Yu, 2011). This was attributed to the fact that the catabolism of levulinic acid is facilitated due to its chemical structure. The glucose and fructose are catabolised through the glycolysis pathway to form acetyl-CoA. In this study, glycerol consumption coincided with levulinic acid consumption as reported in various literature-cited publications conducted with various carbon sources evaluating levulinic acid as precursor for P(3HB-*co*-3HV) production (Chung et al., 2001; Jang and Rogers, 1996; Keenan et al., 2006).

Levulinic acid utilisation as precursor for 3HV production leads to better levulinic acid to 3HV conversion yields than propionic acid (Jang and Rogers, 1996). The combined consumption of sugars and levulinic acid leads also to improved microbial growth and PHA production than the case that only sugars (e.g. glucose, fructose, xylose) are used as carbon sources (Jang and Rogers, 1996; Keenan et al., 2006). Chung et al. (2001) reported that levulinic acid addition during shake flask fermentations of *Ralstonia eutropha* KHB-8862 using fructose as carbon source led to a maximum 3HV content of 75 mol% by regulating the concentration of levulinic acid added. Repeated additions of levulinic acid in bioreactor cultivations enhanced P(3HB-*co*-3HV) content to 85 % of TDW. However, in this study a lower maximum 3HV content (up to 31 mol%) was achieved. Table 7.2 presents selected references for copolymer production employing *Cupriavidus necator* strains, previously classified as *Ralstonia eutropha*. The intracellular P(3HB-*co*-3HV) content (up to 78%, w/w) and concentration (up to 23.4 g/L) achieved in this study with levulinic acid addition (Figure 7.2) was among the highest values reported in the literature when crude glycerol or various sugars were used as the main carbon sources. Wang et al. (2013) reported the production of 15.53 g/L TDW and 12.61 g/L P(3HB-*co*-3HV) (53.9 mol% 3HV) during bioreactor cultures of *R. eutropha* H16 employing glucose and levulinic acid as substrates.

Table 7.2 Production of PHAs from various *Cupriavidus necator* strains (previously classified as *Ralstonia eutropha*) cultivated on various carbon sources and pre-cursors for the production of 3HV

Fermentation media	Strain	PHA type	TDW (g/L)	PHA concentration (g/L)	PHA content (%)	Productivity (g/L/h)	Reference
Crude glycerol and γ-butyrolactone [a]	*C. necator* DSM 545	P(3HB-*co*-4HB)	30.19	10.9	36.1	0.17	Cavalheiro et al., 2012
Crude glycerol, γ-butyrolactone and propionic acid [a]	*C. necator* DSM 545	P(3HB-*co*-4HB-*co*-3HV)	45.25	16.7	36.9	0.25	
Crude glycerol (81%) and rapeseed meal hydrolysates [b]	*C. necator* DSM 545	P(3HB-*co*-3HV)	19.6	10.9	55.6	0.12	Garcia et al., 2013
Crude glycerol (92.4%) and sunflower hydrolysates [b]	*C. necator* DSM 545	P(3HB-*co*-3HV)	19.8	9.9	50	0.09	Kachrimanidou et al., 2013
Crude glycerol (92.4%), SFM hydrolysate and LA	*C. necator* DSM 7237	P(3HB-*co*-3HV)	26.8	20.4	76.2	0.26	Kachrimanidou et al. 2014
		P(3HB-*co*-3HV)	35.2	23.4	66.4	0.24	
LA (1 g/L) and sodium propionate (2.5 g/L)	*C. necator* DSM 545	P(3HB-*co*-3HV)	1	0.3	30	0.0039	Berezina et al. 2016
Glucose, fructose and LA	*C. necator*	P(3HB-*co*-3HV)	3.5	2.4	69	0.05	Jaremko et al. 2011
Glucose and LA	*Ralstonia eutropha* H16	P(3HB-*co*-3HV)	15.53	12.61	81.2	0.23	Wang et al. 2013

Ashby et al. (2012) reported TDW and P(3HB-*co*-3HV) concentrations in the range of 2.3 – 2.7 g/L and 1.1 – 1.2 g/L, respectively, produced by *Pseudomonas oleovorans* NRRL B-14682 in bioreactor fermentations carried out with different ratios of glycerol to levulinic acid. Although the concentrations achieved are significantly lower than this study, a wide range of 3HB to 3HV ratios (100% 3HB to 100% 3HV) can be achieved with the strain *P. oleovorans* (Ashby et al., 2012). In an effort to regulate the 3HV content produced in P(3HB-*co*-3HV) by *C. necator*, enhanced 3HV content (80 %) was obtained by Berezina et al. (2016) using combined feeding of levulinic acid and sodium propionate, yet rather low TDW production (1 g/L) was reported. However, it may not be necessary to reach a 3HV content higher than 30 mol% since the properties of P(3HB-*co*-3HV) copolymers containing 20 – 30 mol% 3HV may be sufficient for many applications as the inclusion of 3HV molar units in the polymer chain entails a decrease in melting temperatures thus facilitating thermal processing (Ashby et al., 2012; Wang et al., 2013)

7.4 Characterization of the PHB and P(3HB-co-3HV) produced in this study

The structure of the produced PHB was confirmed using ^{1}H and ^{13}C NMR (Figures 7.4a and 7.4b). Typical $_{1}H$ NMR shifts of methyl ($-CH_3-$), methylene ($-CH_2-$) and methine group (-CH-) were observed at 1.24, 2.48 and 5.24 ppm respectively (Figure 7.4a). The produced PHB had a satisfactory solubility in $CDCl_3$ and it was found possible to record a ^{13}C NMR spectrum illustrating shifts at 19.9 for methyl carbon, 41 methylene carbon, 67.8 methine carbon, 169.3 carbonyl carbon (Figures 7.4b). The structure of the produced P(3HB-*co*-3HV) was also confirmed using ^{1}H and ^{13}C NMR (Figure 7.5a and 7.5b). From the ratio of the CH_3 (HB) at 1.26 ppm and the CH_3 (HV) at 0.89 ppm, as well as the CH signals at 5.13-5.27 ppm, one can determine an HV content in the copolymer of 19.8 – 21.2 %. The P(3HB-*co*-3HV) samples analyzed by ^{1}H and ^{13}C NMR were taken at the end of the fermentation presented in Figure 7.2. The 3HV contents analyzed by NMR and GC-FID are in close agreement.

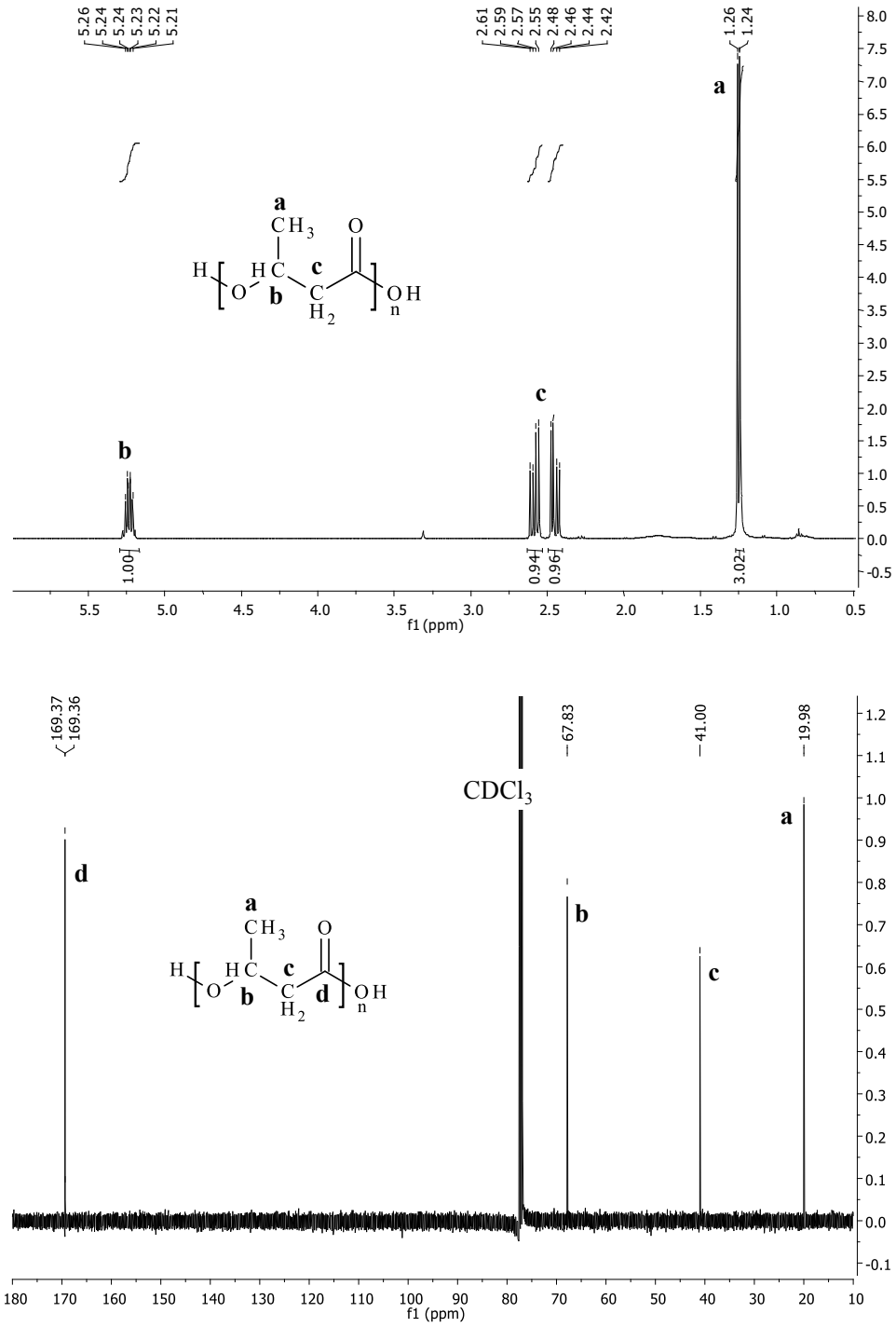

Figure 7.4: ^{1}H NMR spectrum (a) and ^{13}C NMR spectrum (b) of PHB produced from sunflower meal hydrolysates and crude glycerol

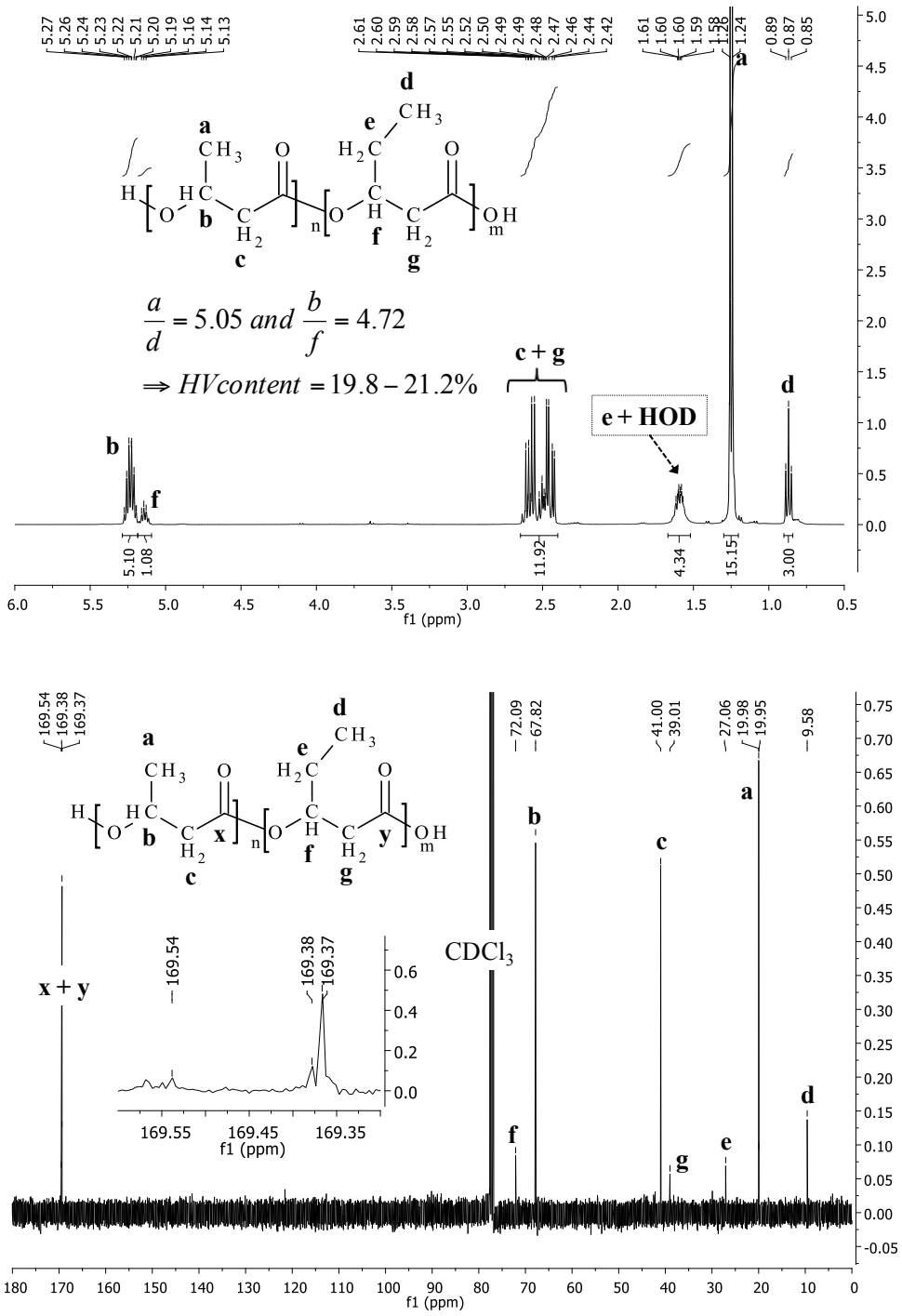

Figure 7.5: ^{1}H NMR spectrum (a) and ^{13}C NMR spectrum (b) of produced P(3HB-*co*-3HV) from sunflower meal hydrolysates, crude glycerol and levulinic acid

Besides the resonances obtained from the solvent, no other chemical shifts were observed implying that only PHB and P(3HB-*co*-3HV) were synthesized by *C. necator* in the presence of LA and crude glycerol.

TGA was carried out to evaluate the thermal stability of the produced PHAs. TGA curves for PHB and P(3HB-*co*-3HV) isolated from bacterial fermentations carried out in the bioreactor are presented in Figure 7.6. The onset temperature of degradation was approximately 180 °C and 200 °C for PHB and P(3HB-*co*-3HV), respectively. More than 90 % of the weight loss is observed in one decomposition stage and specifically between 240 and 280 °C, values that agree with literature cited reports (Pan et al., 2012; Wang et al., 2013). Moreover, the degradation curve corresponding to the copolymer is slightly shifted, as expected, indicating the higher molar mass considering the 3HV molar units. Elevated degradation temperatures with increasing 3HV molar contents were also reported during TGA analyses of P(3HB-*co*-3HV) produced by various bacterial strains (Zakaria et al., 2010; Reddy et al., 2016).

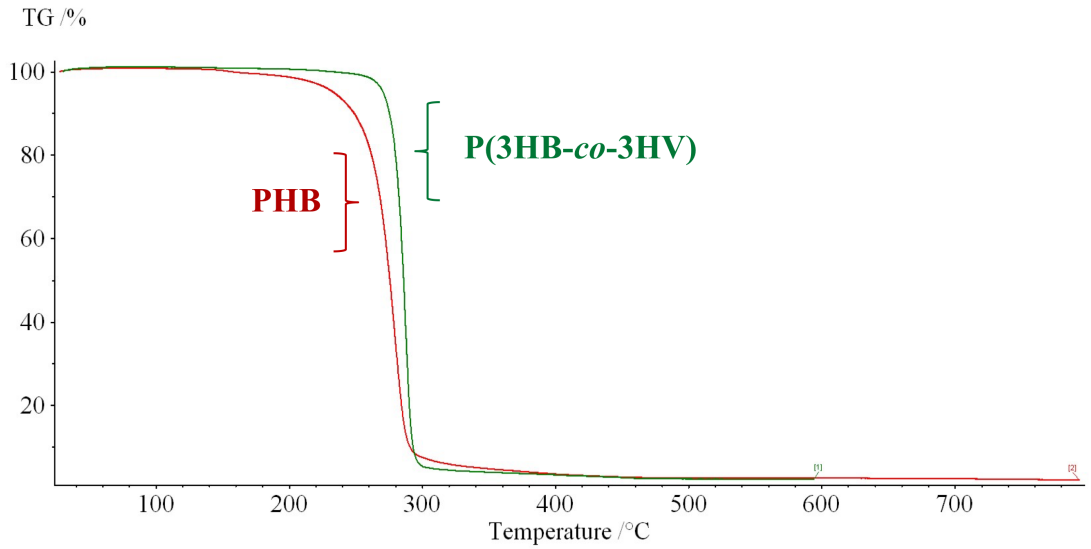

Figure 7.6: Thermogravimetric analysis curve for PHB and P(3HB-*co*-3HV) produced from sunflower meal hydrolysates and crude glycerol

7.5 Conclusion

The results presented in this chapter demonstrated the fermentative production of the copolymer P(3HB-*co*-3HV) when SFM hydrolysates, crude glycerol and levulinic acid were used. Addition of levulinic acid as a precursor led to the production of high P(3HB-*co*-3HV) concentrations with a significant final 3HV content (around 22.5 %). The production of P(3HB-*co*-3HV) could be included in a sunflower based biorefinery. The levulinic acid could be produced from various lignocellulosic feedstocks incorporated in the proposed biorefinery concept, thus providing a cheap substrate for enhanced P(3HB-*co*-3HV) synthesis.

The results presented in Chapter 7 have been published in Bioresource Technology journal:

Kachrimanidou, V., Kopsahelis, N., Papanikolaou, S., Kookos, I., De Bruyn, A., Clark, J.H., Koutinas, A.A. Sunflower-based biorefinery: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production from crude glycerol, sunflower meal and levulinic acid. Bioresource Technol. (2014) 172:121-130.

CHAPTER 8

DEVELOPMENT OF AN INTEGRATED SUNFLOWER-BASED BIOREFINERY CONCEPT FOR THE PRODUCTION OF ANTIOXIDANTS, PROTEIN ISOLATE AND POLY(3-HYDROXYBUTYRATE)

8.1 Introduction

In the previous chapters the capability to implement crude glycerol and sunflower meal as the sole raw materials for the production of nutrient-rich fermentation media suitable for the production of polyhydroxyalkanoates (PHA) was demonstrated. A poly(3-hydroxybutyrate) (PHB) concentration of more than 27 g/L with an approximate content of 72.9% (w/w) was achieved in fed-batch cultures, whereas continuous supplementation of levulinic acid led to the production of up to 23.4 g/L of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), with an intracellular content of 66.4% (w/w) and a 3HV content of 22.5 mol%. Nevertheless, the development of integrated biorefineries could only be sustainable when diversified end-products are generated deriving from the exploitation of all residual streams. The following chapter demonstrates that SFM fractionation constitutes a feasible refining concept leading to the production of an antioxidant-rich stream, protein isolate and nutrient-complete fermentation media for PHB production (Figure 8.1).

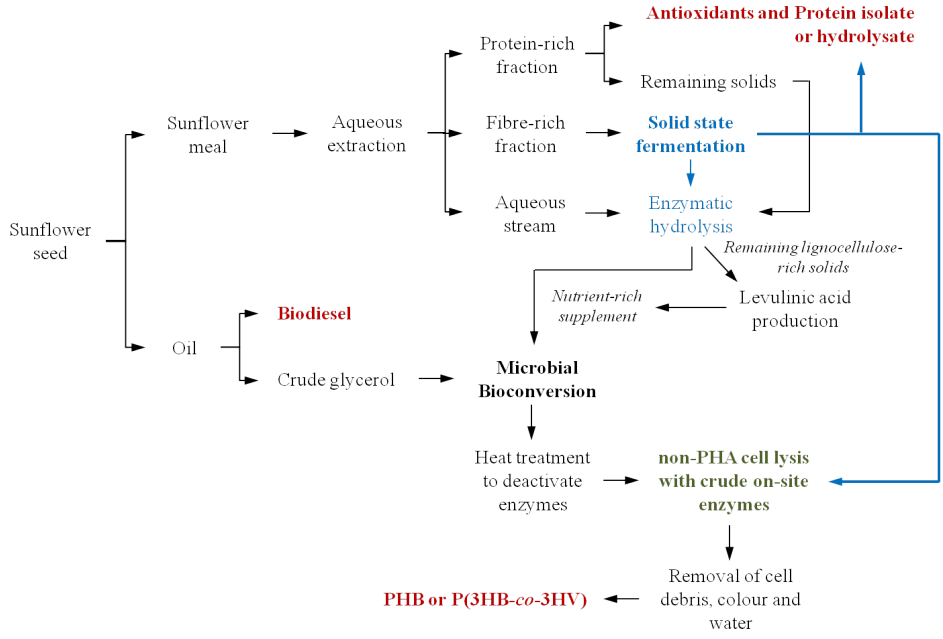

Figure 8.1 Sunflower-based biorefinery concept: Utilization of SFM for the production of antioxidants, protein isolate and PHB or P(3HB-*co*-3HV)

Fermentation media were produced from remaining fractions that resulted during SFM fractionation (Figure 8.2). This approach is based on the principle of optimised resource utilisation preventing the use of commercial nutrient supplements.

