ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΕΠΙΣΤΗΜΗ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ
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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

«Ανάπτυξη και σχεδιασμός καινοτόμου βιοδιυλιστηρίου που βασίζεται στην αξιοποίηση της οινολάσπης και της ακατέργαστης γλυκερόλης προς παραγωγή προϊόντων προστιθέμενης αξίας και πολυ-3-υδροξυ-βουτυρικού εστέρα μέσω μικροβιακών ζυμώσεων»

Χαραλαμπία Δήμου

Αθήνα, 2016

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

PhD THESIS

«Biorefinery development and design using wine lees and crude glycerol for the production of value-added products and poly-(3-hydroxybutyrate) via fermentation»

Charalampia Dimou

Athens, 2016

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ

ΑΠΟΣΤΟΛΟΣ ΚΟΥΤΙΝΑΣ: Επίκουρος Καθηγητής Μηχανικής Τροφίμων, Τμήμα: Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου, Γεωπονικό Πανεπιστήμιο Αθηνών

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

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ABSTRACT

The transition to the bio-economy era and the replacement of petrochemical processes for the production of chemicals and polymers require the valorization of industrial waste and by-product streams through the development of novel and integrated biorefinery concepts. Furthermore, restructuring the conventional fermentation industry is essential because the production of chemicals and polymers via microbial bioconversion is not sustainable through single stage conversion of commercial and purified raw materials (e.g. glucose, yeast extract). The sustainable development of integrated biorefineries will encompass the production of energy, food, feed, chemicals, biodegradable polymers and biomaterials. Within this context, valorization of industrial food waste and by-product streams could lead to reduced environmental impact and additional revenue to existing industrial sectors.

This PhD thesis has focussed on the development of a novel integrated biorefinery concept using wine lees as raw material for the production of ethanol, calcium tartrate, antioxidant-rich extracts and nutrient-rich fermentation supplement. The material balances were determined when wine lees from a Merlot wine variety were used. The process begins with the separation of the liquid and solid fractions via centrifugation. The solid fraction is fractionated into an antioxidant-rich fraction and calcium tartrate. After the separation of phenolic compounds and tartrate salts from the solid fraction of wine lees, the remaining solids were rich in yeast cells. The yeast cells were subsequently lysed using crude enzyme-rich extracts derived via solid state fermentation of *Aspergillus oryzae* carried out on wheat milling by-products. The optimal temperature, pH, reaction duration, initial solid concentration and initial proteolytic activity of the hydrolysis reaction were optimized. The highest free amino nitrogen and inorganic phosphorus concentration achieved were around 1400 mg/L and 130 mg/L, respectively, at pH value of 5.5, temperature of 40 °C, duration of 48 h, initial solid concentration of 75 g/L and initial proteolytic activity of 12 U/mL.

This PhD thesis identified that it is feasible to combine the extraction of value-added products with the production of a nutrient-rich generic feedstock that could be used as fermentation supplement for the production of poly(3-hydroxybutyrate) (PHB). Various feedstocks were initially evaluated for the production of PHB via fermentation including hydrolysates from flour-rich wastes produced by a confectionery industry, rapeseed meal

hydrolysates and crude glycerol derived from biodiesel production processes and wine lees derived hydrolysates. Optimisation of fed-batch bioreactor cultures mainly focused on PHB production using crude glycerol and wine lees derived hydrolysates using the bacterial strain *Cupriavidus necator* DSZM 7237. Shake flask and bioreactor cultures showed that PHB production was triggered by phosphorus depletion. The highest fermentation efficiency was achieved when the crude glycerol and wine lees derived hydrolysates were supplemented with trace elements using an initial carbon to free amino nitrogen ratio of 12.3 g/g leading to a total dry weight of 47.8 g/L, a PHB concentration of 36.9 g/L and a productivity of 0.56 g/L/h. Therefore, wine lees derived hydrolysates could be used as supplement for the production of PHB when crude glycerol is used as carbon source.

The biorefinery concept developed based on the refining of wine lees for the production of ethanol, antioxidant-rich extract, calcium tartrate and yeast cells was evaluated via process design and preliminary techno-economic evaluation. The material and energy balances were validated using the commercial process simulator UniSim (Honeywell). A sensitivity analysis was carried out, based on discounted cash flow analysis, so as to estimate the minimum selling price of the antioxidant-rich extract that should be achieved at different plant capacities in order to develop a profitable wine lees refining process. The minimum selling prices of the antioxidant-rich extract was identified in the range of 122 – 11.06 $/kg in order to develop profitable refining schemes with wine lees processing capacities varying from 500 to 5000 kg/h considering 120 days of annual plant operation. The final products could be used in various industrial segments including food, chemical and cosmetic industries.

ΠΕΡΙΛΗΨΗ

Η μετάβαση στην εποχή της βιο-οικονομίας και η αντικατάσταση των πετροχημικών διεργασιών για την παραγωγή χημικών ουσιών και πολυμερών απαιτούν την αξιοποίηση βιομηχανικών αποβλήτων και παρα-προϊόντων ως πρώτες ύλες για την ανάπτυξη καινοτόμων βιοδιυλιστηρίων. Το γεγονός ότι η συμβατική βιομηχανία ζυμώσεων χρησιμοποιεί εμπορικές πρώτες ύλες (π.χ. γλυκόζη, εκχύλισμα ζύμης) για την παραγωγή χημικών ουσιών και πολυμερών καθιστά τις διεργασίες αυτές μη αειφόρες. Η αειφόρος ανάπτυξη βιοδιυλιστηρίων θα πρέπει να συμπεριλάβει την συνδυασμένη παραγωγή ενέργειας, τροφίμων, ζωοτροφών, χημικών ουσιών, βιοαποικοδομήσιμων πολυμερών και βιοϋλικών. Σε αυτά τα πλαίσια, η αξιοποίηση των παραπροϊόντων και των αποβλήτων της βιομηχανίας τροφίμων μέσω της δημιουργίας βιοδιυλιστηρίων θα βελτιώσει το περιβαλλοντικό αποτύπωμα και τα έσοδα των συμβατικών διεργασιών.