8.2 Fractionation of sunflower meal (SFM)

Fractionation of SFM was performed following a simple sedimentation-flotation method as described by Villanueva et al. (1999). Figure 8.2 presents the mass balances and the protein content of each fraction achieved during SFM fractionation. The lignocellulose-rich fraction (LF) constituted around 31% of the whole SFM dry mass and contained almost 20% of the initial protein, while the protein-rich fraction (PF) constituted around 50% of the SFM mass (db) with a 69.5% of the initial protein content. The soluble fraction contained soluble proteins (10.5% of the initial protein content). After the extraction of phenolic compounds, the protein-rich fraction was sequentially treated under alkaline and acidic conditions to produce the protein isolate (PI) that constituted 34.6% of the initial protein contained in SFM.

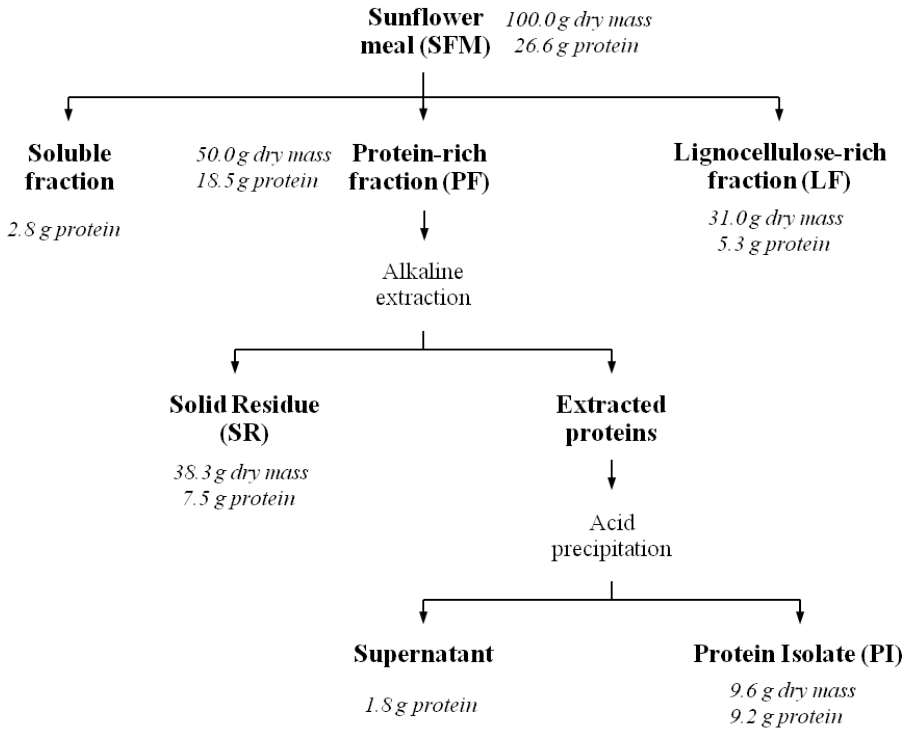

Figure 8.2 SFM fractionation protocol followed for the production of protein isolate (adapted from Villaneuva et al. 1999)

The co-product streams produced during protein isolate separation were the solid residue (SR) with a significant protein content of 28.2% of the initial protein content and the supernatant. The purity of protein isolate was 95.8%. The protein isolate could be used in the food industry or in novel applications including biopolymers, amino acid isolation and edible films (Ordonez et al., 2008; Rodrigues et al., 2012; Salgado et al., 2012). More specifically, protein isolates or modified protein isolates have been evaluated as adhesives for wood-based panel applications (Liu and Li, 2007; Nordqvist et al, 2013). Protein hydrolysis constitutes a method to enhance water resistance and reactivity, enhancing the mechanical properties of wood adhesives (Gao et al., 2015). Unfolding of the protein results in the release of more reactive side-chain groups that interact and form covalent bonds.

On the other hand the high purity of sunflower protein isolate could bestow its implementation as food additive in human nutrition, proposing an alternative to animal proteins. Protein addition in food entails acceptable functional properties including emulsifying activity and stability, whippability, gelation and foaming properties (Moure et

al., 2006; Lacou et al., 2016). However, as stated by Rodrigues et al. (2012), modifications of “native proteins” by physical, chemical and enzymatic treatments are a prerequisite to enhance the functional properties and meet the food manufacture and consumers’ demands. Enzymatic hydrolysis constitutes an enzymatic modification method that results in the breakdown of peptidic bonds to yield peptides and amino acids, that also enhance the nutritional value. For instance, rapeseed protein isolates enzymatically treated with alcalase demonstrated improved functional properties (Vioque et al., 2000). Soybean, sesame and peanut flours were employed in the production of protein hydrolysate by enzymatic hydrolysis, presenting improved functional properties (Radha et al., 2007).

An enzymatic hydrolysis step was implemented in order to evaluate the maximum hydrolysis yield. Figure 8.3 presents the profile change of hydrolysis degree obtained with an initial concentration of 15 g/L of protein isolate employing crude enzymatic extracts produced via solid state fermentation carried out with *Aspergillus oryzae*. Different initial proteolytic activities were implemented targeting to maximize the degree of hydrolysis that reached 55% after 48 h of reaction. The degree of hydrolysis was controlled through determination of free amino nitrogen (FAN) during hydrolysis. The FAN concentration was compared with the Total Kjeldahl Nitrogen (TKN) of the protein isolate used at the beginning of hydrolysis. Therefore, the degree of hydrolysis is expressed as the ratio of FAN to TKN (Figure 8.3).

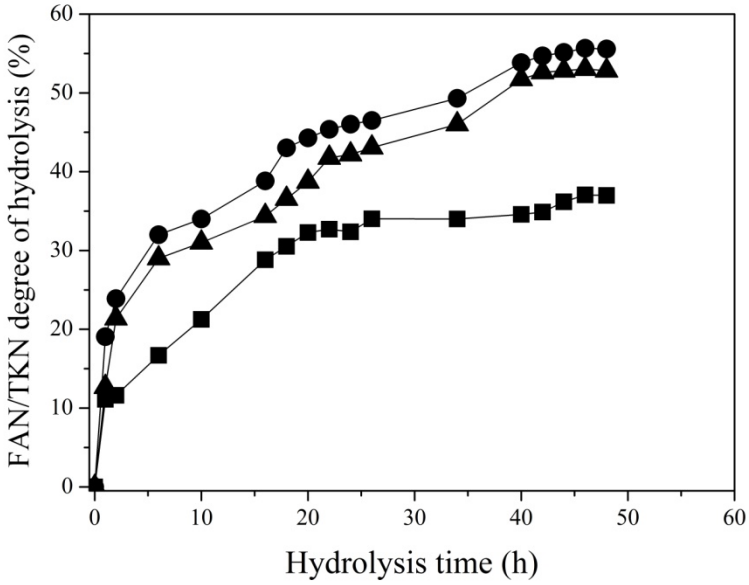

Figure 8.3 Degree of hydrolysis expressed as increasing FAN to TKN ratio during hydrolysis of protein isolate utilizing different initial proteolytic activities of crude enzyme consortia produced via SSF carried out with *A. oryzae*. (■) 6 U/mL, (▲) 24 U/mL, (●) 30 U/mL

8.3 Evaluation of antioxidant activity

Methanol/water or ethanol/water mixtures result in optimum phenolic extraction yields, low protein losses and low protein denaturation when phenolic compounds are extracted from sunflower (González-Pérez and Vereijken, 2007). Based on the fractionation process of SFM (Figure 8.1), the extracts from four different fractions (SFM, PF, LF and SR) were evaluated with regard to the total extracted mass and the total phenolic content (TPC), which was expressed as chlorogenic acid equivalents (CGAE). As presented in Figure 8.4, the extract from SFM showed the highest TPC (444.6 mg CGAE per 100 g, db) followed by the extracts obtained from the protein fraction (380 mg CGAE per 100 g, db), the lignocelullose-rich fraction (80.2 mg CGAE per 100 g, db) and the solid residue fraction (37.7 mg CGAE per 100 g, db).

Depending on the extraction method and the variety of sunflower, TPC values vary significantly. Szydłowska-Czernial et al. (2011) studied the variations in TPC of sunflower shell extracts depending on the extraction method and the obtained values ranged from 58.2 to 341.2 mg CGAE per 100 g (db). The phenolic content of SFM is higher than

respective values reported for well-known plant methanolic extracts, such as *Salvia officinalis*, *Origanum vulgare*, *Rosmarinus officinalis* and *Achillea millefolium* (Wojdyło et al., 2007).

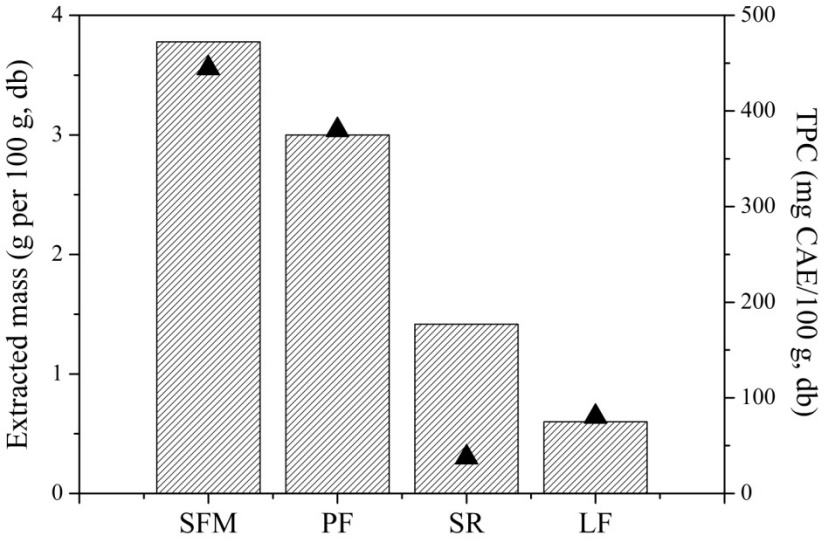

Figure 8.4 Total Phenolic Content (TPC) measured by the Folin-Ciocalteu method and total quantity of material extracted from various streams including sunflower meal (SFM), protein-rich fraction (PF), lignocellulose-rich fraction (LF) and solid residue (SR)

The lowest IC_{50} value, corresponding to the highest antioxidant activity, was measured in the protein-rich fraction (0.035 mg/mL) followed by the SFM (0.05 mg/mL) (Figure 8.5). In the case of the lignocellulose-rich fraction (0.15 mg/mL) and the solid residue fractions (0.15 mg/mL), the IC_{50} values were significantly higher. The IC_{50} value of the protein-rich fraction was equal to those of *R. officinalis* and *S. fruticosa* methanolic fractions (Papageorgiou et al., 2008) and eleven times higher than that of oregano essential oil's phenolic fraction (Kulisic et al., 2004). For comparison purposes, 0.0041 mg/mL of ascorbic acid and 0.0145 mg/mL of butylated hydroxytoluene (BHT) were able to scavenge 50% of DPPH. These results indicate that the SFM extracts have a potential use as antioxidants.

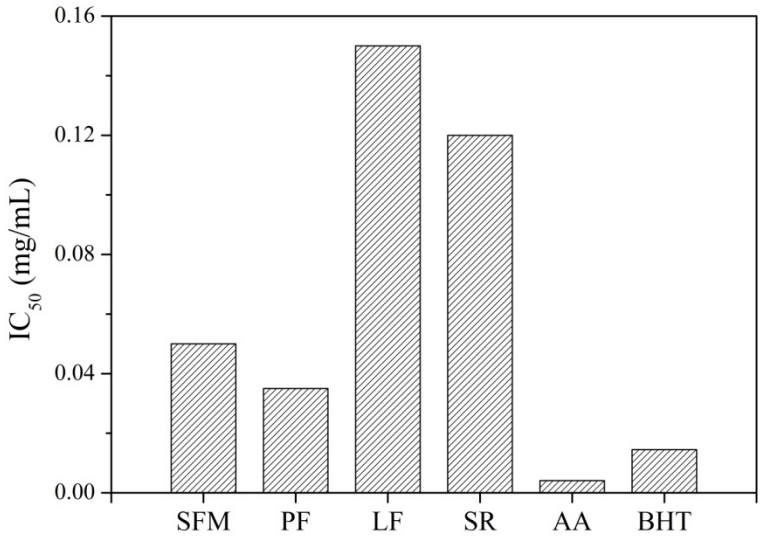

Figure 8.5 IC_{50} values of SFM, PF, LF and SR compared to the standard antioxidants ascorbic acid (AA) and BHT

In a biorefinery concept, the extraction of phenolic compounds should be carried out from the SFM stream in order to increase the quantity of extracted antioxidants and the applications of protein-rich fraction. Furthermore, phenolics and chlorogenic acid in particular should be removed because their presence cause reduction in protein solubility, reduced nutritional value of protein isolate, problems in storage life, stability and organoleptic properties, and dark coloration of sunflower protein products (González-Pérez and Vereijken, 2007; Weisz et al., 2009). Furthermore, chlorogenic acid constitutes more than 50% of the total phenolic compounds, which means that it could be easier to produce an extract with a high chlorogenic acid content. Phenolic extracts from natural resources with 50% chlorogenic acid content are commercialized products with a market price higher than 40 $/kg (source: www.alibaba.com).

Purification of the antioxidant-rich extract facilitates commercialization. Gourdin et al. (2006) presented an efficient method for purification of phenolic compounds based on treatment with ion-exchange resins with recovery yields up to 90%. Weisz et al. (2013) produced an extract rich in polyphenolic compounds from a protein rich sunflower fraction using ion-exchange resins that exhibited strong antioxidant activity. Supercritical fluid extraction and pressurized liquid extraction are promising methods due to conservation of

the biological properties of phenolic compounds, utilization of green solvents, selectivity and low processing time (Fernández-Ponce et al., 2013).

8.4 Solid state fermentation and enzymatic hydrolysis

After the extraction of phenolic compounds and protein isolate, the remaining fractions were evaluated for the production of nutrient supplements for fermentative production of PHB. All residual fractions (especially the solid residue and the lignocellulose-rich fraction) contain significant quantities of protein. The lignocellulose-rich fraction was the only fraction that could be used as substrate for SSF for the production of crude enzyme consortia. These enzymes were subsequently evaluated for hydrolysis of macromolecules (mainly protein) contained in lignocellulose-rich fraction or other fractions. These hydrolysates could be subsequently used as nutrient-rich supplements in fermentation processes that could substitute for yeast extract and inorganic chemicals.

Figure 8.6 presents the profile change of protease activity during SSF of *A. oryzae* cultivated on LF as the sole solid substrate. The protease activity increased up to 57 h when the highest activity is obtained (165 U/g) followed by a decreasing trend. The highest protease activity achieved in this study using lignocellulose-rich fraction as substrate (Figure 8.6) was lower than the respective highest activity (400 U/g at 48 h) produced when whole sunflower meal was used as substrate (Kachrimanidou et al., 2013; Chapter 5). Nevertheless, the protease activity produced was sufficient in order to achieve efficient hydrolysis of protein in the remaining fractions (Figures 8.7 and 8.8). Protein and phytic acid hydrolysis resulted in the production of FAN and IP. Besides hydrolysis of residual stream components, nutrients are also produced due to fungal autolysis initiated by oxygen depletion.

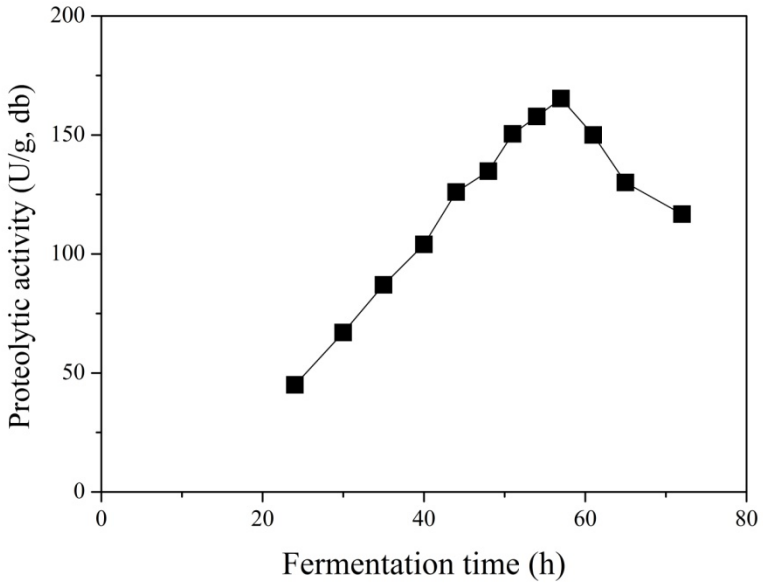

Figure 8.6 Proteolytic activity produced during SSF with *A. oryzae* carried out on lignocellulose-rich fraction

Figure 8.7 presents the profile change of FAN and IP when two different initial concentrations of LF (50 and 100 g/L) were used. These concentrations include both the lignocellulose-rich fraction used in SSF and the unprocessed lignocellulose-rich fraction added prior to initiation of hydrolysis. The concentrations of FAN and IP at the beginning of hydrolysis were subtracted from each experimental result. Both FAN and IP production reached a plateau after approximately 40 h. The maximum FAN production (505 mg/L) was achieved when an initial concentration of 100 g/L of lignocellulose-rich fraction was employed. IP production follows a similar trend with a maximum concentration of 93.7 mg/L at 46 h. The concentrations of FAN (1.5 g/L) and IP (246 mg/L) obtained via hydrolysis of the lignocellulose-rich fraction are significantly lower compared to those obtained when whole sunflower meal was used as substrate under the same experimental conditions. The hydrolysate produced by using 100 g/L of initial lignocellulose-rich fraction concentration was used as hydrolysate I in subsequent fermentations.

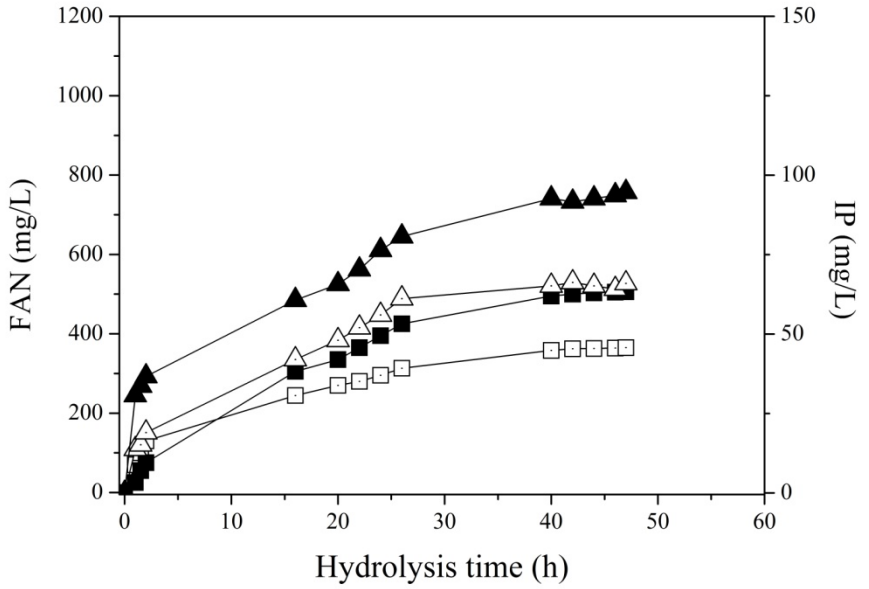

Figure 8.7 FAN and IP production during enzymatic hydrolysis of 50 g/L (□, FAN; △, IP) and 100 g/L (■, FAN; ▲, IP) initial concentration of solids from the lignocellulose-rich fraction

The hydrolysate II was produced using remaining SSF solids mixed with the solid residue fraction that were suspended in the soluble fraction (Figure 8.2). Therefore, tap water and lignocellulose-rich fraction unprocessed solids employed in the case of hydrolysate I production were replaced by the soluble fraction and the solid residue fraction. Figure 8.8 shows the profile change of FAN and IP during hydrolysis for hydrolysate II production using 50 and 100 g/L of initial solid concentrations (based on fermented SSF solids and solid residue). The highest FAN and IP production achieved were approximately 1 g/L and 123 mg/L when the initial solid concentration was 100 g/L. The obtained values are lower than those achieved from whole sunflower meal (Kachrimanidou et al., 2013; Chapter 5) but higher than hydrolysate I (Figure 8.7). In particular, FAN production increased two fold in the case of hydrolysate II. This means that the exploitation of all remaining liquid and solid streams lead to the production of hydrolysates with higher nutrient content.

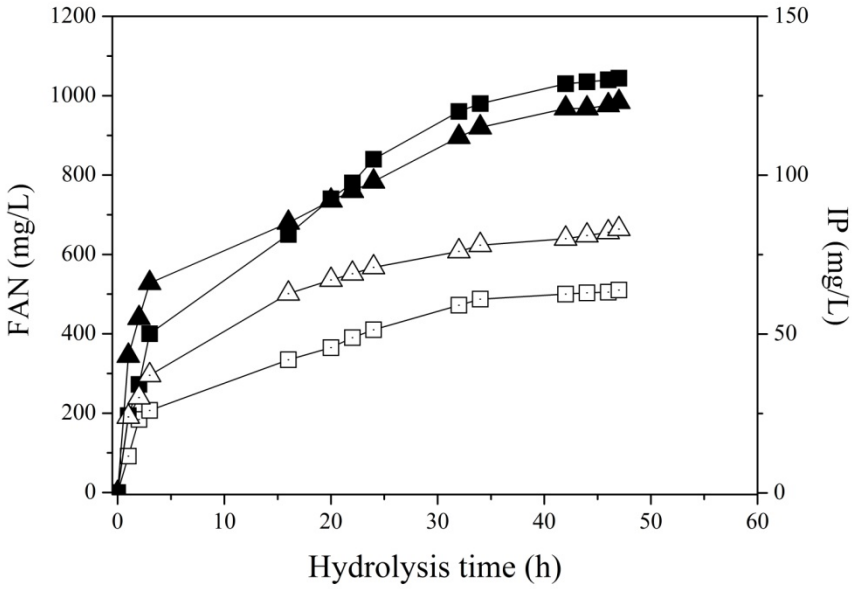

Figure 8.8 FAN and IP production during enzymatic hydrolysis of 50 g/L (□, FAN; ∆, IP) and 100 g/L (■, FAN; ▲, IP) initial concentration of solids using the solid residue fraction and the soluble fraction

8.5 Bacterial bioconversions

In Chapter 6, crude glycerol and whole SFM hydrolysates were successfully employed for the production of up to 27 g/L of PHB. In this chapter, the remaining streams resulting after the extraction of antioxidant-rich fractions and protein isolate have been used for the production of nutrient-rich media for PHB production. In the bio-economy era, it is vital to exploit all nutrients contained in renewable resources including carbon sources, nitrogen sources, phosphorus as well as various minerals and growth factors. SFM contains all necessary nutrients required to produce PHB via fermentation. To prove this concept, three fed-batch bioreactor fermentations were conducted using crude glycerol and three different hydrolysates as nutrient supplements with the strain *C. necator* DSM 7237. The production of hydrolysate I (Figure 8.7) and hydrolysate II (Figure 8.8) was described in section 8.4. Hydrolysate II was diluted with tap water in order to adjust the initial FAN concentration. Hydrolysate III was produced as in the case of hydrolysate II but dilution of this stream was carried out with water employed for washing of remaining hydrolysis solids. The solids that remain after the recovery of hydrolysate II via vacuum filtration contain

significant quantities of nutrients and washing these solids with water leads to the production of a medium rich in nutrients.

In the fermentation that was carried out with hydrolysate I, the initial FAN concentration was 413 mg/L and the initial glycerol concentration was 24.3 g/L. During the first 6 h, PHB production and bacterial growth (Figure 8.9) occurred simultaneously with the consumption of FAN and IP. After complete IP consumption, both bacterial growth and PHB accumulation were terminated. The maximum TDW (7.1 g/L) and PHB concentration (1.2 g/L) were significantly lower than those achieved (up to 37 g/L and 27 g/L, respectively) when whole SFM hydrolysates were used as nutrient supplements (Kachrimanidou et al., 2014; Chapter 6). This means that hydrolysate I is deficient in nutrients and cannot sustain PHB production.

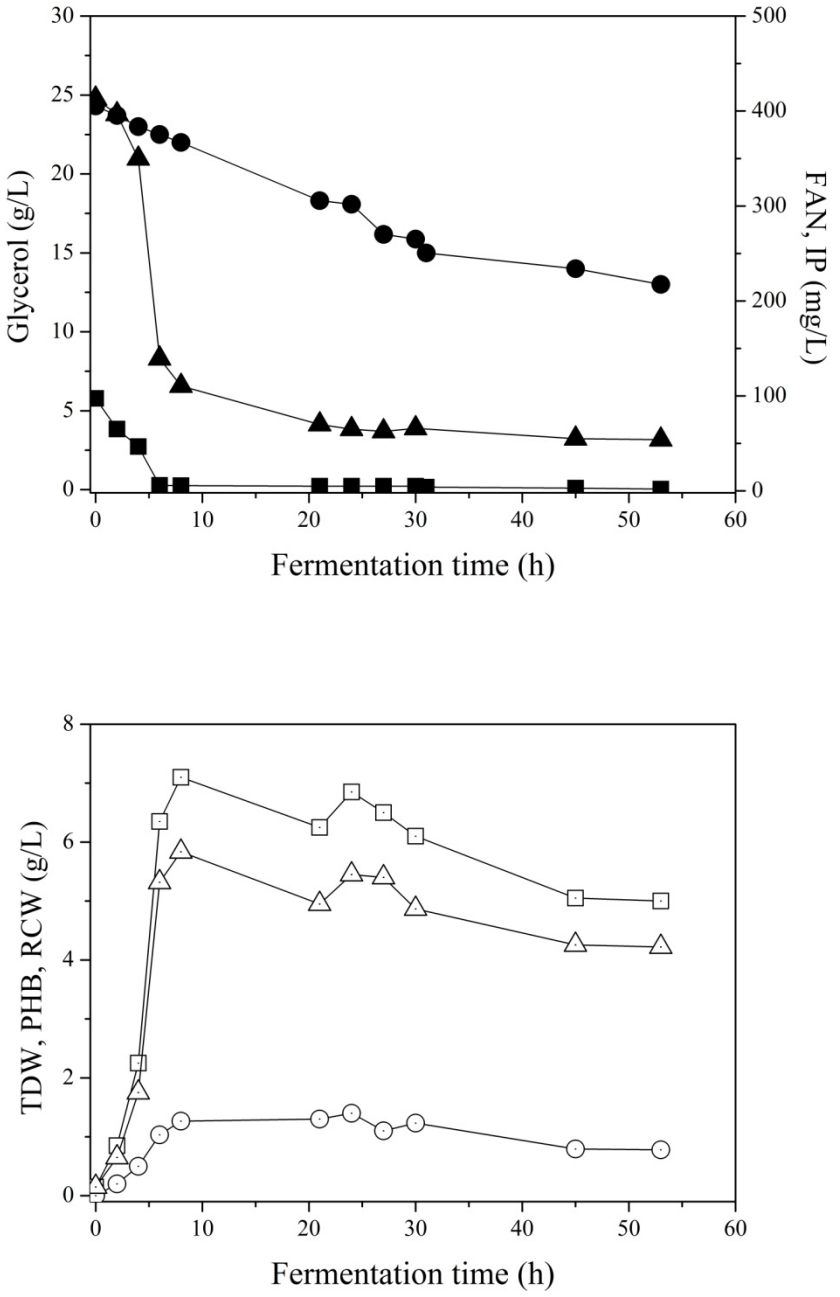

Figure 8.9 Profile change of FAN (▲), glycerol (●), IP (■), TDW (□), PHB (○) and RCW (△) during batch bioreactor fermentation of *C. necator* cultivated on crude glycerol and Hydrolysate I (obtained from the lignocellulose-rich fraction)

The final FAN concentration of hydrolysate II was higher than 1 g/L (Figure 8.8), which is higher than the optimum FAN concentration range (585 - 710 mg/L) identified by Kachrimanidou et al. (2014) for PHB production (see Chapter 6). For this reason, hydrolysate II was diluted with tap water to adjust the FAN concentration at 704 mg/L at the beginning of fermentation. This fermentation (Figure 8.10) resulted in a maximum TDW of 28.5 g/L at 54 h with a PHB content of 66.7 % (w/w). These results indicate that hydrolysate II contains higher nutrient content than hydrolysate I. However, the PHB concentration achieved was lower than the one achieved by whole SFM hydrolysates. This could be attributed to the dilution of hydrolysate II with tap water that reduces the concentration of micronutrients that are necessary to prolong PHB accumulation.

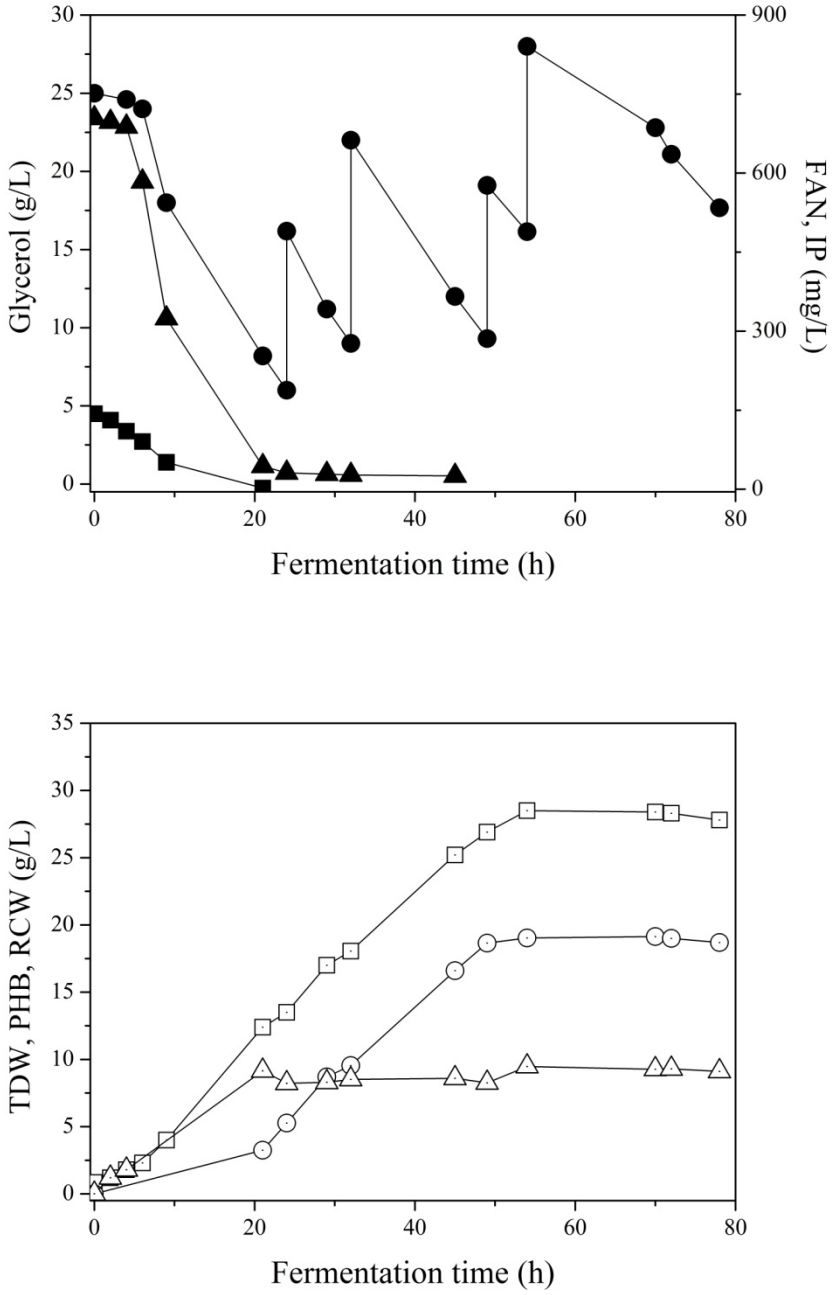

Figure 8.10 Profile change of FAN (▲), glycerol (●), IP (■), TDW (□), PHB (○) and RCW (△) during batch bioreactor fermentation of *C. necator* cultivated on crude glycerol and Hydrolysate II

The fermentation carried out with hydrolysate III (Figure 8.11) was initiated with initial FAN concentration of 739 mg/L. Dilution to adjust the initial FAN concentration was carried out with washing water of remaining hydrolysis solids. Two distinct phases

can be observed. During the first phase, until IP is depleted (approximately 9 h), microbial proliferation takes place in parallel with FAN and IP consumption. The next phase begins after complete IP consumption that initiates PHB formation. Therefore, PHB accumulation occurs due to phosphorus limitation. The glycerol to PHB conversion yield is 0.47 g/g that is significantly higher than other studies ranging from 0.22 g/g to 0.37 g/g (Cavalheiro et al., 2009; Ibrahim and Steinbuchel 2009; Hermann-Krauss et al., 2013; Kachrimanidou et al., 2014). The high yield should be attributed to the consumption of other carbon sources that may have been produced during SFM fractionation. The productivity obtained in the present study was 0.4 g/L/h, while the highest PHB concentration was 57 g/L with an intracellular content of 86.2 % (w/w). These results are among the highest achieved from media based on crude glycerol. Hermann-Krauss et al. (2013) reported an intracellular PHA content of 75.4 % and a volumetric productivity of 0.12 g/L/h. The efficiency of hydrolysate III for PHB production could be attributed to optimum balance of nutrients in the medium. Optimum resource utilisation, process integration and biomass refining leads to efficient separation of value-added products from SFM and PHB production from the remaining streams.

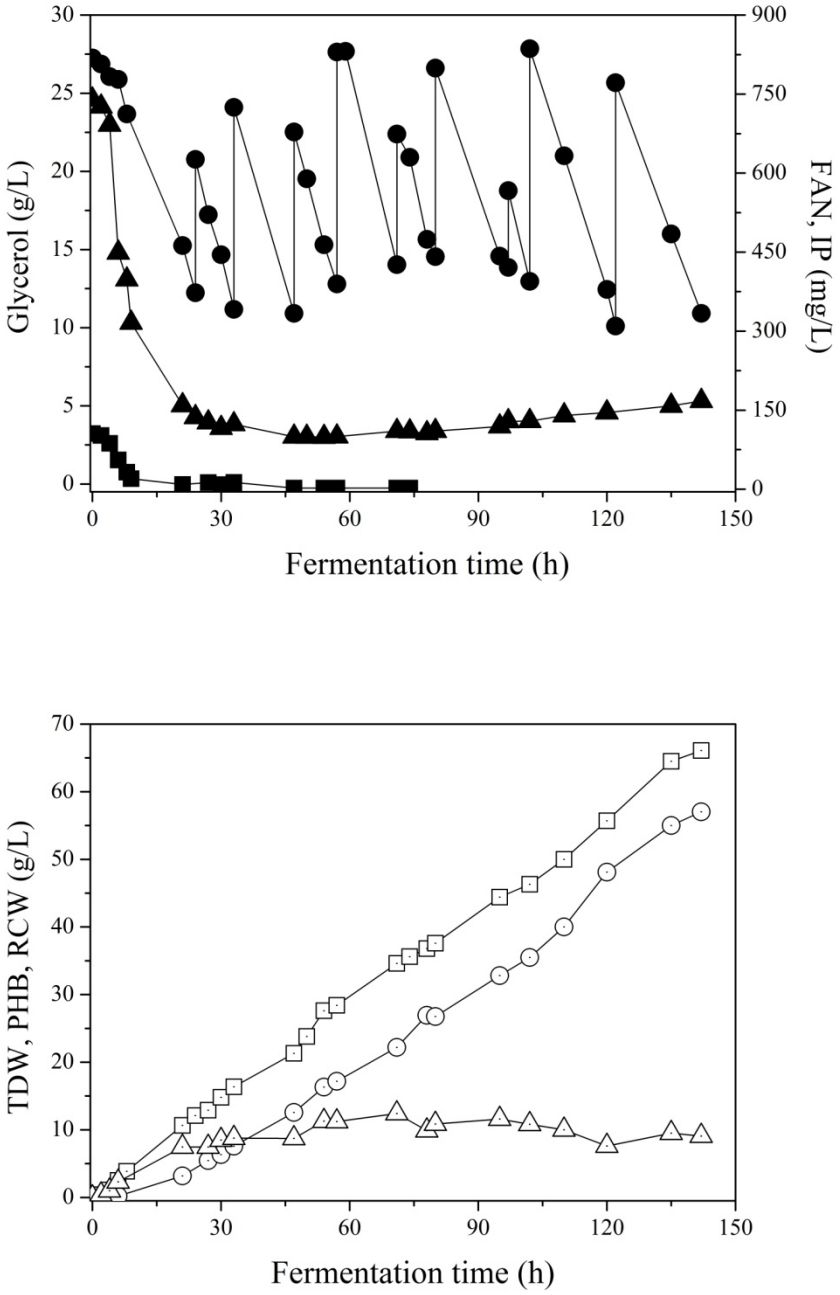

Figure 8.11 Profile change of FAN (▲), glycerol (●), IP (■), TDW (□), PHB (○) and RCW (△) during batch bioreactor fermentation of *C. necator* cultivated on crude glycerol and Hydrolysate III

In this study, the lignocellulosic fraction of SFM has not been exploited, which means that the proposed biorefinery concept could be further expanded. However, the utilisation of C5 and C6 sugars for PHB production will require the use of a microorganism, such as

Burkholderia sacchari (Cesario et al., 2014), that can consume all or at least most of these sugars. It should be stressed that the production of biofuels, biopolymers and chemicals should rely on the utilisation of resources that are not used primarily for food consumption as this leads to significant ethical issues. Furthermore, cellulose could be used for the production of levulinic acid as precursor for the production P(3HB-*co*-3HV) as demonstrated in Chapter 7.

Industrial implementation of PHB is hindered by the high production cost that is attributed mainly to the cost of raw materials. Several studies have focused on the estimation of PHA production costs from different feedstocks (Choi and Lee 1997; van Wegen et al., 1998; Posada et al., 2011). It is nowadays evident that traditional fermentation processes should be restructured and integrated in advanced biomass refining schemes aiming to reduce process economics, improve environmental impact, generate many products for diversified market outlets and create synergies between different industrial sectors. Oilseeds and cereal crops constitute potential feedstocks for the development of cascade bioprocesses that could be integrated in the existing agri-industrial infrastructure (Koutinas et al., 2014a). Feasibility and sustainability will be accomplished only by the utmost exploitation of all residual streams after fractionation targeting the minimisation of waste streams generation. Currently, bio-based products including fuels, chemicals and biopolymers are manufactured in single production chains instead of focusing on the fractionation of the initial raw material combining the production of PHAs with the extraction or production of value-added co-products within a complete biorefinery concept (Cherubini et al., 2010; Koutinas et al., 2014b).

A wheat-based biorefinery concept was developed in the Satake Centre for Grain Process Engineering implementing diversified upstream processes to produce various value-added products through wheat fractionation. Briefly, pearled wheat, containing mainly starch, was utilised to formulate a glucose-based nutrient supplement either by enzymatic hydrolysis by amylolytic enzymes or by fungal autolysis. The feedstock was further employed in the fermentative production of succinic acid, ethanol or PHB (Du et al., 2008; Arifeen et al., 2007; Koutinas et al., 2007b; Xu et al., 2010). Whole wheat flour, pearled wheat flour and wheat milling by-products served as substrates for fungal cultures during SSF or submerged fermentations to generate enzymes for the production of fermentation media (Du et al., 2007; Du et al., 2008). Furthermore, wheat bran deriving from the first fractionation step (pearling) constitutes an end-product with various

applications. Accordingly, it is envisaged that oilseed meals, specifically SFM could be implemented in the development of a complete biorefinery concept. Until now, fractionation of rapeseed meal has been undertaken for the extraction of protein isolate and the production of hydrolysates (Bagger et al., 2004; Pedersen and Gylling, 2001) that could be used as adhesives or additives in the food and feed industry.

The sunflower-based biorefinery concept developed during this study employs simple fractionation steps for the production of protein isolate and antioxidants. All residual streams could be used for the production of generic fermentation media. Bacterial bioconversions were conducted to assess the effectiveness of this supplement in the fermentative production of PHB. Crude glycerol was utilised as carbon source to support PHB synthesis. Therewith, the results obtained during this study, including a high conversion yield of glycerol to PHB (0.47 g/g) in parallel with a significant intracellular PHB content (86%) demonstrated that SFM hydrolysates provided a highly efficient fermentation feedstock. The final PHB concentration achieved is lower than the PHB concentration produced in wheat-derived media (162.8 g/L PHB) reaching a productivity of 0.89 g/L/h (Xu et al. 2010). However, protein isolate and crude phenolic extracts represent two additional value-added products with diversified end uses. As previously stated the remaining solids after enzymatic hydrolysis were not exploited during this study, still they could be valorised to produce levulinic acid as a platform chemical or to generate energy through combustion or pyrolysis. In the following chapter utilisation of crude enzymes produced on-site will be implemented in downstream separation of the recovery and purification of intracellular PHB, thus enhancing the sustainability of the proposed concept.

8.6 Conclusion

This chapter demonstrates the implementation of SFM and crude glycerol to produce antioxidants, protein isolate and PHB. Integration of the proposed processing scheme in a sunflower-based biodiesel plant could lead to the development of a sustainable biorefinery. Future research could focus on the isolation of chlorogenic acid at high purity, further optimisation of PHB or even PHA production, downstream processing and techno-economic evaluation to assess the economic viability of the proposed biorefinery concept.

The results presented in Chapter 8 have been published in the Industrial Crops and Products journal:

Kachrimanidou, V., Kopsahelis, N., Alexandri, M., Strati, A., Gardeli, C., Papanikolaou, S., Komaitis, M., Kookos, I.K., Koutinas, A.A. Integrated sunflower-based biorefinery for the production of antioxidants, protein isolate and poly(3-hydroxybutyrate). Ind. Crop Prod. (2015) 71:106-113.

CHAPTER 9

DOWNSTREAM SEPARATION OF POLY(HYDROXYALKANOATES) USING CRUDE ENZYME CONSORTIA PRODUCED VIA SOLID STATE FERMENTATION INTEGRATED IN A BIOREFINERY CONCEPT

9.1 Introduction

The development of an advanced sunflower-based biorefinery concept was demonstrated leading to the production of antioxidants, protein isolate and nutrient-rich supplements for the production of PHB and P(3HB-*co*-3HV). This process could lead to cost competitive production of PHB and P(3HB-*co*-3HV) by implementing renewable resources as fermentation media. However, to further extend the sustainability of the process, a downstream separation method is proposed that does not involve the utilisation of chemicals.