Η παρούσα διδακτορική διατριβή επικεντρώθηκε στην ανάπτυξη ενός καινοτόμου βιοδιυλιστηρίου με στόχο την αξιοποίηση της οινολάσπης ως πρώτη ύλη για την παραγωγή αιθανόλης, τρυγικών αλάτων, ενός εκχυλίσματος πλούσιο σε φαινολικά συστατικά και ενός υδρολύματος πλούσιο σε θρεπτικά συστατικά το οποίο είναι κατάλληλο ως θρεπτικό μέσο για μικροβιακές ζυμώσεις. Τα ισοζύγια μάζας κατά την βελτιστοποίηση της ανάπτυξης του βιοδιυλιστηρίου υπολογίστηκαν στην περίπτωση που χρησιμοποιήθηκαν οινολάσπες που προήλθαν από την ερυθρά οινοποίηση σταφυλιών της ποικιλίας Μερλότ. Κατά το πρώτο στάδιο της διεργασίας διαχωρίζονται τα στερεά από την υγρή φάση των οινολασπών μέσω φυγοκέντρησης. Ακολούθως, τα στερεά συστατικά χρησιμοποιούνται για τον διαχωρισμό ενός εκχυλίσματος πλούσιο σε φαινολικά συστατικά και τρυγικών αλάτων. Το στερεό υπόλειμμα είναι πλούσιο σε κύτταρα ζυμών, τα οποία παρήχθησαν κατά τη διάρκεια της οινοποίησης. Το στερεό αυτό κλάσμα χρησιμοποιήθηκε για την βελτιστοποίηση διεργασίας παραγωγής υδρολύματος πλούσιο σε θρεπτικά συστατικά που δύναται να χρησιμοποιηθεί ως θρεπτικό μέσο για μικροβιακές ζυμώσεις. Η λύση των κυττάρων ζύμης πραγματοποιήθηκε με την χρήση ακατέργαστων ενζύμων που παρήχθησαν μέσω ζύμωσης στερεάς κατάστασης από το μύκητα *Aspergillus oryzae*. Η μέγιστη παραχθείσα συγκέντρωση αζώτου που περιέχεται στις ελεύθερες αμινομάδες αμινοξέων και πεπτιδίων ήταν 1400 mg/L, ενώ η μέγιστη παραχθείσα συγκέντρωση φωσφόρου ήταν 130 mg/L. Οι βέλτιστες συνθήκες υδρόλυσης ήταν pH 5,5, θερμοκρασία 40 ^{o}C, 48 ώρες διάρκεια υδρόλυσης, 75 g/L αρχική συγκέντρωση του στερεού κλάσματος

των οινολασπών, και 12 U/mL αρχική πρωτεολυτική ενεργότητα.

Η παρούσα διδακτορική διατριβή επικεντρώθηκε επίσης στην παραγωγή του βιοαποικοδομήσιμου πολυμερούς πολυ-3-υδροξυ-βουτυρικού εστέρα (ΠΥΒ) από διάφορα βιομηχανικά παραπροϊόντα και απόβλητα. Αξιολογήθηκε η χρηση υδρολυμάτων που παρήχθησαν μέσω υδρόλυσης αλευρούχων υπολλειμάτων της βιομηχανίας παραγωγής προϊόντων ζαχαροπλαστικής, η ακατέργαστη γλυκερόλη, υδρολύματα κραμβαλεύρων και υδρολύματα που παρήχθησαν από οινολάσπες προς την μικροβιακή παραγωγή ΠΥΒ. Πραγματοποιήθηκε βελτιστοποίηση ζυμώσεων παραγωγής ΠΥΒ σε βιοαντιδραστήρα με χρήση του βακτηριακού στελέχους *Cupriavidus necator* DSZM 7237 που αναπτύχθηκε σε μείγμα ακατέργαστης γλυκερόλης και υδρολυμάτων πλούσιων σε θρεπτικά συστατικά που παρήχθησαν από οινολάσπες. Η μέγιστη συγκέντρωση ΠΥΒ που επιτεύχθηκε ήταν 36.9 g/L και η παραγωγικότητα ήταν 0.56 g/L/h. Επομένως, τα υδρολύματα που προκύπτουν από την διύλιση των οινολασπών δύνανται να χρησιμοποιηθούν για την μικροβιακή παραγωγή του ΠΥΒ.

Το βιοδιυλιστήριο που αναπτύχθηκε με βάση την ολιστική αξιοποίηση των οινολασπών αξιολογήθηκε μέσω σχεδιασμού διεργασιών και εκπόνηση προκαταρκτικής τεχνο-οικονομικής μελέτης. Τα ισοζύγια μάζας και ενέργειας της διεργασίας πραγματοποιήθηκαν μέσω της χρήσης του λογισμικού UniSim (Honeywell). Ακολούθως, αξιολογήθηκε η επίδραση της τιμής πώλησης των αντιοξειδωτικών συστατικών προκειμένου να αξιολογηθεί η βιωσιμότητα της διεργασίας στην περίπτωση που επεξεργάζονται διαφορετικές ποσότητες οινολασπών σε ετήσια βάση. Η ελάχιστη τιμή πώλησης του ρεύματος που είναι πλούσιο σε αντιοξειδωτικά συστατικά κυμαίνεται από 122 $/kg έως 11.06 $/kg όταν η ολική ποσότητα οινολασπών που επεξεργάζονται ετησίως κυμαίνεται από 500 kg/h έως 5000 kg/h.

«Rien ne se perd, Rien ne se crée, Tout se transforme»

“Antoine-Laurent De Lavoisier, 1789”

ΕΥΧΑΡΙΣΤΙΕΣ

Θα ήθελα να ευχαριστήσω τον επιβλέποντα της διατριβής μου Επίκουρο Καθηγητή κ. Απόστολο Κουτίνα για την επίβλεψη σε θέματα μηχανικής βιοδιεργασιών, παραγωγής βιοπολυμερών μέσω μικροβιακών ζυμώσεων, ανάπτυξη και σχεδιασμό βιοδιυλιστηρίων, και εκπόνηση τεχνο-οικονομικών μελετών. Επίσης θα ήθελα να ευχαριστήσω τον Αναπληρωτή Καθηγητή κ. Σεραφείμ Παπανικολάου για την άριστη και πολύ σημαντική συνεργασία. Αξιοσημείωτη ήταν επίσης η συμβολή της Επίκουρης Καθηγήτριας κα. Μαντάλα Ιωάννας που με στήριξε καθόλη τη διάρκεια της εκπόνησης της διδακτορικής μου διατριβής και η οποία ήταν πάντα διαθέσιμη προκειμένου να την συμβουλευτώ σε θέματα που άπτονται της μηχανικής τροφίμων. Φυσικά θα ήθελα να ευχαριστήσω τον Αναπληρωτή Καθηγητή κ. Ιωάννη Κούκο για την παρα πολύ σημαντική συμβολή του κατά την εκπαίδευσή μου στο αντικείμενο του σχεδιασμού διεργασιών.

Δεν θα μπορούσα να παραλείψω να αναφέρω και τον μεταδιδάκτορα ερευνητή Δρ. Κοψαχείλη Νικόλαο για την άριστη συνεργασία που είχαμε κατά την πραγματοποίηση της ερευνητικής δουλειάς. Αξιοσημείωτη ήταν επισης η συμβολή και η συνεργασία με τον μεταδιδάκτορα ερευνητή Δρ. Ανέστη Βλυσσίδη. Επίσης, θα ήθελα να ευχαριστήσω τον Λέκτορα κ. Αθανάσιο Μαλούχο και την Δρ. Αυγή Γαρδέλη για την βοήθεια που μου πρόσφεραν κατά την εκπαίδευση της χρήσης της αέριας χρωματογραφίας.

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PUBLICATIONS AND PRESENTATIONS

Peer reviewed international scientific journals

1. **Dimou C**, Vlysidis A, Kopsahelis N, Papanikolaou S, Koutinas AA, Kookos IK. 2016. Techno-economic analysis of wine lees valorisation for the production of high value added products. Biochemical Engineering Journal. 116: 157 - 165.

2. **Dimou C**, Kopsahelis N, Papadaki A, Papanikolaou S, Kookos IK, Mandala I, Koutinas AA. 2015. Wine lees valorization: Biorefinery development including production of a generic fermentation feedstock employed for poly(3-hydroxybutyrate) synthesis. Food Research International. 73: 81-87.