The aim of this chapter is to demonstrate the ability to recover the PHB and P(3HB-*co*-3HV) produced during fermentation of crude glycerol, SFM hydrolysates and levulinic acid through enzymatic cell lysis using the crude enzyme preparation produced during solid state fermentation by *A. oryzae*. In this way, the enzymes produced via SSF could be employed for downstream separation, besides the production of nutrient-rich hydrolysates for the fermentation stage. A central composite design was applied in order to optimise the temperature and pH during enzymatic cell lysis. After the separation of P(3HB-*co*-3HV), the cell lysate was recycled as nutrient-rich supplement together with crude glycerol for the production of PHB, enhancing the sustainability of the proposed biorefinery.

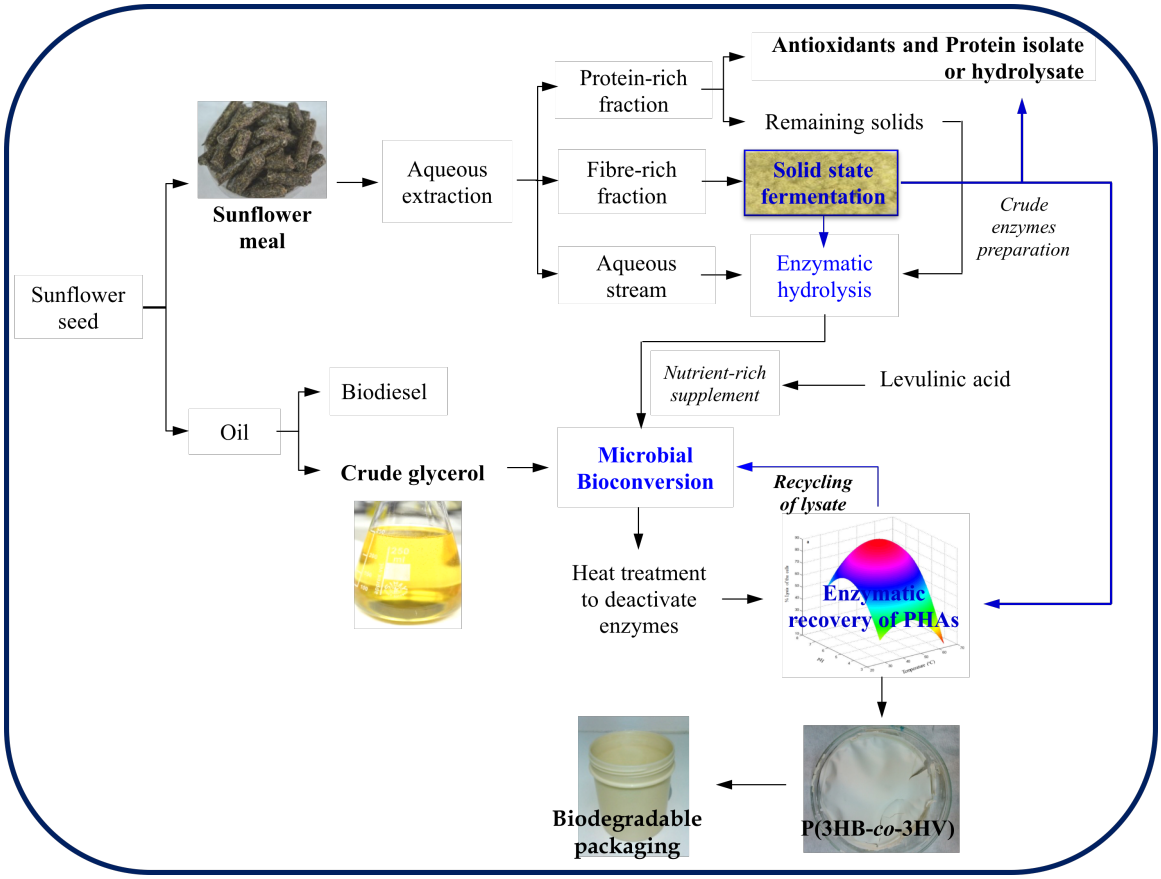

Figure 9.1 Advanced sunflower-based biorefinery including the use of crude enzyme consortia for downstream separation of PHB and P(3HB-*co*-3HV)

9.2 Optimisation of enzymatic cell lysis

The lysis of non-PHA bacterial mass is accomplished via synergistic action of various enzymes produced by *A. oryzae* during SSF. Therefore, identifying the optimum temperature and pH is essential in order to maximise bacterial cell lysis. Results from the Central Composite Design (CCD) are presented in Tables 9.1 and 9.2. Table 9.1 shows the obtained experimental and predicted values together with their corresponding absolute difference. Table 9.2 presents the results in the form of analysis of variance (ANOVA) and the measurement of the b_j coefficients of Eq. 4.3. Moreover, Figure 9.2 shows the graphical representation of model predictions versus experimental points.

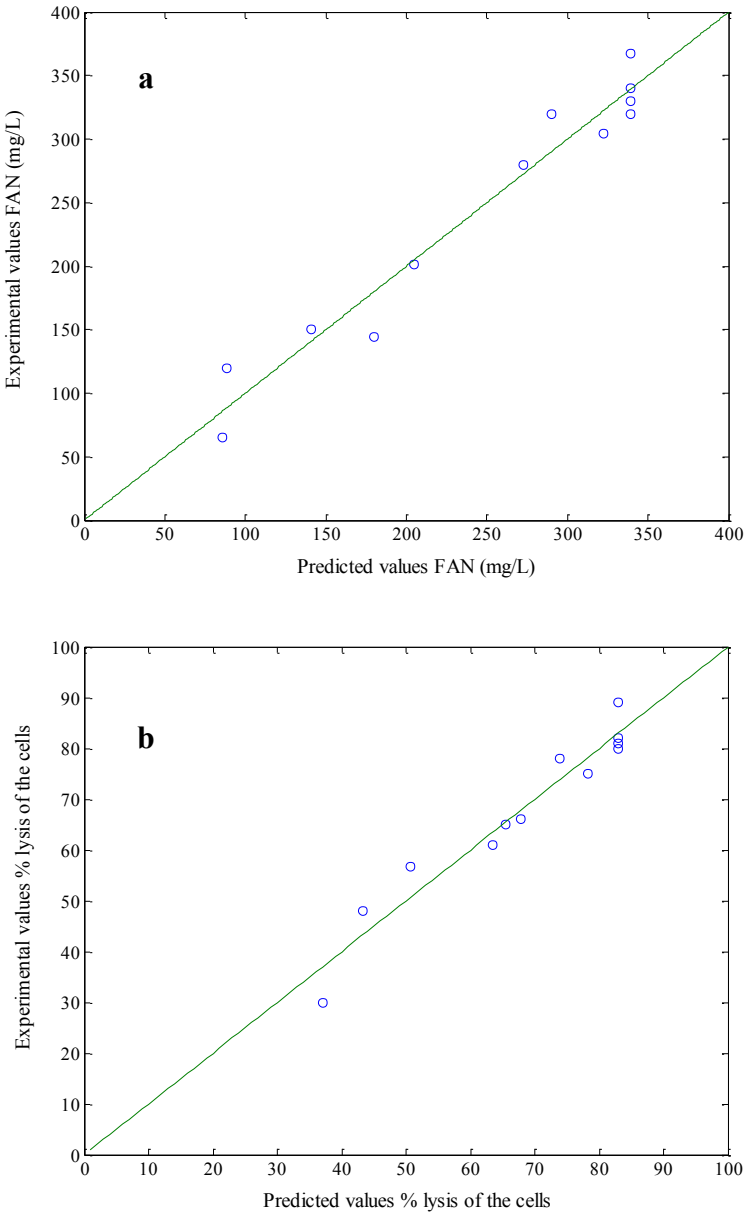

Figure 9.2 Predicted values versus experimental results of FAN production (a) and of percentage of cell lysis (b)

Table 9.1 Experimental results and model predictions obtained from the CCD

	Coded Values		Real Values		FAN concentration at the end of hydrolysis (mg/L)			Percentage of cell lysis		
Run	**x_1 (T)**	**x_2 (pH)**	**X_1 (T)**	**X_2 (pH)**	**Experimental Values**	**Predicted values**	**|error|**	**Experimental values**	**Predicted values**	**|error|**
1	1	1	60.0	7.0	320.0	290.2	29.8	75.0	78.2	3.2
2	1	-1	60.0	4.0	120.0	88.2	31.8	48.0	43.2	4.8
3	-1	1	35.0	7.0	280.0	272.8	7.2	66.0	67.8	1.8
4	-1	-1	35.0	4.0	150.0	140.8	9.2	56.8	50.7	6.1
5	0	1.4142	47.5	7.6	304.0	322.1	18.1	78.0	73.9	4.1
6	0	-1.4142	47.5	3.4	65.0	85.9	20.9	30.0	37.1	7.1
7	1.4142	0	65.2	5.5	144.0	179.5	35.5	65.0	65.5	0.5
8	-1.4142	0	29.8	5.5	201.0	204.5	3.5	61.0	63.4	2.4
9	0	0	47.5	5.5	330.0	339.3	9.3	82.0	83.0	1.0
10	0	0	47.5	5.5	320.0	339.3	19.3	81.0	83.0	2.0
11	0	0	47.5	5.5	340.0	339.3	0.7	80.0	83.0	3.0
12	0	0	47.5	5.5	367.0	339.3	27.7	89.0	83.0	6.0

Table 9.2 ANOVA for the CCD and estimation of parameter values for Y1 response

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P > F
Model	5	110973.90	22194.80	25.112	0.0006
Error	6	5303.02	883.80		
Lack Of Fit	3	4076.27	1358.76	3.3228	0.1752
Pure Error	3	1226.75	408.92		
Total	11	116276.92			

Parameters	Estimate	Standard error	t Ratio	Prob > |t|
b_1 (intercept)	339.250	14.865	22.82	<.0001
b_2 (X_1)	-8.826	10.511	-0.84	0.4332
b_3 (X_2)	83.500	10.511	7.94	0.0002
b_4 (X_1X_2)	17.500	14.865	1.18	0.2837
b_5^2 $(X_1)^2$	-73.625	11.752	-6.27	0.0008
b_6^2 $(X_2)^2$	-67.625	11.752	-5.75	0.0012

R^2 = 0.954, R^2 Adjusted = 0.916, RMSE = 29.73

The response variable Y_1 corresponds to FAN concentration. In this case, the *F*-value, $F_{(5,6)}$, is equal to 25.112, which is higher than the tabulated *F*-value with level of importance 0.01 [$F_{(5,6)}$=10.672], showing that the model is significant. The prediction capability of the model is also acceptable as shown by the R^2 and adj R^2 values, which are equal to 0.954 and 0.916, respectively. The importance of the coefficients of Eq. 4.3 was assessed from the Student's *t*-distribution together with their corresponding probability. Higher *t*-values and lower *p*-values give more significant coefficients. According to the *t*-test, the linear effect of parameter X_2 (pH), is the most important as it has the highest *t* ratio which is 7.94. The quadratic terms of Eq. 4.3 also affect strongly the response as they have high *t* ratio values. The *t* ratio of the quadratic term of temperature and pH are equal to -6.27 and -5.75, respectively. The linear interaction coefficient and the linear coefficient of X_1, which is the temperature, are insignificant as they both have low *t* ratios equal to 1.18

and -0.84, respectively. According to the parameter estimations of Table 9.2, Eq. 4.3 becomes:

$$Y_1 = 339.25 - 8.826x_1 + 83.5x_2 + 17.5x_1x_2 + 73.625x_1^2 + 67.625x_2^2$$

The response variable Y_2 corresponds to the percentage of cell lysis. In this case, the *F*-value, $F_{(5,6)}$, is equal to 17.899, which is also higher than the tabulated *F*-value with level of importance 0.01 [$F_{(5,6)}$=10.672] illustrating that the model is significant. The prediction capability of the model is also acceptable as shown from the R^2 and adj R^2 values which are equal to 0.94 and 0.89, respectively. The importance of the coefficients of Eq. 4.3 was assessed, as in Y_1, from the Student's *t*-distribution and their corresponding probability. According to the *t*-test, as in Y_1 response, the linear effect of parameter X_2, which is the pH, is the most important as it has the highest *t* ratio which is 6.43. The quadratic terms of Eq.4.3 also affect strongly the response as they have high *t* ratio values which are equal to -6.08 and -4.09 for pH and temperature, respectively. The linear interaction coefficient and the linear coefficient of X_1, which is the temperature, are insignificant as they both have low *t* ratios equal to 1.55 and 0.36, respectively. According to the parameter estimations of Table 9.3, Eq. 4.3 becomes:

$$Y_2 = 83 + 0.732x_1 + 13.01x_2 + 4.45x_1x_2 - 9.263x_1^2 - 13.763x_2^2$$

Model predictions of the quadratic equation were interpreted in Figures 9.3a and 9.3b for the Y_1 response. Figure 9.3a presents the entire spectrum of FAN predictions for the whole range of values of the two parameters as a 3D graph, while Figure 9.3b presents the FAN production as a counter plot. Figure 9.3, shows that pH values lower than 4.9 result in low hydrolysis yields (less than 300 mg/L of FAN concentration). Furthermore, pH values higher than 5 give high FAN response (higher than 300 mg/L) for all temperatures apart from the side values (higher than 59 ^{o}C and less than 36 ^{o}C). The maximum FAN concentration of 365 mg/L occurs for an optimum temperature (X_1^{OPT} = 47.7 ^{o}C) that is close to the center point and an optimum pH (X_2^{OPT} = 6.43) that is higher than the center point.

Table 9.3 ANOVA for the CCD and estimation of parameter values for Y2 response

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P > F
Model	5	2932.37	586.474	17.899	0.0015
Error	6	196.60	32.766		
Lack Of Fit	3	146.60	48.8659	2.932	0.2003
Pure Error	3	50.00	16.67		
Total	11	196.60			

Parameters	Estimate	Standard error	t Ratio	Prob > |t|
b_1 (intercept)	83.300	2.862	29.00	<.0001
b_2 (X_1)	0.732	2.024	0.36	0.7299
b_3 (X_2)	13.010	2.024	6.43	0.0007
b_4 (X_1X_2)	4.450	2.862	1.55	0.1770
b_5^2 $(X_1)^2$	-9.263	2.263	-4.09	0.0064
b_6^2 $(X_2)^2$	-13.763	2.263	-6.08	0.0009

R^2 = 0.94, R^2 Adjusted = 0.89, RMSE = 5.72

Regarding the Y_2 response, similar results, as expected, have been generated. Figure 9.4a presents the percentage of non-PHA cell lysis predictions for the whole range of values of the two parameters as a 3D graph, while Figure 9.4b presents the percentage of non-PHA cell lysis as a counter plot. As in the case of Y_1, pH values lower than 4.5 result in cell lysis lower than 70 %. On the contrary, pH values in the range of 5.5 and 7.0 result in higher than 80% of cell lysis for a temperature range near the center point ($40^oC<T<60^oC$). The optimum parameter values for Y_2 are also close to the center point for the temperature (X_1^{OPT} = 49.5 ^oC) and higher than the center point for the pH (X_2^{OPT} = 6.25) and correspond to a maximum percentage of non-PHA cell mass lysis of 86.3%.

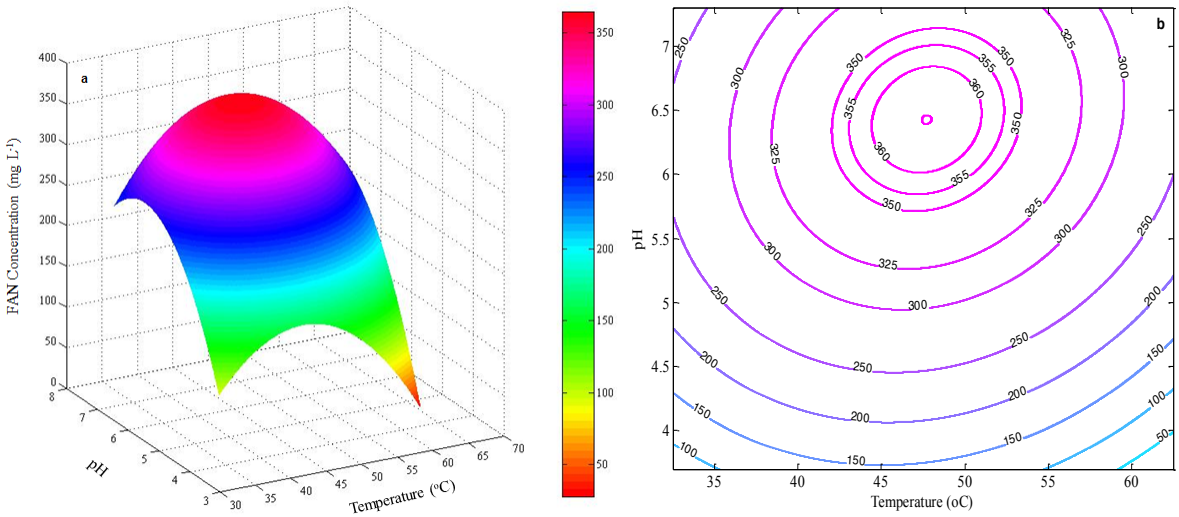

Figure 9.3 Graphical representation of the model predictions for the response variable Y_1 (FAN concentration) for the entire range of parameter values as a 3D graph (a) and as a counter plot (b)

Subsequently, the model was validated by carrying out three experimental runs at the optimum conditions for the non-PHA cell lysis response (temperature of 49.5 ºC and pH value of 6.25). These conditions were chosen over the ones that lead to optimum FAN production since the latter is an indicator mainly of the hydrolysis of protein by proteolytic enzymes. Cell disruption constitutes a complex hydrolytic reaction caused by different crude enzymes produced during SSF. Nonetheless, the two optimal points are close (see Figures 9.3b and 9.4b). The optimal runs resulted in 88.9 ± 0.4% of cell lysis, which was close to the optimum model prediction (86.3%). The corresponding experimental FAN concentration at the end of cell lysis was 364 mg/L, which was also close to the model prediction (362 mg/L).

The optimum temperature for the hydrolysis of bacterial cell protein (47.7 ºC) is very close to the optimum temperature for SFM hydrolysis (45 ºC) using the crude enzymes produced by *A. oryzae* (Kachrimanidou et al., 2013). SFM hydrolysis led to the highest FAN production when uncontrolled pH conditions were used (Kachrimanidou et al., 2013). For this reason, the enzymatic lysis of non-PHA cell mass was also investigated at uncontrolled pH conditions and 48 °C using two initial TDW concentrations (30.9 g/L and 50.1 g/L). Figure 9.5 presents FAN production and percentage of non-PHA cell mass lysis, when an initial TDW of 30.9 g/L was employed with 78.9 % (w/w) intracellular P(3HB-

co-12mol% 3HV) content. The FAN concentration at the end of the reaction was around 344 mg/L and the extent of cell lysis was 94.3 %. FAN production reached a plateau at around 5 h, whereas cell lysis was completed after 15 h.

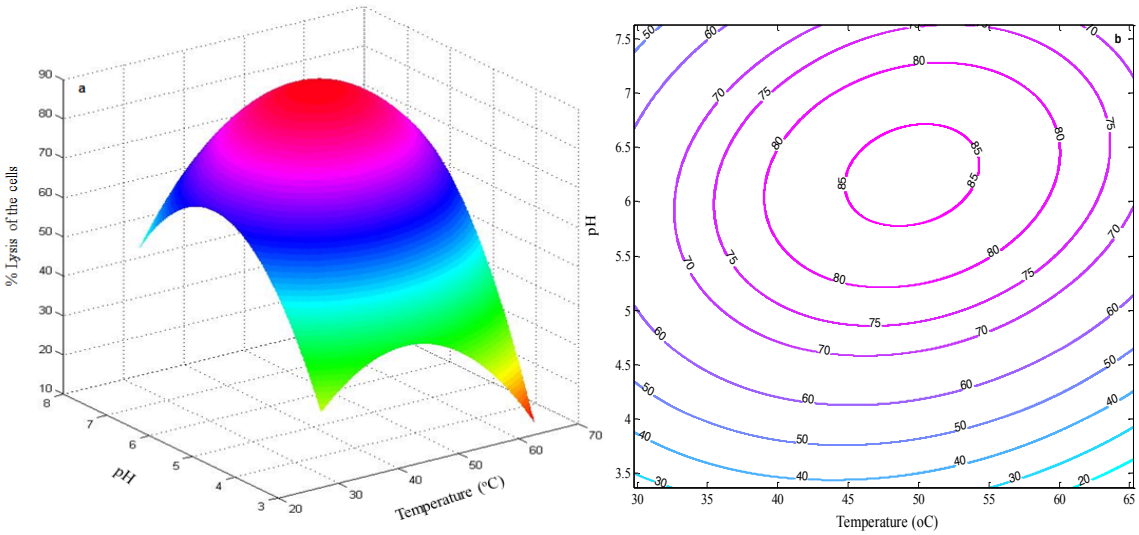

Figure 9.4 Graphical representation of the model predictions for the response variable Y_2 (percentage of non-PHA cell lysis) for the entire range of parameter values as a 3D graph (a) and as a counter plot (b)

The following experiment of bacterial cell lysis (Figure 9.6) was carried out using a higher initial TDW concentration of 50.1 g/L with 78.9 % (w/w) P(3HB-*co*-12mol% 3HV) content under the same reaction conditions (uncontrolled pH and 48 °C) as the previous cell lysis reaction presented in Figure 9.5. FAN production (around 600 mg/L) reached a plateau after approximately 8 h, while the non-PHA cell lysis (87.5 %) was also concluded at around 8 h. The FAN production and percentage of non-PHA cell lysis obtained under uncontrolled pH conditions were higher than the respective values obtained with the optimum temperature and pH conditions identified by the central composite design. This indicates that uncontrolled pH is a favourable operational condition.

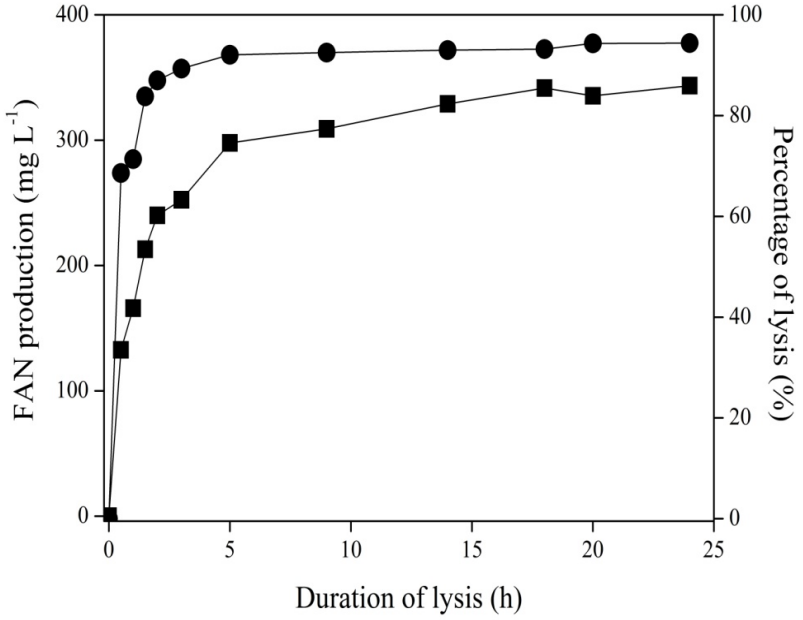

Figure 9.5 FAN production (■) and percentage of non-PHA cell lysis (●) during enzymatic lysis of 30.9 g/L initial TDW using crude enzymes produced by *A. oryzae*

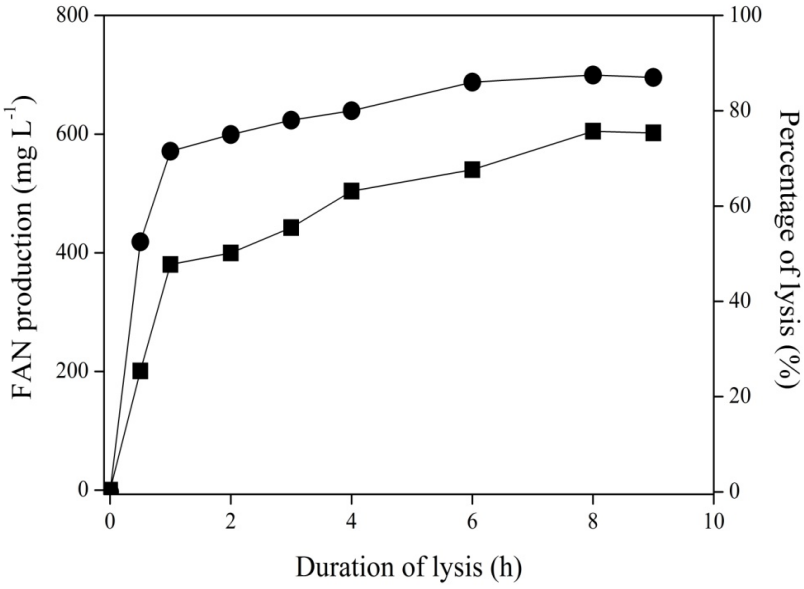

Figure 9.6 FAN production (■) and percentage of non-PHA cell lysis (●) during enzymatic lysis of 50.1 g/L initial TDW using crude enzymes produced by *A. oryzae*

The recovery yield of P(3HB-*co*-12mol% 3HV) obtained at the end of the two experiments presented in Figures 9.5 and 9.6 was in the range of 98 – 99% indicating that enzymes acted specifically on the lysis of non-PHA cell mass, leaving the intracellular

biopolymer intact. Furthermore, the purity of the obtained P(3HB-*co*-3HV) was 98.6 % and 96.7 % for the experiments performed with 30.9 g/L and 50.1 g/L of initial TDW, respectively. The purity could be possibly further increased by using a more concentrated enzyme-rich extract from the SSF where higher enzyme activities are present.

Enzymatic digestion has been previously studied as an alternative downstream process to recover PHA, implementing mainly the use of proteases. An industrial method implementing proteolytic enzymes and surfactant treatment (sodium dodecyl sulfate, SDS) for PHB extraction was established by Holmes and Lim (1990), which was employed in industrial production of Biopol®. The combined action of proteolytic enzymes with phospholipase enhanced cell wall disruption compared to the separate action achieved when individual enzymes (e.g. Alcalase or Lecitase) were used (Holmes and Lim, 1990). Yasotha et al. (2006) employed Alcalase for enzymatic digestion combined with surfactant (SDS and ethylene diamine tetra-acetic acid) treatments achieving 90 % recovery and 92.6% purity of PHA. A high PHA recovery yield (94 %) and purity (92%) was reported via PHA separation from 5 g/L of thermally inactivated cell mass of *Sinorhizobium meliloti* using a process involving the application of lytic enzymes from *Microbispora* sp. (Lakshman et al., 2006).

A heat treatment often precedes the enzymatic reaction in order to deactivate nucleic acids and PHB depolymerase that inhibit the recovery of intracellular biopolymers (Jacquel et al., 2008; Steinbüchel 1996). Kapritchkoff et al. (2006) employed a heat treatment step prior to the assessment of various enzymes including trypsin, bromelain and lysozyme, for PHB extraction from *Ralstonia eutropha* DSM 545 cells. Bromelain proved to be the best among the enzymes tested yielding 88.8% of PHB purity, though high purity (90 %) after 8 h reaction was also obtained when pancreatin was used.

In the literature-cited studies presented above, the capability of various enzymes to hydrolyse non-PHA cell mass has been demonstrated. In this study, crude enzyme-rich extracts produced via solid state fermentation of SFM were used successfully for the recovery of PHA, thus enhancing the sustainability of the proposed biorefinery concept.

9.3 Characterisation of the produced P(3HB-co-3HV)

The P(3HB-*co*-3HV) was produced via bioreactor cultures using SFM hydrolysates, crude glycerol and levulinic acid as fermentation media. The thermal properties of the P(3HB-*co*-3HV) extracted with the conventional chloroform-based method and the novel process involving enzymatic cell lysis were evaluated using DSC. Table 9.4 presents the melting temperature (T_m), glass transition temperature (T_g), crystallisation temperature (T_c), cold crystallisation temperature (T_{cc}), enthalpy of fusion (ΔH_m) and percentage of crystallinity (%X) of the produced PHAs. These constitute parameters of crucial importance for further thermal processing like extrusion or thermoforming, closely associated with the final product formed from biopolymers. Melting temperature and cold crystallisation temperature were obtained from the second heating cycle where the polymer history was eliminated. The degree of crystallinity was calculated based on the corresponding values of melting enthalpy obtained from the first heating cycle. The melting temperatures of the P(3HB-*co*-3HV) samples separated by the conventional method and the enzymatic method were similar (157.2 ^{o}C and 153.9 ^{o}C, respectively).

The melting temperature and the ΔH_m reported by Garcia et al. (2013) and Wang et al. (2013) are similar to the respective properties of the enzymatically recovered P(3HB-*co*-12mol% 3HV) sample. Garcia et al. (2013) used crude glycerol and rapeseed meal hydrolysates for the production of P(3HB-*co*-12mol% 3HV). However, the crystallinity of the enzymatically extracted PHA is higher than all other P(3HB-*co*-3HV) samples presented in Table 9.4. A lower melting temperature of 99 °C was attained in the case of *Pseudomonas oleovorans* NRRL B-14682 cultivated on crude glycerol and levulinic acid for P(3HB-*co*-3HV) synthesis containing a much higher 3HV content of 27 mol% (Ashby et al. 2012).

Table 9.4 Thermal properties of the produced P(3HB-*co*-3HV) and comparison with literature-cited studies utilising crude glycerol

Polymer	3HV content (%)	T_m (°C)	T_g (°C)	T_c (°C)	ΔH_m (J/g)	ΔH_c (J/g)	T_{cc} (°C)	ΔH_{cc} (J/g)	%X
P(3HB-*co*-3HV) (chloroform-based method) [1]	12	157.2	-1.4	-	8.8	-	94.1	11.9	42.6
P(3HB-*co*-3HV) (Enzymatic recovery) [1]	12	153.9	-	61.4	62.7	-21.4	73.4	-43.3	66
P(3HB-*co*-3HV) commercial (Sigma) [1]	12	154.4	-	84.5	65.6	-56.9	-	-	42.9
P(3HB-*co*-3HV) [2]	27	99	-1	-	3.8	-	-	-	-
P(3HB-*co*-3HV) [3]	16	150.2	-	-	73.5	-	-	-	50.3
P(3HB-*co*-3HV) [4]	12	155	-1.9	-	72.6	-	-	-	49

[1] This work; [2] Ashby et al., 2012; [3] Wang et al., 2013; [4] Garcia et al., 2013

As mentioned above, the melting temperature (157.2 °C) of the enzymatically extracted P(3HB-*co*-12mol% 3HV) was calculated from the second heating cycle corresponding to the maximum values observed. In particular, for the copolymer P(3HB-*co*-3HV) two melting transitions were observed deriving from the presence of 3HV molar units in the copolymer, indicative of the isodimorphism presented in P(3HB-*co*-3HV). Similar observations were also reported both by Modi et al. (2011) for commercial P(3HB-*co*-3HV) and Keenan et al. (2004) for P(3HB-*co*-3HV) produced by *Burkholderia cepacia*. Overall, the P(3HB-*co*-3HV) recovered by employing crude enzymatic extracts produced on-site presented similar thermal properties to P(3HB-*co*-3HV) obtained with conventional

methods, either during this study or those reported in literature cited publications. Further optimisation of this method is required in order to increase the purity of the obtained biopolymer.

Table 9.5 compares the weight average molecular mass (M_w), the number average molecular weight (M_n) and the polydispersity index (PI) obtained for the co-polymers extracted by the conventional protocol and the enzymatic protocol with the same properties reported in the literature for P(3HB-*co*-3HV). The presence of glycerol in fermentation media acts as a chain-terminating agent during the polymerisation process by inhibiting the action of PHA synthase, thus leading to the production of biopolymers with low molecular weights (Madden et al., 1999; Ashby et al., 2012; Koller and Marsalek, 2015). The weight average molecular mass of P(3HB-*co*-3HV) was 130 kDa with a PI equal to 2.1 when the conventional extraction method was used. Lower values (110 and 1.96, respectively) were achieved for the copolymer recovered after enzymatic lysis of non-PHA cell mass.

The M_w and PI of the P(3HB-*co*-3HV) produced by *Burkholderia cepacia* ATCC 17759 (Zhu et al., 2012) had similar values to the present study. However, in the other two publications (Ashby et al., 2012; Kumar et al., 2015) presented in Table 9.5, the M_w and PI values of the reported P(3HB-*co*-3HV) are significantly higher. Glycerol accumulation in the fermentation medium could also comprise a crucial factor resulting in low molecular weight of P(3HB-*co*-3HV) (Tanadchangsaeng et al., 2012). Zhu et al. (2009) employed *Burkholderia cepacia* to produce PHB from crude glycerol where a prolonged interaction of bacterial cells with glycerol resulted in a decrease in M_w. The low M_w values reported in this study could restrain lamination and extrusion processes for packaging applications that require high molecular weight biopolymers. However, the biopolymer produced in this study could be used in novel applications including scaffold production for tissue engineering applications or microencapsulation of drugs for slow delivery (Chen and Wu, 2005). Mixing with other bio-based polymers (e.g. polylactic acid, thermoplastic starch) could overcome the drawbacks of the low molecular weight of P(3HB-*co*-3HV) (Shen et al., 2009).

Table 9.5 Molecular weight distribution of the produced P(3HB-*co*-3HV) and comparison with literature-cited studies

Polymer	3HV content (%)	M_w (kDa)	M_n (kDa)	PI	Reference
P(3HB-*co*-3HV)	12	130	61	2.1	this work
P(3HB-*co*-3HV) (Enzymatic recovery)	12	110	56	1.96	
P(3HB-*co*-3HV)	27	-	511	1.97	Ashby et al., 2012
P(3HB-*co*-3HV)	11.4	115	61.8	1.9	Zhu et al., 2012
P(3HB-*co*-3HV)	14.7	107.8	69	1.6	
P(3HB-*co*-3HV)	13.4	385	183	2.1	Kumar et al., 2015

9.4 Shake flask fermentations for PHB production

After the enzymatic treatment of the bacterial cells the hydrolysate was rich in nitrogen sources, as indicated by the high FAN content, as well as other nutrients. Thus, the possibility to provide a fermentation nutrient supplement was evaluated. The resulting hydrolysate was centrifuged to separate the P(3HB-*co*-3HV) granules and the supernatant obtained was filter sterilised and combined with crude glycerol as a carbon source. Figure 9.7 presents the consumption of FAN, IP and glycerol as well as the production of TDW and PHB during shake flask fermentations carried out with the bacterial strain *C. necator* DSM 7237. The initial FAN and glycerol concentrations were approximately 330 mg/L and 21 g/L, respectively. It is easily observed that both FAN and crude glycerol were consumed while microbial proliferation takes place reaching a final TDW of 5.75 g/L. The PHB concentration at the end of fermentation was 1.04 g/L corresponding to an intracellular content of 18 % (w/w). The low concentration of IP in the hydrolysate (~28 mg/L) indicates that addition of some micro-nutrients may be necessary in order to

formulate a nutrient-complete fermentation media for PHB production in bioreactor cultures.

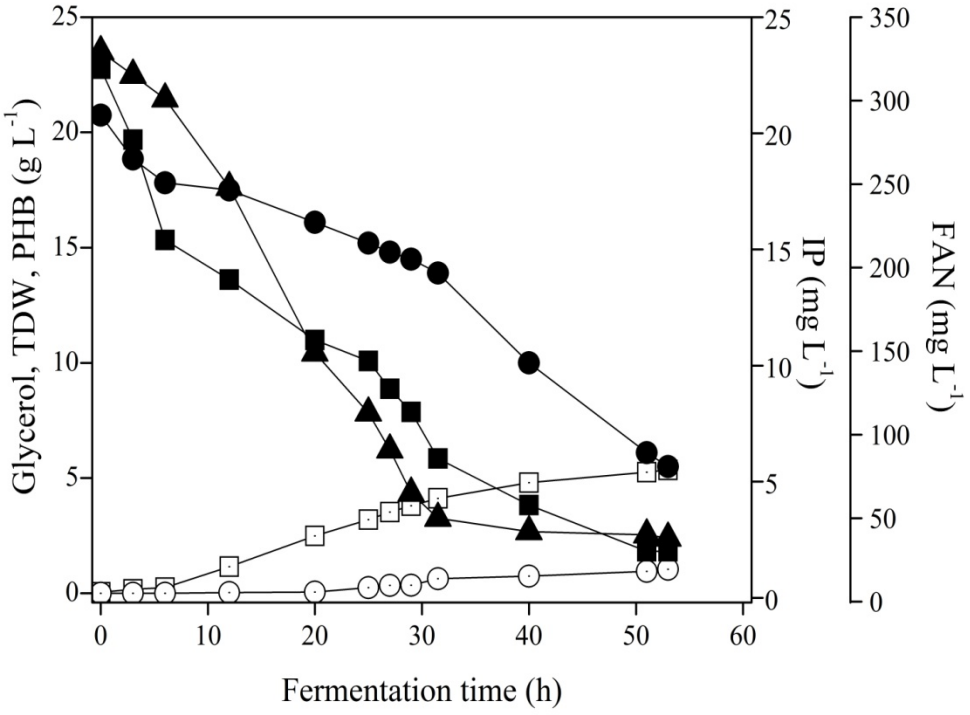

Figure 9.7 Consumption of FAN (○), IP (△) and glycerol (□) as well as production of TDW (■) and PHB (▲) during shake flask fermentation of *C. necator* cultivated on crude glycerol and the lysate obtained by the enzymatic lysis of non-PHA cell mass

The results presented in Figure 9.7 show that the bacterial lysate produced after non-PHA cell lysis could be recycled in the sunflower-based biorefinery in order to support microbial growth reducing the use of commercial sources of nutrients during PHB production.

9.5 Conclusion

This chapter evaluated the enzymatic recovery of intracellular P(3HB-*co*-3HV) granules using crude enzyme consortia produced on-site via SSF with a fungal strain of *A. oryzae*. High recovery yields (98%) and purities (96.7 %) were achieved under uncontrolled pH conditions and 48 °C using high initial TDW concentrations. Thermal properties and molecular weight of the bacterial P(3HB-*co*-3HV) were also evaluated to

assess the effect on polymer properties of the proposed method in relation to the conventional extraction protocol. The lysate could be recycled as a nutrient supplement for the production of PHB using crude glycerol as carbon source, thus minimizing the generation of waste streams.

The results presented in Chapter 9 are under revision in the Food and Bioproducts Processing journal:

Kachrimanidou, V., Kopsahelis, N., Vlysidis, A., Papanikolaou, S., Kookos, I.K., Martínez, B., Rondán, M.C., Koutinas, A.A. Downstream separation of poly(hydroxyalkanoates) using crude enzyme consortia produced via solid state fermentation integrated in a biorefinery concept.

CHAPTER 10

TECHNO-ECONOMIC EVALUATION OF THE PROPOSED SUNFLOWER-BASED BIOREFINERY CONCEPT

10.1 Introduction

In the previous chapters, the development of an advanced sunflower-based biorefinery concept leading to the production of antioxidants, protein isolate and nutrient rich supplements for the production of PHB and P(3HB-*co*-3HV) was presented. The biopolymers were produced using only crude renewable resources as well as levulinic acid that could be also produced from renewable resources. Moreover, a downstream separation method was proposed based on the utilisation of crude enzyme consortia produced through solid state fermentation of *A*. *oryzae* with the lignocellulose-rich fraction as substrate.

In this chapter, a preliminary techno-economic evaluation of the complete sunflower-based biorefinery (Figure 10.1) concept will be presented. The estimation of the fixed capital investment and the cost of manufacture were performed based on the fractionation of sunflower meal to generate antioxidants, protein isolate and PHB using fundamental chemical engineering principles. The annual production capacity of PHB was calculated based on the annual biodiesel production and the respective availability of crude glycerol. The fractionation of sunflower meal was carried out according to the results presented in Chapter 8 The experimental results used in the preliminary techno-economic evaluation were selected based on the optimal fermentation results presented in Chapter 8. The recovery of intracellular PHB is conducted by employing enzymatic cell lysis.

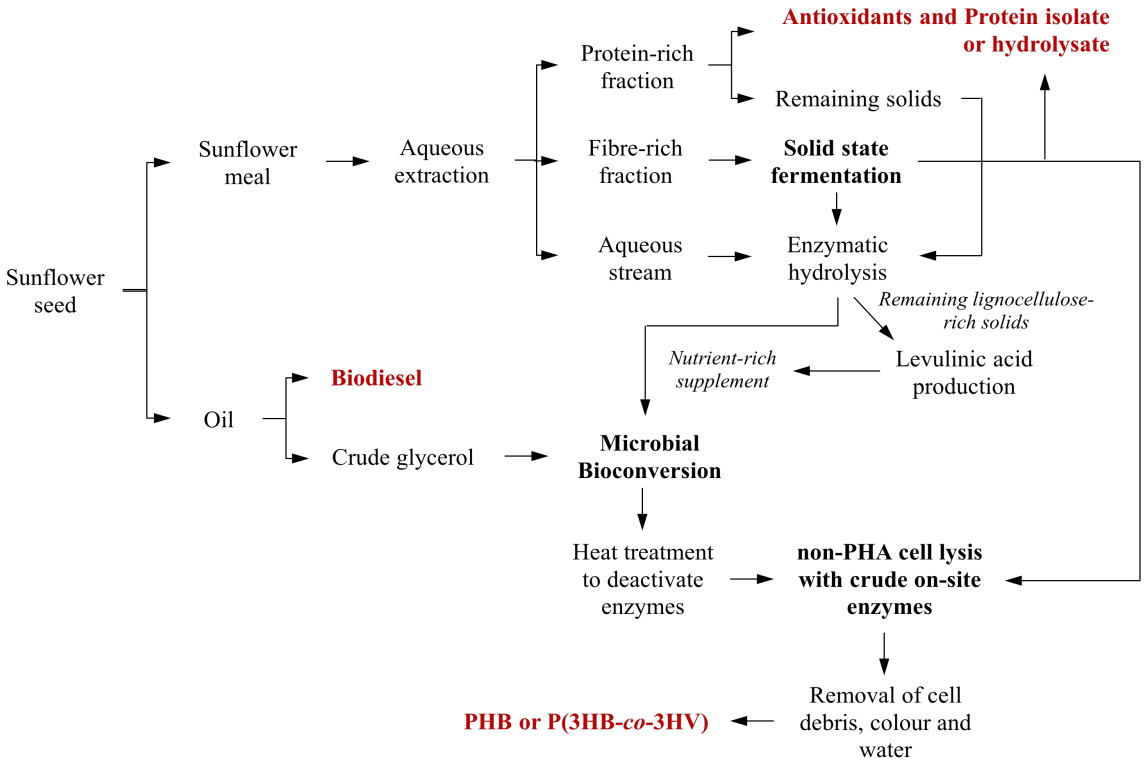

Figure 10.1 Sunflower-based biorefinery concept for PHA production. Utilisation of SFM for the production of antioxidants, protein isolate and PHB or P(3HB-*co*-3HV)

10.2 Description of the design

Costing studies were performed based on preliminary economic analysis with accuracy up to ±30%, aiming to estimate the total capital investment and the cost of manufacture for process flow sheets leading to the production of antioxidants, protein isolate and PHB. The industrial plant is assumed to operate 7,920 h/y to process the by-products obtained from a sunflower-based biodiesel production plant. More specifically, the annual biodiesel production was set at 50,000 t/y, deriving from the reaction of 50,789.14 t/y of oil. Considering that the oil content of sunflower seed is approximately 40 % (w/w), 126.982,8 t/y of sunflower seed are processed generating 76,183.7 t/y of sunflower meal as a side stream. Moreover, the annual production of crude glycerol was estimated to be 5,176.927 t/y. All stoichiometric calculations were carried out considering that triolein is the triglyceride in sunflower oil. The material and energy balances were carried out using well known procedures and rules of thumb (Koutinas et al., 2014d; Turton et al., 2003). Sizing of the process equipment was carried out employing well

established engineering principles. Moreover, the free on board (fob) cost, corresponding to the purchased cost (C_p), was calculated that was subsequently used for the estimation of the fixed capital investment (FCI). The cost of manufacture was calculated by taking into consideration the fixed capital related costs and well as the cost of labour, raw materials and utilities.