Conferences and Workshops

1. Papadaki A, Kopsahelis N, **Dimou C**, Xenopoulos E, Papanikolaou S, Koutinas AA. 2015. Biorefinery development based on integrated cheese whey and wine lees valorisation. Dairy Waste Valorisation Workshop, Cost Action WG4, 10-11 March, Semmering, Austria.

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3. **Dimou C**, Mantala I, Papanikolaou S, Mantalaris A, Koutinas A, Kopsahelis N. 2012. Valorization of wine lees as renewable resource for biorefinery development. The 5th International Conference on Industrial Bioprocesses (IFIB-2012), Taipei, Taiwan, 7-10 October.

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

Αφιερωμένο,

στην οικογένεια μου που με υπομονή με στήριξε όλα αυτά τα χρόνια

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CHAPTER 1

INTRODUCTION

1.1 Sustainable development

In general, sustainability can be considered as "*the interaction among the biological or environmental system, the economy system and the society system*" (Barbier, 1987). The most common definition attributed to sustainability is defined as "*Sustainable development is the development that meets the needs of the present without compromising the ability of future generations to meet their own needs*" (www.un-documents.net/ocf-02.htm, accessed 05/2016). In fact, this definition contains two key elements: 1) The concept of basic needs of the world's poor, in which absolute priority must be given. 2) The idea of limitations induced from technological ability and social state to the environment's capability to satisfy present and future needs (Butlin, 1989).

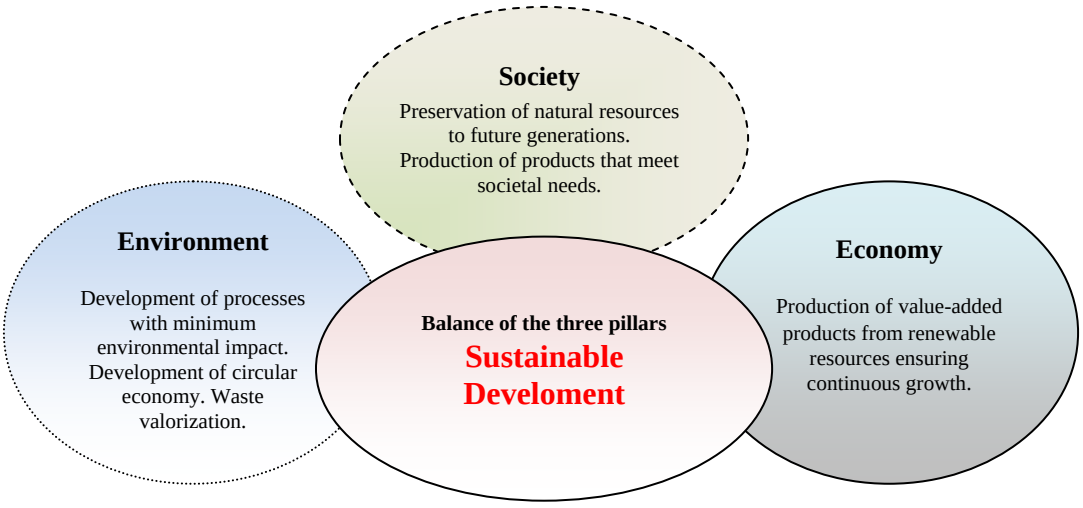


Figure 1.1: The three pillars of sustainability

Many authors have described sustainability as a three domain system: "Society, Environment and Economy" (sustainabledevelopment.un.org/globalsdreport/, accessed 9/2015) or "Economy, Equity, Ecology" (Figure 1.1), which occurs at the point of equilibrium. This three-dimensional system has been expanded to a fourth-dimensional

system trying to embody other pylons such as “Culture” or “Governance and Insitutions” (Scerri and James, 2010).

1.2 Biorefineries: Definition and concept

It is well known that the utilization of fossil fuels results in increasing greenhouse gas emissions (GHG), such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). The Earth’s climate is affected by GHG emissions, overexploitation of land and various human activities. Awareness of environmental pollution, concerns about fossil fuel supplies on global level, increasing demand for energy and consumer products along with problematic recycling poses the need of re-examination of petroleum economy (IPCC. 2007). As a viable alternative to fossil refineries, the development of biorefineries demonstrate new routes for the production of energy, fuel and chemicals using renewable resources as raw materials.

One of the definitions of biorefining is the following “*Biorefining is the transfer of the efficiency and logic of fossil-based chemistry and substantial converting industry as well as the production of energy onto the biomass industry*” (Kamm *et al.*, 2005). A definition reported by the National Renewable Energy Laboratory defines that “*A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass*” (www.nrel.gov/biomass/biorefinery.html, accessed in 9/2015).

The biorefinery concept is based on the exploitation of organic carbon molecules extracted from plants, so as to replace carbon from oil and gas. Biorefinery strategies aim to minimise GHG emissions and the formation of waste promoting the use of renewable resources having as ultimate goal the protection of the environment. Renewable resources are consisted of different components. Each one can be extracted and functionalized leading to the production of various intermediate products. In general, renewable resources are mainly consisted of carbohydrates, lignin, proteins and fats/oils. They also contain in lower quantities various minerals, vitamins and bioactive compounds. On that basis, many biorefineries can be developed based on the fractionation of different renewable resources.

As described in Figure 1.2, biomass could be processed in different stages in order to produce different products. Various biomass sources are initially processed for the extraction of various components as end-products. Biomass could be fractionated into different fractions via physical, biological and/or thermochemical technologies. These steps constitute the conventional agro-industrial sector providing raw materials in the form of end-products or residues and wastes for the development of the second industrial sector that will flourish in the bio-economy era. The future sustainable processes will utilize renewable resources as raw materials for the production of fuels (e.g. biodiesel, ethanol), bioactive compounds, bio-based polymers, chemicals, materials and energy.

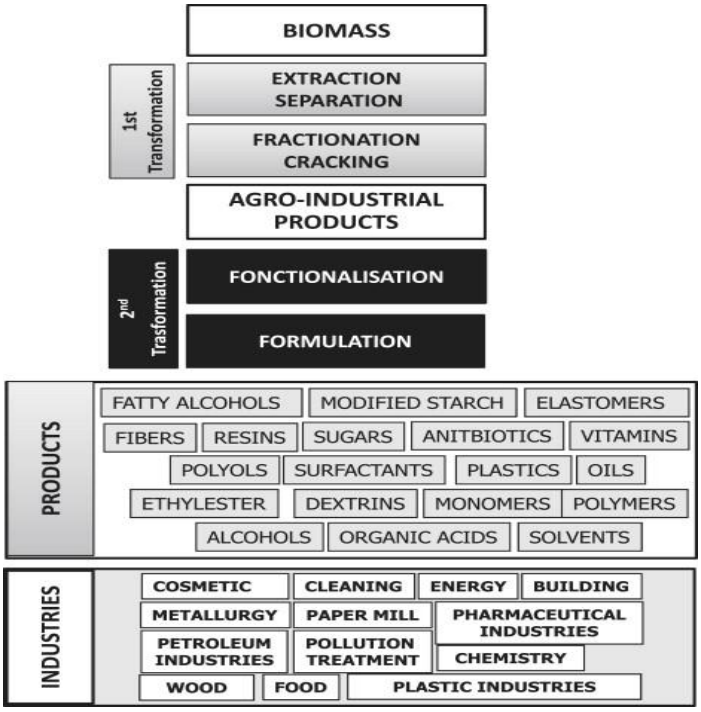


Figure 1.2: Global schematic diagram of biorefineries starting with the fractionation of biomass components followed by the utilization of agro-industrial products as raw materials by second transformation industries leading to the production of end-products with various industrial applications (Industries and Agroresources Competitiveness: www.iar-pole.com, accessed 6/2015)

The aim of this thesis is to develop an innovative biorefinery concept for the production of several value-added products from wine lees and crude glycerol, such as nutrient-rich supplements for fermentations, ethanol, tartrate salts, antioxidants and bio-

degradable polymers. This thesis embraces the idea of sustainable development by implementing a biorefining strategy for holistic valorization of agri-industrial wastes and by-products.