10.3 Extraction of antioxidants-rich fraction from sunflower meal

Figure 10.2 presents the process flow sheet describing the unit operations employed for the separation of the antioxidant-rich fraction from the sunflower meal. Initially, sunflower meal and ethanol are fed into the leaching vessel (T-101), where extraction of antioxidants is performed assuming a residence time of 1 h. The mixed effluent is directed towards a centrifugation step (CF-101) to separate the solid from the liquid fraction. The liquid stream produced after centrifugation that contains the antioxidant-rich fraction is heated (E-101) and fed to the vessel V-101 to separate the antioxidant-rich fraction from ethanol that is recycled (stream 10). A fluidised bed drier (T-102) is employed in order to remove water and ethanol from the sunflower meal that contains a moisture content of 10% (w/w). The sunflower meal is subsequently directed to the fractionation section (AREA 200).

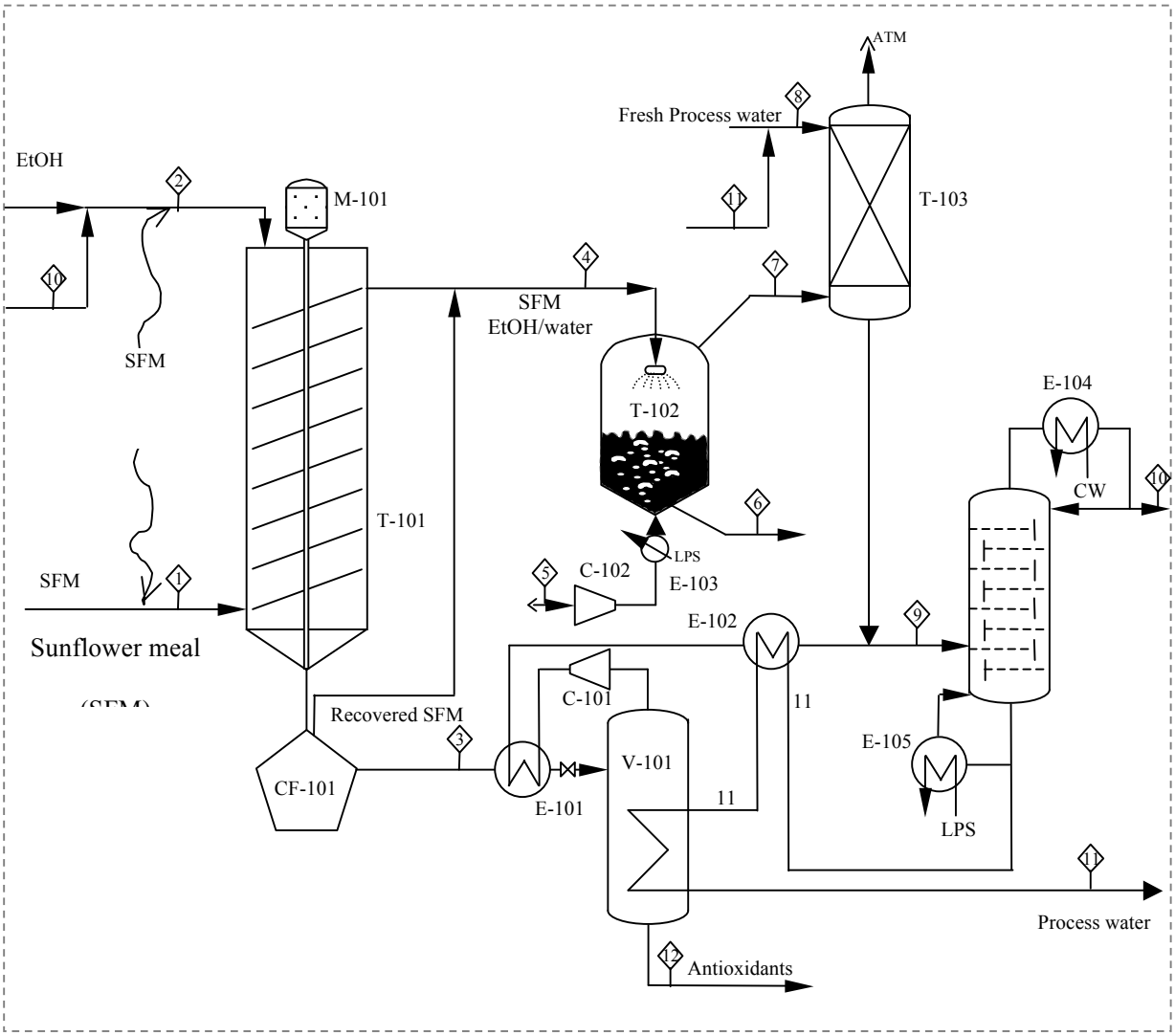

Figure 10.2 Process flow diagram for AREA 100: antioxidants extraction

The cost of raw materials (C_{RM}) in the AREA 100 is mainly attributed to the annual consumption of ethanol and more specifically estimated at M$ 1.232 (1,232.4 t EtOH/y × 1,000 $/t). The electricity consumption in the compressor constitutes 99 % of the total consumption in AREA 100. Figure 10.3 presents the total quantities of input and output streams for AREA 100 for the extraction of antioxidants. The annual production of antioxidant-rich fraction is 2,785 t/y, with an antioxidant content of 12% that consists mainly of chlorogenic acid (50.5 %) (Kachrimanidou et al., 2014).

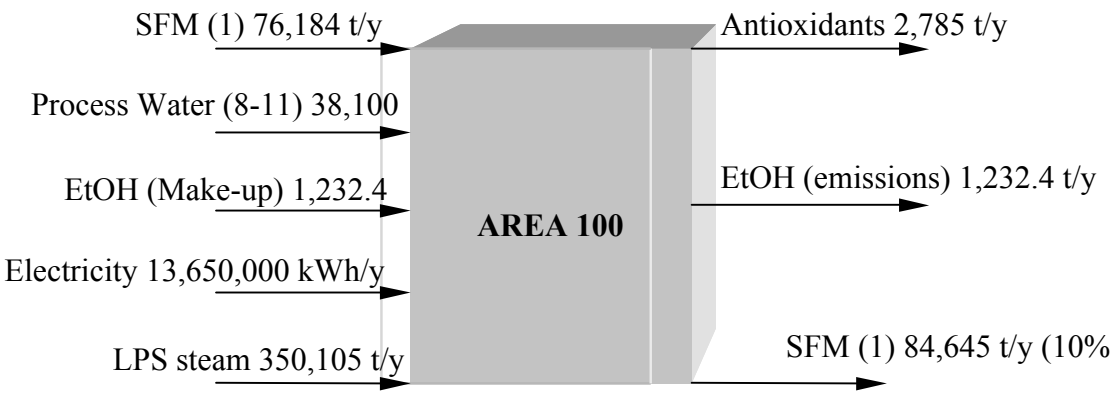

Figure 10.3 Summary of total quantities of input and output streams employed in the extraction of the antioxidant-rich stream (AREA 100)

The costs related with the extraction of antioxidants from sunflower meal (AREA 100) are presented in Tables 10.1 and 10.2. Table 10.1 presents the equipment specifications (i.e. type of equipment, characteristic size) and the fob cost for each equipment in 2013 of the process flow diagram illustrated in Figure 10.2. The total purchased equipment cost (C_p) is M$ 4.864. It is easily noticed that approximately half of the C_p is attributed to the compressor, entailing also the highest annual electricity consumption. The operation of this process requires 4-5 workers per shift.

10.4 Fractionation of protein isolate from sunflower meal

Figure 10.4 presents the process flow sheet followed for the fractionation of sunflower meal to generate protein isolate. Sunflower meal after the removal of antioxidants is fed into a mixing tank (T-201) where it is mixed with water in order to reach a final concentration of 200 kg/m^3. The residence time is defined at 10 min. The effluent is transferred to a settling tank (T-202) where the lignocellulose-rich fraction is removed. The remaining stream is fed to a centrifugal compressor (CF-201), where the protein-rich and soluble fractions are separated. The protein-rich fraction is treated with NaOH in a mixing tank (T-203) for 1 h to solubilise the proteins. After a centrifugation step (CF-202), the solid residue is removed and the liquid effluent is fed into a mixing tank (T-204), in order to precipitate the protein via HCl addition to reduce the pH value to 4.3.

A subsequent centrifugation step is implemented to remove water and the remaining fraction is pumped to a spray drier to obtain the protein isolate.

Table 10.1 AREA 100: Summary of equipment specifications and purchased equipment cost (C_p) for antioxidants extraction

Equipment		Material	Char. Size	fob cost ($)	CEPCI	fob cost ($@2013)
T-101	Cylindrical tank 2m×6.4m	SS304	20 m^3	21,100	238.7	50,100
M-101	Electrical motor	CS	1.7 hp	5,000		11,900
V-101	Vertical tank	SS304	3.05×15.15 m	52,000		123,600
E-101	Shell & Tube heat exch.	SS304	6.3 m^2	6,800		9880
C-101	Compressor	SS304	530 hp	247,000		359,000
E-102	Shell & Tube heat exch.	SS304	305 m^2	83,000		120,600
C-102	Compressor	CS	1530 hp	1,667,000		2,422,400
E-103	Shell & Tube heat exch.	CS	170 m^2	30,700		44,600
T-102	Fluidised bed drier	SS304	2.67 kg/s	270,000	390.4	392,350
T-103	Absorption (tray) column	SS304	D=4.9 m H=21.3m	387,000		562,360
	30 sieve trays	SS304	D=4.9 m	110,000		159,840
T-104	Distillation (tray) column	SS304	D=2.7 m H=6.6m	231,000		335,700
	10 sieve trays	SS304	D=2.7 m	26,100		37,950
E-104*	Condenser	SS304	A=250 m^2	40,700		59,150
E-105	Reboiler	SS304	A=125 m^2	25,000		36,350
CF-101	Disc stack centrifuge	SS304	8.5 m^3/h	95,000	444.0	138,050
						TOTAL M$ 4.864

* E-104 consumes 9,726,820 t CW/y

Table 10.2 AREA 100: Summary of the utilities cost and labour cost calculation for the extraction of antioxidants

Unit operation	Equipment	Workers/shift [a]	Electricity [b] (10^6 kW h/y)	lps [c] (t/y)	Utilities cost (M$/y)
T-101	Cylindrical tank 2m×6.4m	0.1			
M-101	Electrical motor	-	10,300		
V-101	Vertical tank	0.1			
E-101	Shell &Tube heat exch.	0.05			
C-101	Compressor	0.1	3,488,320		
E-102	Shell &Tube heat exch.	0.05			
C-102	Compressor	0.1	10,032,000		
E-103	Shell &Tube heat exch.	0.05		76,105	
T-102	Fluidised bed drier	0.1			
T-103	Absorption (tray) column	0.1			
	30 sieve trays	-			
T-104	Distillation (tray) column	0.1			
	10 sieve trays	-			
E-104*	Condenser	0.05			
E-105	Reboiler	0.05		274,000	
CF-101	Disc stack centrifuge	0.1	118,800		
Total		**5 Workers C_{OL}= 0.2 M$/y**	**13,650,000**	**350,105**	**C_{UT}= 11.47 M$/y**

[a] Labour cost $40,000/(yr/worker); [b] Electricity cost: $0.06/kW h; [c] lps Cost: $30/t; * E-104 consumes 9,726,820 t CW/y, $0.015/t.

Figure 10.5 presents the summary of total quantities of input and output streams of AREA 200. The annual protein isolate production is 7,314 t/y. Furthermore, the lignocellulose-rich fraction and the solid residue are generated during the fractionation process and are subsequently implemented in solid state fermentation and enzymatic hydrolysis (AREA 300).

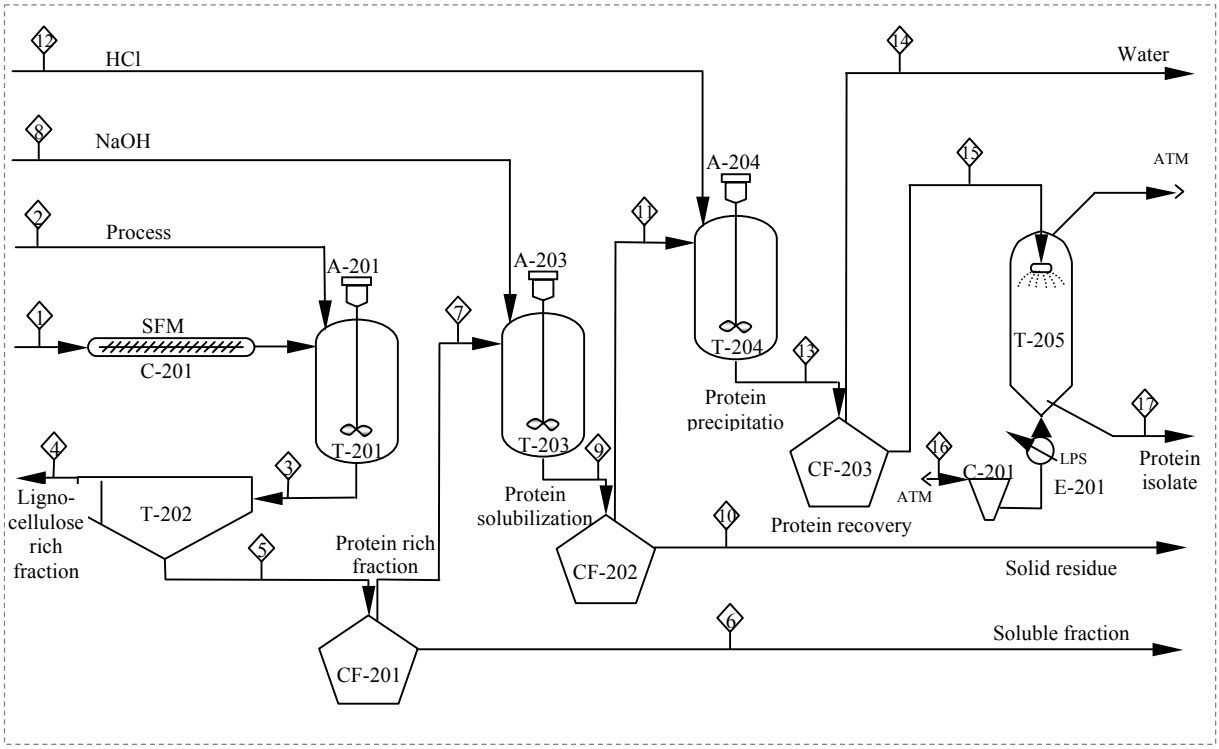

Figure 10.4 Process flow diagram for AREA 200: fractionation of sunflower meal for protein isolate production

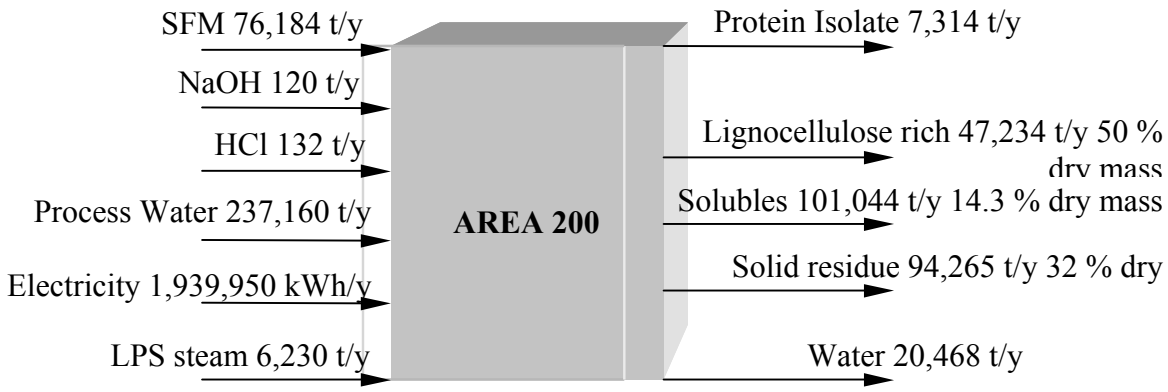

Figure 10.5 Summary of total quantities of input and output streams during fractionation of sunflower meal for protein isolate production (AREA 200)

The C_p value for the fractionation process of SFM (AREA 200) is M$ 1.315 (Table 10.3), with the cost of spray dryer and disk stack centrifuge being the predominant ones. It could be observed that fractionation of SFM constitutes a cost-effective process,

demonstrating a relatively low value for the purchased equipment cost. Nevertheless, in order to combine the extraction of protein isolate with the production of PHB, the side streams produced from this process will be used for the production of nutrient rich supplements that will be further employed in bacterial bioconversions for PHB production.

Table 10.3 AREA 200: Summary of equipment specifications and purchased equipment cost (C_p) for sunflower meal fractionation

Equipment		Material	Char. Size	fob cost ($)	CEPCI	fob cost ($@2013)
C-201	Screw conveyor		0.23×100 m	64,800		94,170
T-201	Mixing tank	CS	13.4 m^3	25,700		37,345
A-201	Agitator	CS	60 bhp	27,000		39,235
T-202	Tank	Concrete	197.5 m^3	26,300		38,220
T-203	Mixing tank	SS304	21 m^3	50,600		73,530
A-203	Agitator	SS316	18.8 bhp	26,150	390.4	38,000
T-204	Mixing tank	SS304	8.14 m^3	29,850		43,380
A-204	Agitator	SS304	7.3 bhp	13,100		19,040
T-205	Spray dryer	SS304	0.256 kg/s	165,000		239,770
E-201	Fixed tube heat exch.	CS	30.3 m^2	6,100		8,870
C-201	Centrifugal compressor	CS	102.1 kW	69,100	----------	100,400
CF-201	Disc stack centrifuge	SS304	39.5 m^3/h	167,000		242,670
CF-202	Disc stack centrifuge	SS304	25.1 m^3/h	133,800	444.0	194,430
CF-203	Disc stack centrifuge	SS304	9.76 m^3/h	98,500		143,140
						TOTAL M$ 1.315

The cost-effectiveness of the above process is strongly correlated with the diversified market outlets of protein isolate. During this study the protein isolate was of high purity (higher than 95%) that could enable various food applications, enhancing the

income generated from the protein isolate. Besides the protein isolate, significant protein content remains in the solid residue fraction after the solubilisation of the protein (19.6 %) and the lignocellulose-rich fraction (17.1 %). Table 10.4 presents the utilities and labour cost calculations for sunflower meal fractionation. The cost of raw materials (C_{RM}) derives from the cost of chemicals employed in solubilisation and precipitation of sunflower protein, assessed at M$ 0.3. Nevertheless, it is undisputable that the utilisation of sunflower meal as renewable feedstock entails the formulation of different value-added products.

Table 10.4 AREA 200: Summary of the utilities cost and operating cost calculation for the fractionation of SFM

Unit operation	Equipment	Workers/shift [a]	Electricity [b] (10^6 kW h/y)	lps[c] (t/y)	Utilities cost (M$/y)
C-201	Screw conveyor	0.1	31,700		
T-201	Mixing tank	0.2			
A-201	Agitator	-	352,000		
T-202	Tank	0.1			
T-203	Mixing tank	0.2			
A-203	Agitator	-	110,880		
T-204	Mixing tank	0.2			
A-204	Agitator	-	43,120		
T-205	Spray dryer	0.1			
E-201	Fixed tube heat exchanger	0.05		6,230	
C-201	Centrifugal compressor	0.1	808,250		
CF-201	Disc stack centrifuge	0.05	237,600		
CF-202	Disc stack centrifuge	0.05	237,600		
CF-203	Disc stack centrifuge	0.05	118,800		
Total		**6 Workers C_{OL}= 0.24 M$/y**	**1,939,950**	**6,230**	**C_{UT}= 0.304 M$/y**

[a] Labour cost $40,000/y/worker; [b] Electricity cost: $0.06/kWh; [c] lps Cost: $30/t

10.5 Costing of PHB production process

The annual production capacity of PHB was calculated based on crude glycerol and the overall conversion yield obtained via optimum experimental results. TDW and PHB final concentrations were 66.1 g/L and 57.02 g/L respectively, corresponding to a PHB content of 86.26 %. The following parameters are also assumed: preparation of the bioreactor requires 1 h, loading time is 4 h, fermentation time is 142 h and uploading of the bioreactor is 5 h, resulting in a cycle time of 152 h/batch, thus 52 batches/y. The overall conversion yield of glycerol to PHB was found to be 0.475 g/g, which entails an annual production capacity of 2.459 t PHB/y. The initial crude glycerol is 0.12 kg/m^3 hence the working volume of the broth (V_{broth}) in the bioreactor will be 829.7 m^3. The total volume of the bioreactor is calculated to 1,037 m^3 (V_{broth} is 80 % of the total volume), which is significantly large, thus 2 bioreactors are selected to operate in parallel, corresponding to a 518.5 m^3 total volume each.

The sunflower meal stream (with a moisture content of 10 % w/w) deriving from the fractionation process (AREA 200) is mixed with process water and fed in the sterilisation tank (T-301) that supplies the feed to the solid state fermentation section (Figure 10.6). Subsequently, the stream is fed in mixing tanks (T-302) where enzymatic hydrolysis is performed. The hydrolysate stream is supplied by two hydrolysis tanks (T-302), each one providing the required volumetric flowrate to the steriliser, for 76 h. At the same time, the other tank is cleaned (2 h), loaded (24 h) and the hydrolysis is performed (50 h). At the end of enzymatic hydrolysis, centrifugation is carried out with a disk stack centrifuge and the hydrolysate is pumped in storage tank (T-303) where it is combined with crude glycerol and process water prior to sterilisation.

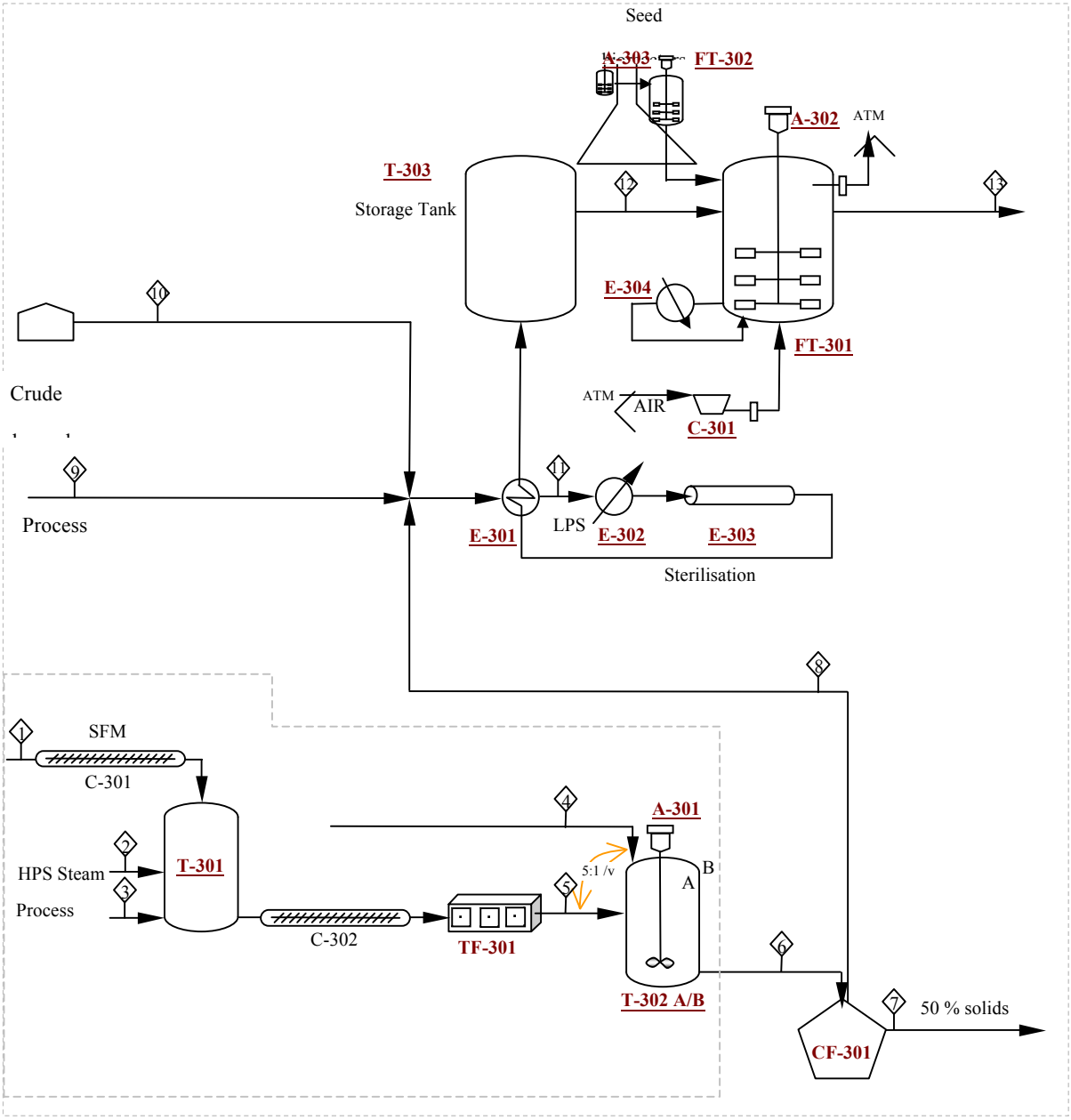

Figure 10.6 Process flow diagram for AREA 300: solid state fermentation, enzymatic hydrolysis and PHB production via fermentation

The sterilisation section includes three heat exchangers, namely E-301, E-302 and E-303. E-301 is the preheating section of the steriliser, exchanging heat between the input and output streams. Low pressure steam (160 °C) is utilised in E-302 to elevate the temperature at 130 °C, while E-303 constitutes the holding section of sterilisation, assuming a residence time of 2 min. Subsequently, the process stream is fed in the bioreactor. As previously stated, 2 bioreactors are required to operate simultaneously. Air is used at a rate of 0.5 vvm to fulfil oxygen demands for microbial proliferation and PHB

synthesis. Uploading of the bioreactor is 5 h during a batch cycle of 152 h. An intermediate storage tank employed prior to downstream separation and purification of PHB enables continuous flow of fermentation broth during the recovery of PHB. The total quantities of input and output streams of AREA 300 are illustrated in Figure 10.7.

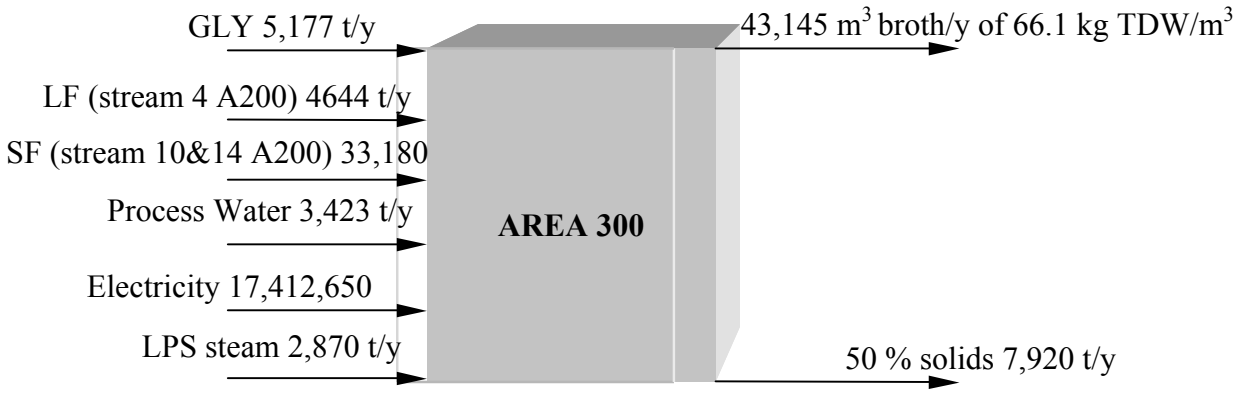

Figure 10.7 Summary of total quantities of input and output streams during solid state fermentation, enzymatic hydrolysis and fermentation (AREA 300)

In Tables 10.5 and 10.6 the costs related to PHB production are presented. Solid state fermentation and enzymatic hydrolysis to produce the fermentation supplement are included along with the bioconversion process. The characteristic size of each equipment is also presented in Table 10.5 along with the purchased cost (fob cost). The costs are assessed for a production capacity of 2,460 t PHB/y.

In order to assess the overall cost of manufacture of PHB, apart from the Fixed Capital Investment that will be further on evaluated, the cost of raw materials (C_{RM}), the cost of operating labour (C_{OL}) and the cost of utilities (C_{UT}) are also implemented, while the cost of waste treatment (C_{WT}) is considered negligible. As it is observed in Table 10.5, C_p is in the order of M$ 8.6. The solid state fermentation and enzymatic hydrolysis account for 42.7 % of the total C_p, while the remaining cost includes the cost of the fermentation area. Glycerol is available without cost and for this reason the cost of fermentation supplements for inoculum preparation was used for the calculation of C_{RM}.

Table 10.5 AREA 300: Summary of equipment specifications and purchased equipment cost for solid state fermentation, enzymatic hydrolysis and PHB fermentation

Equipment		Material	Char. Size	fob cost ($)	CEPCI	fob cost ($@2013) CEPCI=567.3
C-301	Screw conveyor		0.23×100 m	64,800		94,300
C-302	Screw conveyor		0.23×100 m	64,800		94,300
T-301	2 Sterilisation tanks	SS304	80 m^3 each	2×195,000		566,720
TF-301	2 Tray SS bioreactors	SS304	638 m^2 each	2×193,620		561,500
T-302	2 Mixing tanks	SS304	480 m^3 each	2×450,000		1,307,800
CF-301	Disc stack centrifuge	SS304	5.04 m^3/h	87,620	390.4	127,330
E-301	s&t heat exhanger	SS304	56.9 m^2	15,000		21,800
E-302	s&t heat exhanger	SS304	1.64 m^2	6,800		9,890
E-303	Sc40 0.1 m×20.7 m tube	SS304	0.1 m×20.7 m	6,100		8.860
T-303	Storage tank	SS304	828.7 m^3	161,700		235,000
E-304	s&t heat exhanger	SS304	44.6 m^2	2× 12,550		36,470
A-301	2 Agitators	SS304	720 hp each	2×317,000		921,280
FT-301	2 bioreactors	SS304	520 m^3 each	2×510,000		1,482,200
A-302	2 bioreactor agitators	SS316	660 hp	2×497,000	521.9	1,444,400
C-301	Centrifugal compressor	CS	8000 scfm	2×350,000		1,017,200
FT-302	2 seed bioreactors	SS304	52 m^3 each	2×235,000		683,000
A-303	2 bioreactor agitators	SS316	66 hp	Included		
C-302	Reciprocating compressor	CS	800 scfm	Included		
						TOTAL M$ 8.6

The consumption of electricity during the process in AREA 300 will be 7.1 kWh per kg PHB reaching a total annual cost of M$ 1,044. Furthermore, the annual low pressure steam consumption is 2872 t/y.

Table 10.6 AREA 300 Summary of the utilities cost and labour cost for solid state fermentation, enzymatic hydrolysis and PHB fermentation

Unit operation	Equipment	Workers/shift [a]	Electricity [b] (10^6 kWh/y)	lps [c] (t/y)	Utilities cost (M$/y)
C-301	Screw conveyor	0.1	31,700		
C-302	Screw conveyor	0.1	31,700		
T-301	2 Sterilization tanks	2×0.1=0.2		1966	
TR-301	2 Tray SS bioreactors	2×1=2			
T-302	2 Mixing tanks	2×0.1=0.2			
CF-301	Disc stack centrifuge	0.1	118,800		
E-301	s&t heat exhanger	0.05			
E-302	s&t heat exhanger	0.05		779	
E-303	Sc40 0.1 m×20.7 m tube	-			
T-303	Storage tank	-			
E-304	s&t heat exhanger	0.05			
A-301	2 Agitators	-	3,120,000		
FT-301	2 bioreactors	2×1=2		104	
A-302	2 bioreactor agitators	-	6,807,230		
C-301	Centrifugal compressor	0.1	7,116,700		
FT-302	2 seed bioreactors	2×1=2		23	
A-303	2 bioreactor agitators	-	108,160		
C-302	Reciprocating compressor	0.1	78,361		
Total		**32 Workers C_{OL}= 1.28 M$/y**	**17,412,650**	**2872**	**C_{UT} = 1.13 M$/y**

[a] Labour cost $40,000/(y/worker); [b] Electricity cost: $0.06/kWh; [c] lps Cost: $30/t

10.6 Downstream processing stage for PHB recovery and purification

PHB recovery is initiated with a heating step to deactivate the enzymes in bacterial cells that are subsequently fed in an intermediate storage tank as presented in Figure 10.8. The broth is heated to 70 °C by a feed-effluent heat exchanger (E-401) and then to 80 ^{o}C by using low pressure steam (E-402). The output stream is directed to centrifugation in order to increase the concentration of the bacterial mass, where the outflow concentration of bacterial mass is 90 kg/m^3. In the mixing tank (T-405), bacterial cells are disrupted with crude enzymes produced on-site via solid state fermentation. Subsequently hydrogen peroxide is used according to the patent published by Holmes and Lin (1991). Water is removed in the evaporator (V-401) and the output stream is fed in a spray dryer (V-402), where dried PHB granules are obtained as end-product.

The production of crude enzyme-rich extracts employed in enzymatic lysis of bacterial cell mass was a similar process to the one presented for the production of SFM hydrolysates in the PHB production section (AREA 300). In the case of PHB recovery, the lignocellulose-rich fraction is mixed with process water and fed in the sterilisation tank (T-401). Subsequently, the stream is fed in mixing tanks (T-402) where the enzyme-rich aqueous solution is produced by mixing the SSF solids with water. Enzymatic extracts are separated from solid residues via centrifugation and the hydrolysate is fed in a mixing tank (T-405) where bacterial cell disruption is carried out.

Figure 10.8 presents the process flow sheet of PHB recovery including the production of crude enzymes used for the disruption of bacterial cells (AREA 400). Figure 10.9 presents the quantities of input and output streams for AREA 400.

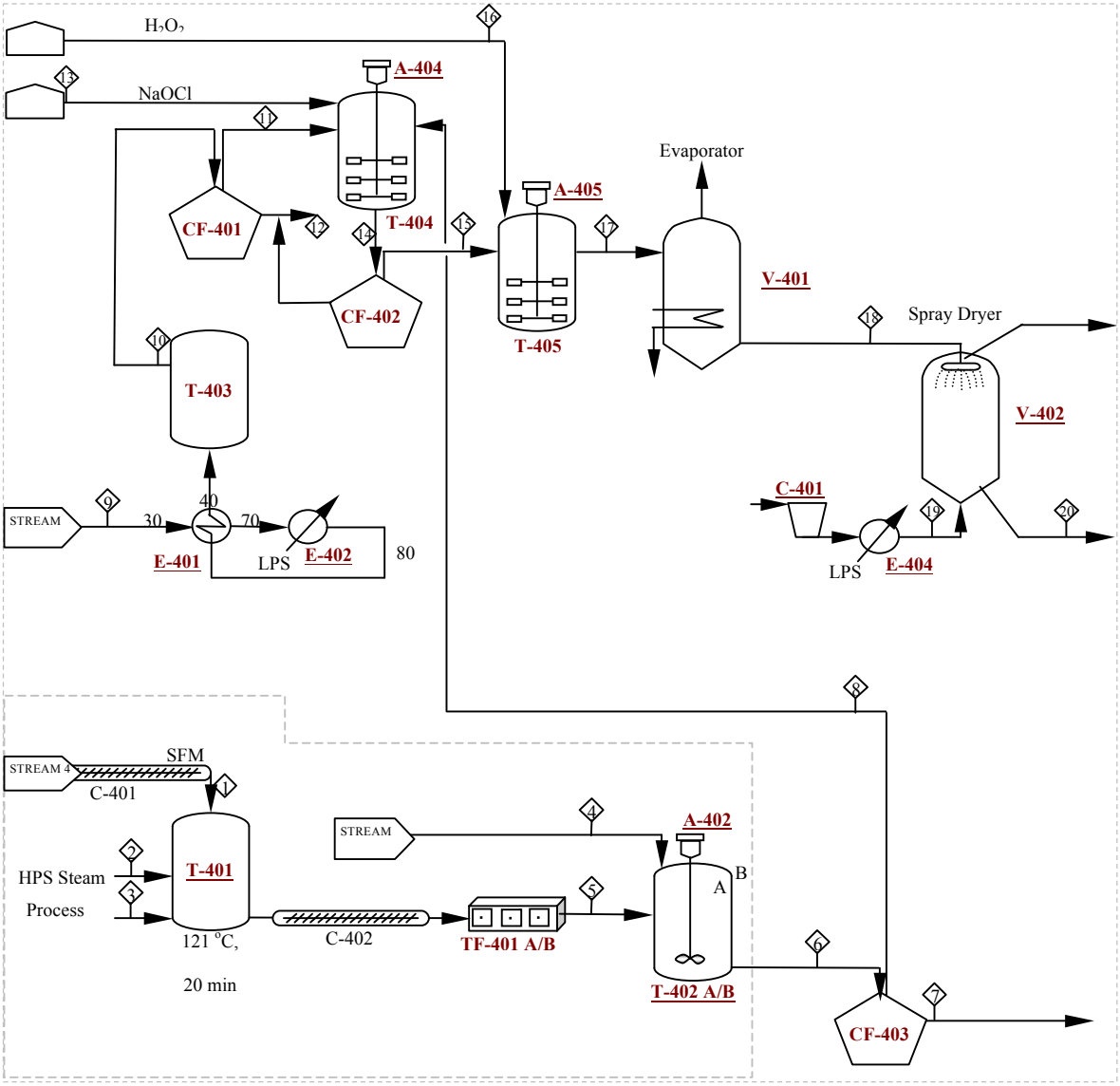

Figure 10.8 Process flow diagram for AREA 400: Production of crude enzyme-rich extract and PHB recovery

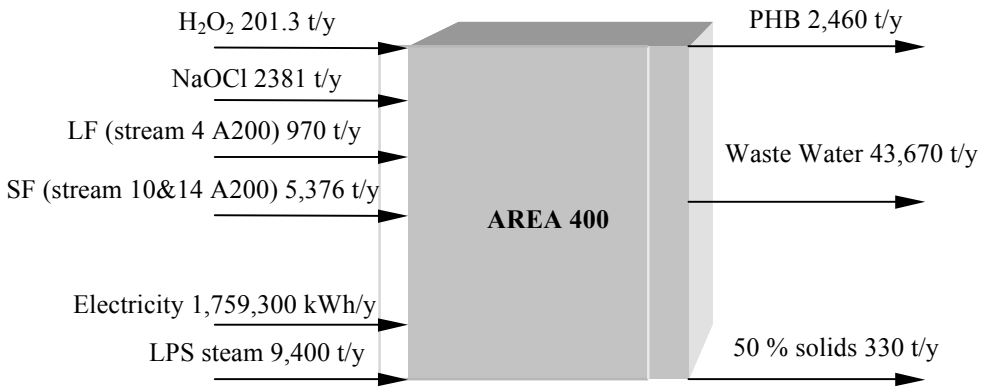

Figure 10.9 Summary of total quantities of input and output streams for PHB recovery including the production of lytic enzymes (AREA 400)

Table 10.7 presents the costs associated with the necessary equipment employed in the process flow diagram of Figure 10.8 along with the characteristic size of each unit operation. The total purchased equipment cost is M$ 2.825, distributed mainly between the mixing tanks, the heat exchanger and the spray dryer. The downstream process for PHB purification requires 9 workers per shift corresponding to an annual labour cost of M$ 1.28. The utilisation of crude enzymes produced on-site from the lignocellulose-rich fraction as substrate for PHB recovery entails a relatively low annual cost of raw materials, considering that only the cost of NaOCl and H_2O_2 are included. Also, the annual cost of utilities is in the order of M$ 0.388.