1.3 Definition of renewable resources

Renewable resources are resources or materials that can be produced naturally in the environment and their utilization in food and non-food applications does not lead to depletion as the can be naturally replenished. These raw materials can derive from the agriculture or the forestry sector and when they are used in non-food applications the carbon emitted to the environment has already been utilized for plant growth. In this way, there is no new emission of carbon to the environment.

The renewable feedstocks used for chemical and polymer synthesis can be classiffied as first and second generation biosources. The first generation biosources are sources that come from plants and are mainly destined for food and feed consumption, such as corn, potatoes and other carbohydrate-rich crops. The second generation of biosources are industrial feedstocks of agri-industrial origin, mainly waste and by-products streams or agricultural residues that are not used for food and feed purposes (Bozell, 2008; Elizondo-Villarreal *et al.*, 2012).

1.4 Definition and classification of bio-based polymers

The vast majority of fossil resources are consumed for fuel production. A small fraction of fossil resources (ca. 10-12%) are used for the production of chemcials and polymers. Polymer production holds the greatest part of this proportion (Clark *et al.*, 2006). Taking into account, the increasingly consumption of fossil fuel, it has been estimated that by 2040 the oil price will increase significantly due to increasing oil demand. Considering that petroleum refining leads to the production of numerous polymers that play a significant role in the economy, it is vital to find sustainable ways for the production of polymers via biorefining of renewable resources.

Polymers from renewable materials or so-called “biopolymers” can be produced mainly by three different routes (Flieger *et al.*, 2003). They can by extracted directly from

biomass (e.g. starch, protein, cellulose. Biopolymers can be produced directly via fermentation by different microorganisms (e.g. polyhydroxyalkanoates). Finally, the monomers can be produced via fermentation followed by concentional or novel polymerization routes (e.g. polylactic acid). Some bio-based polymers are also biodegradable.

1.5 Valorization of renewable sources

The usage of renewable sources, such as waste or by-product streams of various industries, as raw materials to produce value-added products could lead to reduced environmental impact due to reduced waste production, GHG emmissions and energy utilisation. A wide range of high value-added products, such as enzymes, biofuels, organic acids, biopolymers, and chemicals, can be obtained by upgrading renewable raw materials by biotechnological processes (Figure 1.3). Future biorefineries will rely entirely on renewable raw materials using all components of biomass in order to produce the least possible amount of waste (Koutinas *et al.*, 2001; 2004; 2005; 2007a; 2007b).

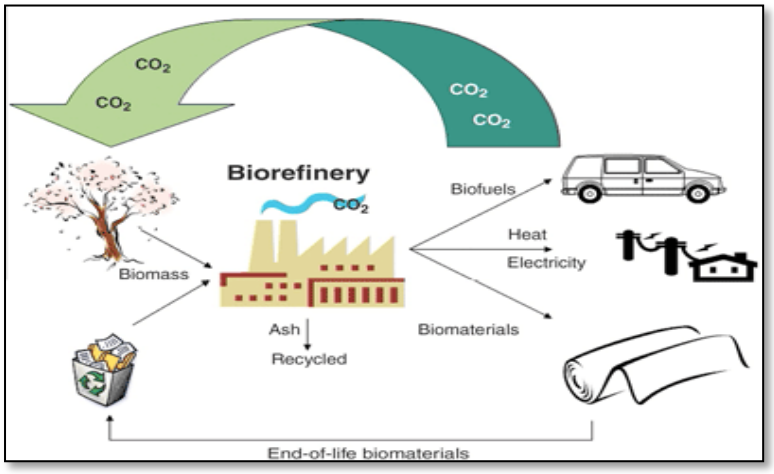


Figure 1.3: Schematic representation of valorization of biomass to produce biofuels, biomaterials and energy in biorefineries (Ragauskas *et al.*, 2006)

For instance, bran-rich wneat milling by-products due to their chemical composition could be evaluated for their ability to produce enzymes, biopolymers and

platform chemicals through microbial fermentations. Wheat milling by-products find various applications in innovative biorefineries as cheap and renewable raw materials (Koutinas *et al.*, 2004). According to Pandey *et al.* (1999), upgrating of wheat bran through the production of enzymes is a possible alternative. Du *et al.* (2008) reported the production of 48 U/g glucoamylase and 64 U/g protease in ideal conditions from wheat milling by-products.

1.6 Major contribution of the thesis

The work presented in this PhD thesis mainly focusses on the following aspects:

1. The development of a novel biorefinery concept using wine lees as raw material derived nowadays as waste stream from conventional wineries. The developed biorefinery led to the production of potable ethanol, antioxidant-rich extracts, tartrate salts and nutrient-rich hydrolysates.

2. The production of the biodegradable polyester poly(3-hydroxybutyrate) (PHB) has been investigated using different industrial waste and by-product streams. Crude glycerol and rapeseed meal were used for the formulation of fermentation medis that were produced by biodiesel production processes. Flour-based waste streams were used for PHB production that were produced by a confectionery industry. Wine lees were also used in combination with aforementioned carbon sources for PHB production.

3. The production of PHB has been optimized in bioreactor cultures using wine lees derived hydrolysates and crude glycerol.

4. Process design and techno-economic evaluation of the novel refining concept has been carried out to evaluate the profitability of the developed concept.

CHAPTER 2

BY-PRODUCTS FROM BIODIESEL PRODUCTION PROCESSES

2.1 Definition of biofuels and biodiesel

Serious environmental issues arise from the increasing demand and consumption of fossil fuels. Air pollution, greenhouse effect, acid rain, and pollution of water and soil resources are just some of them. Taking into account that fossil fuels are not renewable, finding and using alternative energy sources, is a matter of high priority. For this reason, the production of biofuels has been the focus of research and industrial development since the 1970s.

Biofuels are liquid or gaseous fuels produced from biomass (Directive 2009/28/ EC, article 2). According to Greek legislation, law number 3468/2006 (as amended by 3734/2009, 3851/2010, 3889/2010) and directive 2009/28/EC, biomass is the biodegradable fraction of products, waste and residues from agriculture, including vegetal and animal substances, forestry and related industrial activities, as well as the biodegradable fraction of industrial waste matter and municipal sewage and garbage.

Biodiesel is the most applicable biofuel in Europe. The European standard EN 14214:2012 defines biodiesel as fatty acid methyl esters (FAME) derived from biologically produced oils or fats, including vegetable oils, animal fats and microalgal oils using methanol for transesterification. When other alcohol is used for fatty acid-alkyl-ester production, the biodiesel formed is not covered by this standard. FAMEs are used, mainly, in compression ignition (diesel) engines. According to the American Standard ASTM D6751, biodiesel or biodiesel blends are designated to mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats. In the case of biodiesel blends, B100 stands for pure biodiesel and B20 stands for 20% biodiesel and 80% fossil diesel.

2.2 Biodiesel production

Biodiesel is a biodegradable, non-toxic, almost sulfurless and non-aromatic environmentally friendly alternative diesel fuel. Its usage brings together serious social, environmental and economic advantages sush as dicreased global warming, rural regrowth

and creation of new jobs (Kiss *et al.*, 2008). Over the last decades, the biodiesel industry is rapidly growing. Figure 2.1 shows that in 2014, US, Brazil and Germany were ranked first, second and third as major biodiesel producers worldwide with a production volume of around 4.7 billion liters for the USA and 3.4 billion liters both for Brazil and Germany. Indonesia, Argentina and France follow with a production volume of 3.1, 2.9 and 2.1 billion liters, respectively.

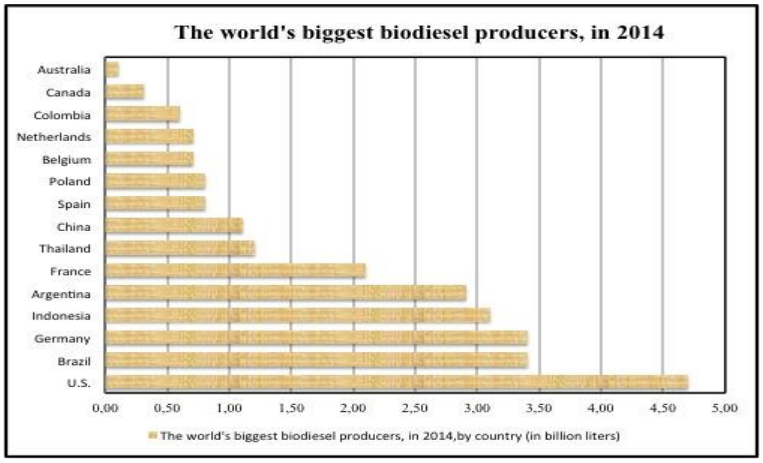


Figure 2.1: Biodiesel production capacity of different countries in 2014 (Statista. The statistics portal. 2015)

2.3 Raw materials for biodiesel production

Biodiesel production is mainly based on catalytic transesterification of vegetable oils or animal fats. These resources are mainly oils extracted from oilseeds, animal fats and used cooking oils. Besides soybeen and cottonseed, rapeseed and sunflower are the main oilseeds used for the industrial production of long chain-(mainly mono)-alkyl-esters (Ma and Hanna, 1999). The Common European Agricultural policies favour the use of rapeseed and sunflower oil for diodiesel production (Panoutsou *et al.*, 2008).

2.3.1 Sunflower seeds

The common sunflower **(***Helianthus annus*) is a large annual forb, cultivated not only for its edible oil but also as raw material for biodiesel production. Many parameters affect the quantity of the extracted oil from sunflower seeds. Growing conditions, post-

harvest seed handling, and the method used for the extaction of oil from the sunflower seed (chemical or mechanical methods) influence the production of sunflower oil (www.farmandranchguide.com/news/markets/sunflower-production-for-up-percent-over-last-year/article_05b5bc92-a24f-11e4-8064-0b8ba7cda9f8.html, accessed 07/2015). The sunflower oil is a mixture of mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids, mostly oleic acid (ca. 30%) and linoleic acid (ca. 60%).

Wan *et al.* (1979) concluded that there were no obvious differences in the composition of the studied sunflower varieties, regarding oil, protein, crude fiber and ash contents (Table 2.1). Sunflower meal is the by-product stream (Figure 2.2) that remains after the extraction of oil. The sunflower meal is used traditionally as animal feed, especially for ruminants (Chuntracort *et al.*, 2014). In recent years, there is a trend towards the fractionation of biomass for the production of value-added products, such as protein concentrates, antioxidants and polysaccharides, having a wide range of different end-uses, such as food and feed additives and bioactive peptides preventing chronic deseases (Kannan *et al.*, 2012).

Table 2.1: Average chemical composition of various sunflower seeds (Wan *et al.*, 1979)

Seed-part	Oil	Nitrogen	Protein (N x 6.25%)	Ash	Crude fiber[1]	Gross pigment content [2]
Achene	37.9-49.1	3.1-4.3	19.6-27.1	3.7- 4.3	15.2-20.4	-
Kernel	49.0-55.0	4.3-4.7	26.7-29.6	3.3-3.9	2.1-2.7	2.8-3.86
Hull	1.9-2.9	0.6-0.9	3.7-5.4	2.9-3.5	60.4 -61.5	-
Achene	31.7-34.3	2.7-3.6	16.8-22.6	-	22.5-27.7	-
Kernel	52.6-56.2	4.6-5.5	28.6-34.2	1.6-1.7	2.0-3.8	3.09-3.4
Hull	0.9-2.9	0.6-0.7	3.7-4.6	1.7-4.1	62.8-68.4	-

[1] represents moisture free basis,
[2] g Clorogenic acid per 100 g

Figure 2.2: Sunflower meal

2.3.2 Rapeseed

Rapeseed (*Brassica napus*) is cultivated for edible oil and biodiesel production, while the pressed cake is used for animal feed (Voća *et al.*, 2005). In 2007, 10.2 billion liters of biodiesel was produced worldwide. Around 60% of the worldwide production capacity was produced in Europe (Baka and Roland-Holst, 2009). Rapeseed is the most important oilseed cultivated in most European countries (Bureau *et al.*, 2009; Thamsiriroj and Murphy, 2010). In 2008, rapeseed accounted for 79% of all raw materials used for biodiesel production in Europe (Ajanovic, 2010).

The rapeseed meal contains anti-nutritional constituents (Table 2.2) that have the possibility to cause negative effects in animal health. It has been reported, that these adverse effects are caused by the constituents interfering with absorption of nutrients or minerals (Mikić *et al.*, 2009). These constituents include phytic acid (Koutinas *et al.*, 2007a), precursors of toxic compounds such as glucosinolates (Tranchino *et al.*, 1983) and fibre content (Pal Vig and Walia, 2001) (Table 2.2). Therefore, rapeseed meal is usually mixed with other supplements and used as animal feed supplement for cattle, swine and poultry (Rutkowski, 1971).

In France, albumins and globulins (proteins) are extracted via a pilot-scale process from rapeseed meal (Chabanon *et al.*, 2007). Membrane filtration and chromatography have been used to purify cruciferin (globulin), napin (albumin) and lipid transfer proteins (LTP). Bérot *et al.* (2005) extracted 200 g of cruciferin, 42 g of napin and 5 g of LTP from 3.5 kg of rapeseed meal. Rapeseed meal has been used as source of nutrients for the production of various enzymes. Enzymes such as laccase (Hu and Duvnjak, 2004), phytase and protease (El-Batal and Karem, 2001; Koutinas *et al.*, 2007a) have been produced by solid state fermentation processes. Rapeseed meal has been used as raw material for the production of a generic fermentation feedstock via combined solid state fermentation

followed by enzymatic hydrolysis. Wang *et al.* (2010) used that feedstock to grow *Saccharomyces cerevisiae* using glucose as carbon source.