Table 10.7 AREA 400: Summary of equipment specifications and purchased equipment cost (C_p) for PHB separation and purification

Equipment		Material	Char. Size	fob cost ($)	CEPCI	fob cost ($@2013) CEPCI=567.3
T-401	2 Sterilization tanks	SS304	13.4 m^3 each	2×38,600		112,180
TR-401	2 Tray SS bioreactors	SS304	133 m^2 each	2×50,000		145,300
T-402	2 Mixing tanks	SS304	100 m^3 each	2×220,000		639,400
C-401	Screw conveyor		0.23×100 m	64,800		94,300
C-402	Screw conveyor		0.23×100 m	64,800		94,300
E-401	s&t heat exhanger	SS304	770 m^2	160,000		232,500
E-402	s&t heat exhanger	SS304	25 m^2	9,400	390.4	13,700
T-403	Storage tank	SS304	1340 m^3	228,200		331,600
T-404	Mixing tank	SS304	17.7 m^3	45,700		66,400
A-404	Agitator	SS304	14.3 hp	included		
T-405	Mixing tank	SS304	4.3 m^3	21,000		30,500
A-405	Agitator	SS304	3.4 hp	included		
V-401	Evaporator	SS304	0.175 kg W/s	70,000		101,720
C-401	Compressor	CS	110 hp	56,000		81,400
E-404	s&t heat exhanger	CS	37.5 m^2	6,800		9,880

V-402	Spray Dryer	SS304	0.087 kgW/s	263,000		382,170
A-401	2 Agitators	SS304	190 hp each	2×72640	521.9	157,600
CF-401	Disc stack centrifuge	SS304	5.45 m^3/h	88,560		113,150
CF-402	Disc stack centrifuge	SS304	7.4 m^3/h	93,050	444.0	118,900
CF-403	Disc stack centrifuge	SS304	1 m^3/h	78,400		100,200
						TOTAL M$ 2.825

Table 10.8 AREA 400: Summary of the utilities cost and labour cost calculation for the PHB purification and separation section

Unit operation	Equipment	Workers / shift[a]	Electricity[b] (10^6 kW h/yr)	lps[c] (t/yr)	Utilities cost (M$/yr)
T-401	2 Sterilization tanks	2×0.1=0.2			
TR-401	2 Tray SS bioreactors	2×0.1=0.2			
T-402	2 Mixing tanks	2×0.1=0.2			
C-401	Screw conveyor	0.1	31,700		
C-402	Screw conveyor	0.1	31,700		
E-401	s&t heat exhanger	0.05			
E-402	s&t heat exhanger	0.05		865	
T-403	Storage tank	0.1			
T-404	Mixing tank	0.1			
A-404	Agitator	-	140,990		
T-405	Mixing tank	0.1			
A-405	Agitator	-	25,145		
V-401	Evaporator	0.2		6,683	
C-401	Compressor	0.1	630,280		
E-404	s&t heat exhanger	0.05		1,850	
V-402	Spray Dryer	0.2			
A-401	2 Agitators	2×0=0	650,000		
CF-401	Disc stack centrifuge	0.05	95,040		

CF-402	Disc stack centrifuge	0.05	95,040		
CF-403	Disc stack centrifuge	0.05	59,400		
Total		**9 Workers C_{OL}= 1.28 M$/yr**	**1,759,300**	**9,400**	**C_{UT} = 0.388 M$/yr**

[a] Labour cost $40,000/(y/worker); [b] Electricity cost: $0.06/kWh ; [c] lps Cost: $30/t.

10.7 Estimation of Fixed Capital Investment and Cost of Manufacture

The cost of manufacture (COM) for PHB was calculated by employing two well established equations, previously used in the techno-economic evaluation of microbial oil (Koutinas et al. 2014) and 2,3-butanediol production (Koutinas et al. 2016). The first one (Eq. 10.1) estimates the COM without the depreciation (Koutinas et al. 2016).

$$COM = 0.18\,FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{UT} + C_{WT}) \quad \text{Eq. 10.1}$$

In the case that depreciation is considered then the following equation can be implemented (Eq. 10.2).

$$COM = 0.28\,FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{UT} + C_{WT}) \quad \text{Eq. 10.2}$$

The Fixed Capital Investment (FCI) is estimated based on the total purchased equipment cost (C_p) for each process flow diagram (AREA 100-400) and multiplying each respective FCI with 5. Table 10.9 summarises the cost calculations for the complete process where it is easily observed that the total C_p is M$ 17,6 and the FCI is in the order of M$ 88. Apart from FCI, the operating cost of labour (C_{OL}), the cost or raw materials (C_{RM}) and the utilities cost (C_{UT}) were also calculated.

Table 10.9 Summary of the purchased equipment cost as well as the costs of labour, raw materials and utilities for the four process flow diagrams (AREA 100-400)

	C_p (M$)	C_{OL} (M$)	C_{RM} (M$)	C_{UT} (M$)
AREA 100	4,863,830	200,000	1,232,000	11,470,000
AREA 200	1,312,200	240,000	300,000	304,000
AREA 300	8,603,199	1,280,000	445,000	1,130,919
AREA 400	2,825,200	360,000	1,035,000	388,000
Total (M$)	17,604,429	2,080,000	3,012,000	13,292,920
FCI	88,022,144			

The application of Eq. 10.1 to calculate the COM without depreciation for AREA 100 and AREA 200 for the production of protein isolate and crude phenolic extract (antioxidants) results in a COM of M$ 23.12, while when Eq. 10.2 is used the COM is estimated at M$ 26. Correspondingly, the COM is evaluated implementing both equations for the complete process including PHB production, entailing a COM in the order of M$ 41.57 (Eq. 10.1) and M$ 50.37 (Eq. 10.2). Specifically, regarding the COM per kg of PHB produced, it is estimated using the costs associated with AREA 300 and AREA 400 that employ the formulation of nutrient supplement, the fermentation process and the recovery of PHB. The COM for the production of PHB without depreciation is estimated at 7.5 $/kg PHB, while by using Eq. 10.2 COM is 9.82 $/kg PHB.

In order to assess the viability of PHB commercialisation it is also important to evaluate the revenues of the proposed processes. Integration of sunflower meal processing in a biorefinery concept leads to the production of protein isolate, crude phenolic extract and PHB. The remaining lignocellulose-rich fraction and the solid residue that are not utilised in solid state fermentation and enzymatic hydrolysis sections can be also used as animal feed supplements due to their protein content. Table 10.10 presents a summary of the annual production capacity of products obtained along with the respective commercial market prices.

Table 10.10 Annual production capacity and market value of the products generated during the proposed biorefinery approach

Product	Annual production (t/y)	Market value	
SFM sold as animal feed	76,184	250	US $/t
Glycerol sold as animal feed	5,176	300	US $/t
Protein isolate	7,314	1,250 – 2,500	US $/t
Crude phenolic extract (antioxidants)	2,785	6 – 15	$/kg
Protein rich fraction[a]	30,920	100	US $/t
Lignocellulose-rich fraction[a]	18,973	100	US $/t
PHB	2,460	3000 – 5000	US $/t

[a] denotes the annual remaining quantities that are not utilised in the biorefinery concept

The currently applied practice involves the commercial utilisation of sunflower meal and crude glycerol in the market as animal feed supplements. Sunflower meal and crude glycerol are sold at 250 US$/t and 300 US$/t, respectively, entailing an annual profit of M$ 20,6 deriving mainly from sunflower meal (92.4% of the total revenues). The profitability of the proposed sunflower based biorefinery is significantly related to the various end-products targeting diversified market outlets. The market prices of these end-products may vary significantly as presented in Table 10.10 based on their market outlet. For instance, the protein isolate could be used as animal feed or food supplement leading to significant variations in the market price. In a similar manner, the purity of crude phenolic extract and the content of chlorogenic acid affect the selling price of the antioxidant-rich extract. For this reason, the revenues were assessed based on different market prices of the products in order to provide a rough estimate on the profitability potential of the whole biorefinery concept.

As previously described, the COM was estimated at M$ 41.57 and M$ 50.37 using Eq. 10.1 and 10.2, respectively, while the currently applied practice leads to an annual profit of M$ 20,6. In order to propose an economically feasible approach, the profitability margin should be not only higher than the COM, but also provide a higher revenue than the current situation. Table 10.11 presents the revenues of four cases based on different

market prices of the end-products including the utilisation of current situation as base-case scenario where crude glycerol and sunflower meal are used as animal feed.

Table 10.11 Estimated revenues for different cases based on various market prices of the end-products (The prices of end-products were taken from Table 10.10)

Proposed approach	Revenues (M$)
Case I: Currently applied practice (base-case scenario)	20,599,078
Case II: Low limit market prices for all products	38,221,820
Case III: Average value market prices	55,844,520
Case IV: High limit market prices	77,349,320

It is easily observed that when the market prices of end-products remain at their lower limit and more specifically, 1,250 $/t for the protein isolate, 6 $/kg for the crude phenolic extract and 3,000 $/t for PHB, then the profitability margin is negative (considering also that the cost of manufacture is around M$ 50). Furthermore, revenues estimation using the average values of market prices for each product entails a profitability of M$ 5.5 when the COM is estimated around M$ 50, rising to 14.26 in the case that COM is calculated including depreciation (E. 10.2). These values are still lower than the currently applied practice (base case scenario). In the case where the highest market prices could be achieved the total revenues are significantly increased, reaching M$ 77,35, which is higher than the annual revenue from selling the sunflower meal and crude glycerol as animal feed and the cost of manufacture of all end-products.

In the preliminary calculations presented above, it can be observed that the variations in the market prices of protein isolate and crude phenolic extract were more related to the profitability of the biorefinery concept compared to the market price of PHB. For this reason, incoming cash flow was also examined by maintaining a low market price for PHB (3,000 US $/t) while varying the market price of protein isolate and crude phenolic extract. In particular, the market price of crude phenolic extract presented a more pronounced effect on the revenues compared to fluctuations in protein isolate market price. Figure 10.10 demonstrates the increase of revenues by altering the market price of crude phenolic extract using two different commercial values for protein isolate (1,250 and 2,500 US $/t) while PHB value remains at 3,000 US $/t. A two-fold increase in the value of

crude phenolic extract entailed an increase in revenues in the order of M$ 16, indicating the significance of this end-product in the profitability of the biorefinery concept.

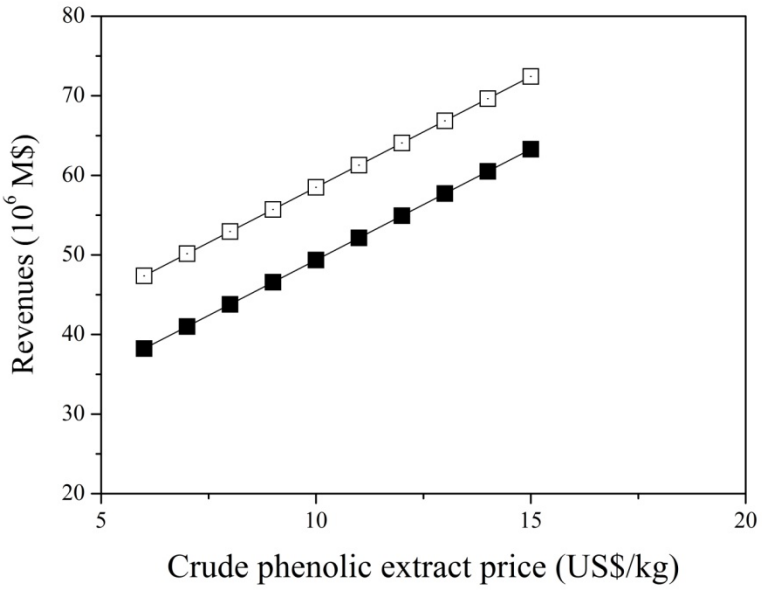

Figure 10.10 Estimation of revenues by increasing the market price of crude phenolic extract by maintaining the market price of protein isolate at 1,250 and 2,500 US $/t and the market price of PHB at 3,000 $/t

The economics of PHB production have been widely studied in the literature. Choi and Lee (1997) presented an economic assessment of PHB production examining different bacterial strains using the respective optimum carbon sources including glucose, sucrose methanol. PHB recovery was evaluated using the sodium hypochlorite digestion method and a dispersion method that lead to high polymer purity (95%). The lowest price (5.58 US$/kg) for an annual production of 2,850 t was attained when *Alcaligens eutrophus* was used, which decreased to 4,75 US$/kg by increasing the capacity to 10^6 t/y. After the process analysis, it was clearly demonstrated that the carbon source accounts for the major operating cost. The PHB yield, intracellular content and volumetric productivity also constitute key step parameters that have to be optimized in order to improve process economics.

Van Wegen et al. (1998) evaluated PHB production from glucose by recombinant *Escherichia coli* for an annual production capacity of 4,300 t, achieving a final price of 5.63 US$@1995 /kg. When whey was evaluated, the final PHB price was reduced to 3.59 – 5.63 US$1995/kg, allowing for further reduction in the case that glucose was combined

with whey as substrate. However, taking into consideration that carbon source is of primary importance in the final PHB market price, recent techno-economic analyses are focused on the evaluation of low-cost substrates. Under this rationale, a techno-economic assessment of PHB production from crude glycerol of two different purities (88% and 98% wt%) was presented by Posada et al. (2011). Lower costs ranging from US$ 1.94 – 2.38 per kg of PHB were achieved when glycerol at 98 wt% was employed depending on the downstream process used. Analysis of PHB production cost from glycerol using *Bacillus megaterium* was also investigated by Naranjo et al. (2013) reaching an industrial price of 2.6 US$/kg, stating a 20% of profit margin. Nonetheless, the majority of the studies conducted in the literature are dedicated solely on the estimation of PHB production cost and very few studies evaluate the profitability of biorefinery concepts that include PHB production among other products.

10.8 Conclusion

The results of this chapter present a preliminary techno-economic assessment of the proposed sunflower-based biorefinery concept. Mass and energy balances were performed to estimate the sizing of the equipment and subsequently the total purchased equipment cost. Calculation of FCI along with C_{OL}, C_{RM} and C_{UT} entailed the estimation of the COM, using well established equations. The overall conclusion that could be drawn from this chapter is that economics of SFM fractionation could be feasible by incorporating the combined production of protein isolate, crude phenolic extract and PHB. The different end-uses of the products will determine their commercial price targeting diversified market outlets, thus significantly affecting the annual revenues and the profitability margin. Therefore, assessing the market demands on specific products would be a key step to develop and further evolve the proposed sunflower-based biorefinery process.

CHAPTER 11

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Biotechnological production of poly(hydroxyalkanoates) exhibits significant importance considering the substitution of conventional petroleum derived plastics. However, as demonstrated in Chapter 2 the main problem hindering the industrial implementation of poly(hydroxybutyrate) production is the high cost of manufacture.

The imminent depletion of fossilised resources has induced a tremendous increase in biofuels production, particularly in biodiesel, as renewable and environmentally benign fuel. The main by-products generated during biodiesel production are oilseed meals and crude glycerol. Consequently, it is evident that novel bioprocessing strategies should encompass the valorisation of these streams for the production of value-added products targeting the development of integrated biorefineries.

Currently, crude glycerol and oilseed meals are implemented as supplements in animal feed. However, the transition towards the bio-economy era will necessitate the exploitation of the abundantly present biomass to formulate specialty chemicals and commodities, along with biofuels. Under this rationale, in the present study, sunflower meal and crude glycerol were evaluated in the development of an integrated sunflower-based biorefinery concept, endeavouring the production of PHB as the major target product. The study was initiated with the development of a two-stage bioprocess that will lead to the formulation of a generic fermentation feedstock for bacterial bioconversions (Chapter 5). Solid state fermentation was carried out to produce crude enzymatic consortia with the fungal strain *Aspergillus oryzae*. Optimisation of solid state fermentation was evaluated based on the optimisation of protease production, considering that protein is the main component in sunflower meal. The maximum proteolytic activity (400 U/g) was observed at 65% (w/w, db) initial moisture content, at 48 h fermentation duration and 30 °C incubation temperature. In the following step, the fermented mass was mixed with unprocessed sunflower meal to perform enzymatic hydrolysis, which was assessed in terms of maximum free amino nitrogen and inorganic phosphorus production. Different parameters affecting enzymatic hydrolysis were evaluated, including pH value, temperature, initial solid concentration and initial enzymatic activity. The maximum free amino nitrogen concentration achieved was 2 g/L when an initial enzymatic activity of 16

U/mL was employed, corresponding to 40 g/L of yeast extract. Apart from amino acids, peptides and phosphorus, the obtained hydrolysate contained significant amount of micronutrients that sustained microbial proliferation.

Subsequent experiments (Chapter 6) focused on the evaluation of sunflower meal hydrolysates combined with crude glycerol as fermentation supplements in bacterial bioconversions with *Cupriavidus necator* sp. An initial screening using three different strains was conducted to select the most promising strain, namely *C. necator* DSM 7237. Optimisation of fermentation conditions was performed in bioreactors operated in fed-batch mode to elucidate the effect of glycerol purity, carbon to nitrogen (C/N) ratio, and assess different bioprocessing strategies. Among the optimum results, total dry weight production reached 37 g/L with an intracellular content of 72.9%, corresponding to a concentration of 27 g/L of PHB when the C/N ratio was 17.05 g/g (Table 6.2). Combined feeding of sunflower meal hydrolysate and crude glycerol led to the production of 32.6 g/L PHB with a 77.2 % (w/w) content.

The effect of levulinic acid as a precursor for the production of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) was evaluated in shake flasks and bioreactor cultivations under the optimum fermentation conditions (Chapter 7). Biosynthesis of 23.4 g/L of P(3HB-*co*-3HV) with a final 3HV content of 22.5 % was accomplished by the additions of a concentrated levulinic acid solution combined with crude glycerol during fed-batch cultures.

Under the viewpoint of developing a sustainable integrated biorefinery, various end-products should be generated, targeting diversified market outlets to increase the economic feasibility of the whole process. Fractionation of sunflower meal (Figure 8.1) was implemented to produce protein isolate of high purity (more than 95%) and antioxidants, consisting primarily of chlorogenic acid. The remaining fractions, namely lignocellulose-rich fraction, solid residue and soluble fraction were combined in solid state fermentation and enzymatic hydrolysis to formulate different nutrient-rich supplements (Figure 8.4 and Figure 8.5) achieving significant concentrations of free amino nitrogen and inorganic phosphrorus. The aforementioned hydrolysates were evaluated in bacterial bioconversions for PHB production. The hydrolysate deriving from the combination of all residual streams during enzymatic hydrolysis exhibited enhanced PHB production up to 57.1 g/L corresponding to an intracellular content of 86.2 % (w/w). The glycerol to PHB

conversion yield accomplished during this study was 0.47 g/g, which is among the highest in the literature.

Apart from utilisation of low-cost feedstock in the bioconversion process, economically feasible production of PHB and P(3HB-co-3HV) could be enhanced by implementing a cost-effective separation process. Thereof, Chapter 9 elaborated the development of a novel downstream separation method based on the utilisation of crude enzymes produced on-site by *A. oryzae* and lignocellulose-rich fraction as substrate in solid state fermentations. Central Composite Design was applied to identify the optimum operating parameters (temperature and pH value) during enzymatic cell lysis. High recovery yields (98 %) and purities (96.7 %) were achieved under uncontrolled pH conditions and 48 °C using high initial TDW concentrations (Figure 9.5 and Figure 9.6). Furthermore, thermal properties and molecular weight for the bacterial P(3HB-*co*-3HV) were evaluated demonstrating properties similar to previously literature-cited publications. The lysate obtained after enzymatic cell lysis was recuperated in bioconversions aiming to minimise waste stream generation.

A preliminary techno-economic evaluation was performed to estimate the costs related with the complete sunflower-based biorefinery leading to the production of antioxidants, protein isolate and PHB (Chapter 10). The Fixed Capital Investment (FCI) that was estimated based on the total purchased equipment cost (C_p) for each designed process was in the order of M$ 88. The cost of manufacture (COM) was calculated using well established equations including FCI along with the cost of raw materials, cost of operating labour and utilities. It was demonstrated that the profitability of the proposed process is closely associated with the different market prices of protein isolate and crude phenolic extract fraction. The overall conclusion that could be drawn from the economics of the developed biorefinery is that integration of sunflower meal fractionation could be feasible by incorporating the combined production of protein isolate, crude phenolic extract and PHB. The different end-uses of the products will determine their commercial price targeting diversified market outlets, thus significantly affecting the overall revenue and the profitability margin.

The results accomplished within the present study on the development of the sunflower-based biorefinery demonstrate a promising approach on the integration of bioprocessing strategies. However, research should be extended under the prospect of

further improving the proposed biorefinery concept. The main subjects that should be addressed include: 1) utilisation of remaining solids after enzymatic hydrolysis, 2) the improvement of bioconversion in terms of enhanced productivity, 3) improvement of thermal properties and molecular weight of the produced polymers, 4) evaluation of the production of levulinic acid from the lignocellulose-rich fraction, 5) Life cycle analysis.

The first research suggestion could target the valorisation of the remaining solid fraction occurring after enzymatic hydrolysis that could be evaluated in bioremediation processes for soil enhancement or as onset material in slow pyrolysis to produce biochar. Another promising approach could be to evaluate the ability to synthesise levulinic acid as platform chemical incorporated in the biorefinery concept. In this way, the levulinic acidrequired for the production of P(3HB-*co*-3HV) could be produced onsite. Levulinic acid could be also formulated by the remaining lignocellulose-rich fraction that is not employed in solid state fermentation for nutrient formulation. Economic analysis should be performed to estimate the feasibility of this process compared to the currently applied practice (i.e. use as animal feed supplement).

Regarding the bacterial bioconversion process for PHB synthesis, even though the results obtained in this study were found among the highest in the literature deriving from crude glycerol, specific aspects could be elaborated to extend the effectiveness. The volumetric productivity could be further improved by substituting the conventional nutrient sources used in inoculum preparation with sunflower meal hydrolysates. It is speculated that by this way the bacterial strain could overcome the lag phase caused by the adaptation in the fermentation stage and enter the exponential phase rapidly.

Regarding the techno-economic analysis performed during this study, a significant correlation among the different end-products was established. Nevertheless, these results comprise of preliminary calculations that include the production of antioxidants, protein isolate and PHB. To better evaluate the feasibility and the profitability margin of the integrated concept, process economics should be extended to include the analysis of the complete process initiated from the oil extraction from the seed that will be used in biodiesel production, along with the utilisation of remaining fractions for heat and energy generation that would lead to reduced utilities cost. A sensitivity analysis should be elaborated together with the estimation of Net Present Value (NPV). The final step towards the complete evaluation on the sustainability of the sunflower-based biorefinery

concept would be the application of Life Cycle Analysis. Development of LCA would enable to pinpoint and modify accordingly energy consuming procedures towards the increased environmental benignness.

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