Table 2.2: Average chemical composition of *Brassica* sp. meals (Bell and Jeffers, 1976)

Composition of rapeseed meal	
Component	**Mean value**
Moisture (%)	7.7
Crude protein (N x 6.25) (%)	35.9
Crude fiber (%)	11.2
Ether extract (%)	3.8
Ash (%)	6.5
Isothiocyanate (mg/g)	
Butenyl	1.9
Pentenyl	1.0
Total	2.9
Oxazolidinethione (mg/g)	4.1
Total glucosinolates	7.0

2.4 Industrial production of biodiesel and crude glycerol

Biodiesel is produced via transesterification of triglycerides. After the production of biodiesel, several processing steps should take place in order to purify the biodiesel, recycle the methanol from the glycerol-rich stream, and increase the content of glycerol in the final crude glycerol stream. The main by-product from biodiesel production is crude glycerol.

Nowadays, industrial processes for biodiesel production mainly utilize the alkali-catalyzed process of vegetable oils, such as rapeseed and sunflower (Figure 2.3). Apart from oils or fats, the transesterification reaction needs an alcohol and a catalyst. The alcohol mostly used is methanol and the reaction of transesterification is called mathanolysis, forming fatty acid methyl esters or biodiesel and crude glycerol. Methanol is more preferrable than ethanol, due to its low market price, fast reaction with triglycerides

and high dissolution in alkali catalyst. The most widely used catalysts are sodium methoxide ($NaOCH_3$), potassium methoxide ($KOCH_3$) and sodium or potassium hydroxide (NaOH, KOH). Of these, the most widely used is sodium hydroxide because is cheaper than the other catalysts (Ma and Hanna, 1999).

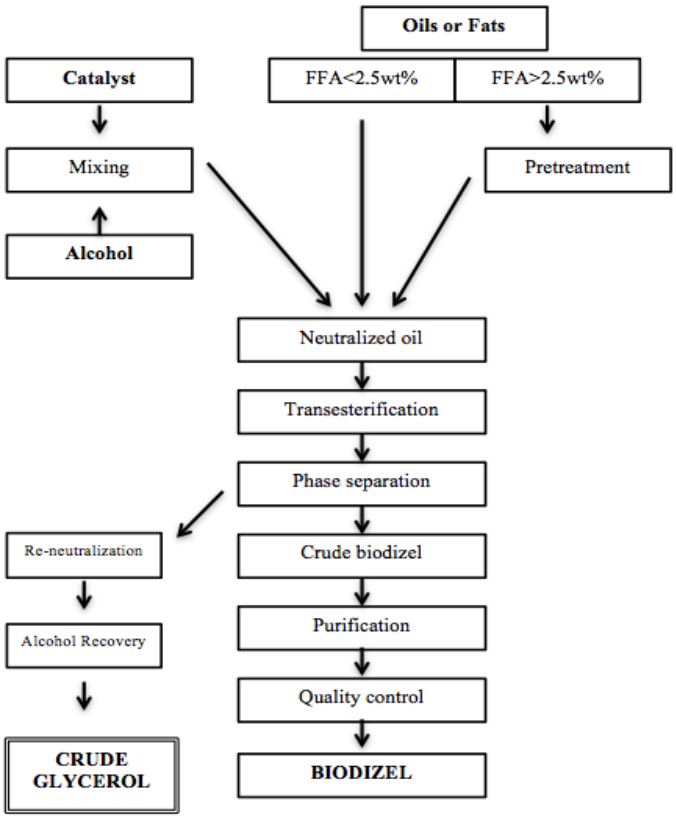


Figure 2.3: Simplified process flow chart of biodiesel production process

The main steps used in the biodiesel production process are presented below:

1) Evaluation of feedstock and pretreatment steps aiming to minimize the content of free fatty acids (FFA): One of the main parameters for commercial biodiesel production is the quality of raw material used and specifically the water and free fatty acid content. If the oil/fat used as feedstock contains more than 2.5% (w/w) FFA, the alkali catalyzed reaction is not the appropriate one. Figure 2.4 shows the reaction that occurs between FFA and NaOH leading to the formation of soaps (www.istc.illinois.edu/tech/small-scale-biodiesel.pdf, accessed 05/2016).

Figure 2.4: Saponification reaction of sodium hydroxide and FFA forming soap and water

Many pretreatment methods have been proposed to reduce the content of FFA. Steam distillation, extraction with an alcohol (Turkay and Civelekoglu, 1991) and esterification by acid catalysis (Zhang *et al.*, 2008) are some of them mentioned in literature-cited publications. The most common pretreatment is esterification of the FFA with methanol in the presence of acid catalysts, usually sulfuric acid (Di Serio *et al.*, 2005).

2) Mixing of methanol with the catalyst: The alkali catalyst, such as NaOH or KOH, is mixed slowly with methanol leading to the formation of methoxide before it is mixed with the vegetable oil or fat. Alkali catalyst must be in excess to avoid reaction with FFA (Weisz *et al.*, 1979).

3) Trasesterification between triglyceride and alcohol followed by separation: When triglycerides, the alcohol and alkali catalyst are mixed in a stirred reactor, transesterification takes place following the reaction presented in Figure 2.5. Once transesterification has been completed, two phases are formed containing the esters and glycerol. The separation of the two phases is achieved via decanting leading to the production of a glycerol-rich phase and a biodiesel-rich phase. Both phases also contain the catalyst, alcohol, oil, and soap. Although glycerol tends to contain a higher percentage of these compounds, significant amounts of these compounds are also present in the biodiesel-rich fraction (www.ampc.montana.edu/documents/policypaper/policy22.pdf, accessed 10/2015).

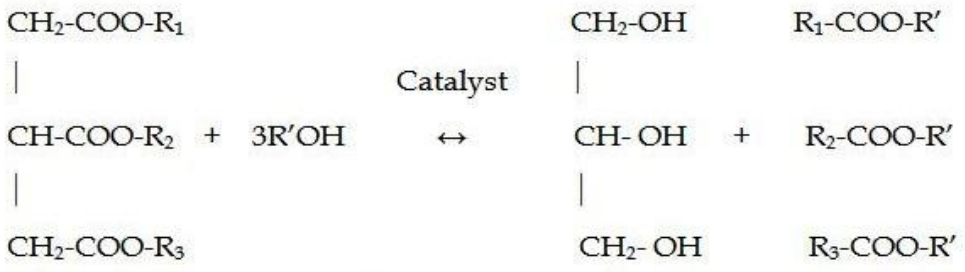


Figure 2.5: Transesterification reaction

4) Purification of the crude glycerol stream: The crude glycerol stream is usually purified in order to increase the final end-uses of this stream, such as cosmetics, soaps and phasmaceuticals (Wen *et al.*, 2008; Da Silva *et al.*, 2009; Wang *et al.*, 2001; Whittington, 2006). The transesterification leads to the production of 10% (w/w) glycerol (Anand and Saxena, 2012). In most biodiesel production plants, the purity of crude glycerol is usually in the range of 77-91% (w/w). High purity glycerol can be achieved via distillation.

The glycerol-rich fraction that is separated after the transesterification process contains the catalyst, unreacted alcohol, soaps, salts, water and various impurities. The remaining alkali catalyst is usually neutralized by an acid. Hydrochloric acid or sulfuric acid is added into the glycerol-rich phase during the neutralization step producing salts, such as sodium chloride or potassium sulfate, the latter can be recovered for use as fertilizer (Duncan, 2003). After the neutralization step, the unused alcohol in the crude glycerol phase is recycled to the transesterification step via distillation. When the alcohol used in the transesterification stage is ethanol, an azeotrope mixture with water is formed. Thus, the complete recovery of ethanol via distillation is not possible and the use of molecular sieves for the removal of water could be applied (Gerpen, 2005).

5) Purification of biodiesel: After the separation of the glycerol-rich phase, the biodiesel-rich phase contains the catalyst, ureacted alcohol, water, free glycerol and soap. Neutrilisation, washing with water, acid treatment and drying are employed for the purification of biodiesel (Gerpen, 2005; Bertram *et al.*, 2005; He *et al.*, 2006).

2.5 Crude glycerol composition

The annual production capacity of crude glycerol follows in an analogous manner the production of biodiesel (Figure 2.1) because based on the stoichiometry crude glycerol is generated as approximately 10% (w/w) of the biodiesel produced. This means that every gallon (3.78 L) of biodiesel produced, around 1.05 pounds (0.475 kg) of glycerol is generated. The composition of crude glycerol produced by different biodiesel production plants varies. The main factors that affect the composition of crude glycerol are the following:

1) The nature of the feedstocks used as oils and fats and the impurities they contain.

2) The efficiency of the transesterification process.

3) The type of catalyst used.

The effect of these factors on crude glycerol composition has been studied in the literature and its variation is presented in Table 2.3. As mentioned earlier, in most cases biodiesel production is carried out by using methanol and sodium or potassium hydroxide as the corresponding alcohol and alkaline catalyst. The main impurities that may be present in crude glycerol streams are methanol, soap, catalysts, salts, various organic components, water and other minor impurities. Thompson and He (2006) reported that different feedstocks led to different glycerol purities. Mustard seed feedstocks had a low purity level of glycerol (62 %), soy oil feedstock had slightly higher purity of glycerol (67.8 %), while waste vegetable oil had even higher level of glycerol purity (76.6 %). González-Pajuelo *et al.* (2005) and Mu *et al.* (2006) reported that crude glycerol purity could be in the range from 65 % to 85 % (w/w). Asad-ur-Rehman *et al.* (2008) reported that the crude glycerol produced during biodiesel synthesis from sunflower oil contained 30 % (w/w) glycerol, 50 % (w/w) methanol, 13% (w/w) soap, 2% (w/w) moisture, approximately 2-3 % (w/w) salts and 2-3 % other impurities.

Table 2.3: Chemical composition of crude glycerol

Components	Percentage (%)	Reference
Ash	0 – 7	Kerr *et al.*, 2009
Glycerol	47 – 91	Mothes *et al.*, 2007; Ooi *et al.*, 2001 Mu *et al.*, 2006; González-Pajuelo *et al.*, 2005
Methanol	0 – 28	Hansen *et al.*, 2009; Pyle, 2008
Moisture	0.3 – 24	Kerr *et al.*, 2009
pH	4 – 9	Kerr *et al.*, 2009
Salts	0 – 7	Cavalheiro *et al.*, 2009; Kerr *et al.*, 2009 Mothes *et al.*, 2007

Mu *et al.* (2006) reported that, despite utilizing identical feedstocks, the crude glycerol formed had differences in concentration depending on the catalyst (alkali or

lipase) employed for the transesterification reaction. The salt content can be as high as 5 % (w/w) and the residual methanol content could be as high as 32 % (w/w) (Rittman *et al.*, 2008). Thompson and He (2006) stated that crude glycerol might contain small quantities of metals such as Na, Ca, K, Mg, Na, P, and S in concentrations varying from 4 to 163 ppm, apart from Na, which could exceed 1%.

2.6 Valorization of crude glycerol

Crude glycerol is produced along with biodiesel during transesterification of triglycerides. Accordingly, since the biodiesel industry is growing, the same production rate is also followed in the case of crude glycerol production. Crude glycerol can be used as feed ingredient in cattle, swine and poultry animals becoming a good source of energy (Mach *et al.*, 2009; Lammers *et al* 2008a; 2008b). Despite the applications of purified glycerol, such as ingredients in personal care (e.g. toothpaste, soap, extracted herbs with glycerol), pharmaceuticals (e.g. sweetener, levigating agent) and food industry products (e.g. food and drink additive, sweetener), the high production capacity of glycerol and the inability of small biodiesel producers to purify the glycerol necessitates the development of processes utilizing crude glycerol of lower glycerol purities. Thus, cost-competitive processes utilizing lower grade glycerol should be identified. This would have several benefits for producers, consumers, the economy and the environment promoting sustainable development. Finding alternative usages of raw glycerol would:

1) Decrease the total biodiesel production cost in the growing global market
2) Enable small biodiesel producers to identify ways of valorizing crude glycerol
3) Lead to the production of bio-based products
4) Convert crude glycerol into a valuable feedstock for chemical and polymer production

The core focus on the innovative valorization of crude glycerol follows two main routes: chemical or biological conversion. It has been reported that chemical conversions of crude glycerol could lead to the production of dihydroxyacetone, ketomalonic acid (a building block for household detergents), propylene glycol, acrolein and chlorinated products (Chiu *et al.*, 2005; Kraft *et al.*, 2007; Koutinas *et al.*, 2007a,b). Etherification can

yield glycerol tertiary butyl ether, which can be used as an additive in transport fuels (including biodiesel) (Johnson and Taconi, 2007). Hydrogen or syngas can be also produced from crude glycerol via gasification (Sereshki *et al.*, 2010; Slinn *et al.*, 2008). Bioconversion leads to the production of a broad spectrum of fine and platform chemicals in milder conditions than those generated via chemical conversion, while in the same time environmental benefits are attained. Some fermentation products derived from crude glycerol that have been widely studied are presented below:

1) 1,3 propanediol by anaerobic fermentation using the strains *Klebsiella pneumonia* (Mu *et al*, 2006; Oh *et al.*, 2008), *Clostridium butyricum* (Papanikolaou *et al.*, 2003; 2008) and mixed cultures from sludge (Dietz and Zeng, 2014).

2) Citric acid by fed-batch fermentation using *Yarrowia lypolytica* (Kamzolova *et al*, 2011; Rymowicz *et al.*, 2008; 2009).

3) Hydrogen and ethanol by *Rhodopseudomonas palustris* (Sabourin-Provost and Hallenbeck, 2009) and *Enterobacter aerogenes*, leading also to simultaneous production of ethanol (Ito *et al.*, 2005).

4) Lipids by *Schizochytrium limacinum* SR21 (microalgal fermentation) and *Cryptococcus curvatus* (fed-batch cultures of yeast) (Liang *et al.*, 2010a; 2010b).

5) Docosahexaenoic acid (DHA) (22:6, n-3) and eicosapentaenoic acid (EPA) (20:5, n-3) by fermentation of the algae *Schizochytrium limacinum* (Ethier *et al.*, 2011; Pyle, 2008).

6) Other metabolites such as succinic acid (Scholten *et al.*, 2009), glyceric acid (Habe *et al.*, 2009), glycolipid-type biosurfactants and glycolipids (Liu *et al.*, 2011), fumaric acid (Zhou *et al.*, 2014), and dihydroxyacetone (Liebminger *et al.*, 2014) among others.

7) Poly(hydroxyalkanoates) (PHAs) from various microorganisms as it will be presented in Chapter 4.

CHAPTER 3

BY-PRODUCT AND WASTE STREAMS FROM WINERIES

3.1 Intoduction

Wine production is one of the most important agri-food sectors in the world. Vinification describes the process of winemaking. Winemaking involves a series of steps carried out during the production of wine from grapes. Wine is the product obtained from the alcoholic fermentation of fresh grapes or must. It is a seasonal activity, mainly performed during autumn. Around 60-70% of the liquid streams generated are obtained 3 months after starting the process (Torrijos and Moletta, 2003).

In 2015, worldwide wine production reached 275.7 million hectoliters. The biggest wine producers were France (48.9 million hectoliters, Mhl) followed by Spain (36.6 Mhl), the United States of America (22.1 Mhl), Argentine (13.4 Mhl), Chile (12.87 Mhl), Australia (12 Mhl) and New Zealand (2.4 Mhl) (www.oiv.int/oiv/info/en_OIV_Press_Conference_23_October_2014, accessed 01/2015). Although European Union is still the world leading wine producer, in recent years, the production of wine in Europe follows a slightly declining trend. For instance, a 6% decline was observed from 2013 to 2014. Yet, the worldwide production is almost constant. This is a concequence of the increasing wine production in other countries from 2013 to 2014 according to OIV statistics, e.g. Argentina 15.2 Mhl (+1%), South Africa 11 Mhl (+4%), New Zeeland 3.2 Mhl (+29%). According to European Union Commision reports, in September 2015, Europe accounts for 45% world wine growing areas, 57% of global production and 70% of exports in global terms (ec.europa.eu/agriculture/wine_en, accessed 01/2016).

3.2 Environmental concerns and management of winery wastes

Along with wine production, large volumes of both liquid and solid waste are produced. The quantity and quality of these waste streams depend on the wine type and the production techniques and practices used. These streams are organic solid waste (e.g.

skins, seeds, marc etc.), wastewater, emissions of greenhouse gases (e.g. CO_2, volatile organic compounds, etc.) and inorganic wastes (diatomaceous earth, bentonite clay, and perlite) (Musee *et al.*, 2007). For instance, grape stalks are traditionally burnt in the field causing significant environmental problems through the release of toxic compounds (polycyclic aromatic hydrocarbons) and greenhouse gases. It has been estimated, that only in Europe around 14.5 million t per year of winemaking waste streams are produced (Chouchouli *et al.*, 2013).

Considering waste derived from the vinification process the main arising problems are: 1) Managing and handling the huge quantities of wine by-products produced during a small period of time (August-October), and 2) Environmental pollulion derived from discharging effluents in open areas (a usual practice followed). Wine residues resist to biological degradation due to specific pollutant characterists (low pH value and high concentration of phytotoxic and antibacterial phenolic compounds). Moldes *et al.* (2007; 2008) and Arienzo *et al.* (2009) stated that wine lees and marc might cause adverse phytoxic effects when applied in crops or wetlands, respectively.

Bustamante *et al.* (2005) exploring uses of winery and distillery effluents in agriculture mentioned that wastewater generated in Spain was six times higher than the total amount formed in France and Italy, mainly due to the low cost of disposal fees. Bustamante *et al.* (2005) attributed incompatibility of agricultural requirements with winery waste properties. Analyzing different remnants from the wine industry found that these streams were charactised by: 1) low pH (mean values ranged from 3.8 to 6.8) and electrical conductivity (1.62 - 6.15 dS/m), 2) high organic matter content (669 - 920 g/kg), 3) high concentrations of macronutrients and especially K (11.9 - 72.8 g/kg), 4) high concentrations of polyphenols (1.2 - 19.0 g/kg), and 5) low concentrations of micronutrients and heavy metals. The environmental issues arising from winery waste disposal include water pollution, soil degradation, damage to vegetation, odours and air emissions, noise from vehicles and equipments (Kumar and Christen, 2010). Thus, enviromental pollution issues and disposal difficulties of winery wastes are attributed to the seasonal production of these streams and their composition.

3.3 Legislative framework and policies for winery waste disposal and managment

According to the European Council Regulation (EC) No 479/2008, on the common organisation of the market in wine and (EC) No 491/2009, amending Regulation (EC) No 1234/2007, establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation), "*grape marc (GM)*" and "*wine lees (WL)*" are considered by-products and must be sent to alcohol distilleries, aiming to extract alcohol and tartrates. The producing outcome streams from distillation are "*exhausted grape marc (EGM)*", the waste in solid state, and "*vinasse (V)*" in liquid form. Nonetheless, low production capacity wineries do not abide by the above regulations and generate grape marc and wine lees together with grape stalk as organic waste. On the other hand, the aerobic depuration of the winery effluents, vinasse and winery wastewater, generates another solid waste, i.e. winery-sludge.

The wine sector, due to the huge quantities of highly concentrated by-products with suspended solids and organic matter, such as wine lees, should find ways to minimize its environmental impact. ISO series and especially 14001:2015 and its supporting standards 14006:2011 focusing on the development of eco-designed systems, have foreseen requirements needed for environmental management and minimization of pollution risks from hazardous industrial waste (www.iso.org, accessed 6/2015). Alternatives like recycling or valorization of winery waste could, if proper employed, contribute by all meens to sustainable development.

3.4 Winery waste and by-product streams

Winemaking involves a sequence of steps for the conversion of grapes to wine, as it is demonstrated in Figure 3.1. Wine is the product obtained from total or partial alcoholic fermentation of fresh raw grapes. Several unit operations are required for the implementation of the winemaking process. Generally, white wine derives from the fermentation of a *clarified must*, obtained after the removal of stems and the subsequent pressing of grape berries. On the other hand, red wines come from the fermentation of a non-clarified must, which in turn comes from destemming of grapes and crushing of grape clusters. Musts, for red winemaking, are mostly macerated with stems, seeds and seeds to extract color and aroma compounds. After the fermentation, decanting, clarification and

maturation follows before bottling, which marks the end of the vinification process (Figure 3.1)

Throughout the duration of relevant steps described, having differences in the technological processes followed for different wine products, various winery by-products and residues are generated (Asselin and Delteil, 2003). These output fractions are characterized by high concentrations of biodegradable compounds and suspended solids (Navarro *et al.*, 2005). The main by-products generated from wineries and distilleries, in different stages are divided into two categories: A) Solid and semi-solid by-products and wastes: grape stalk, grape marc, wine lees and B) Liquid waste: wastewater.

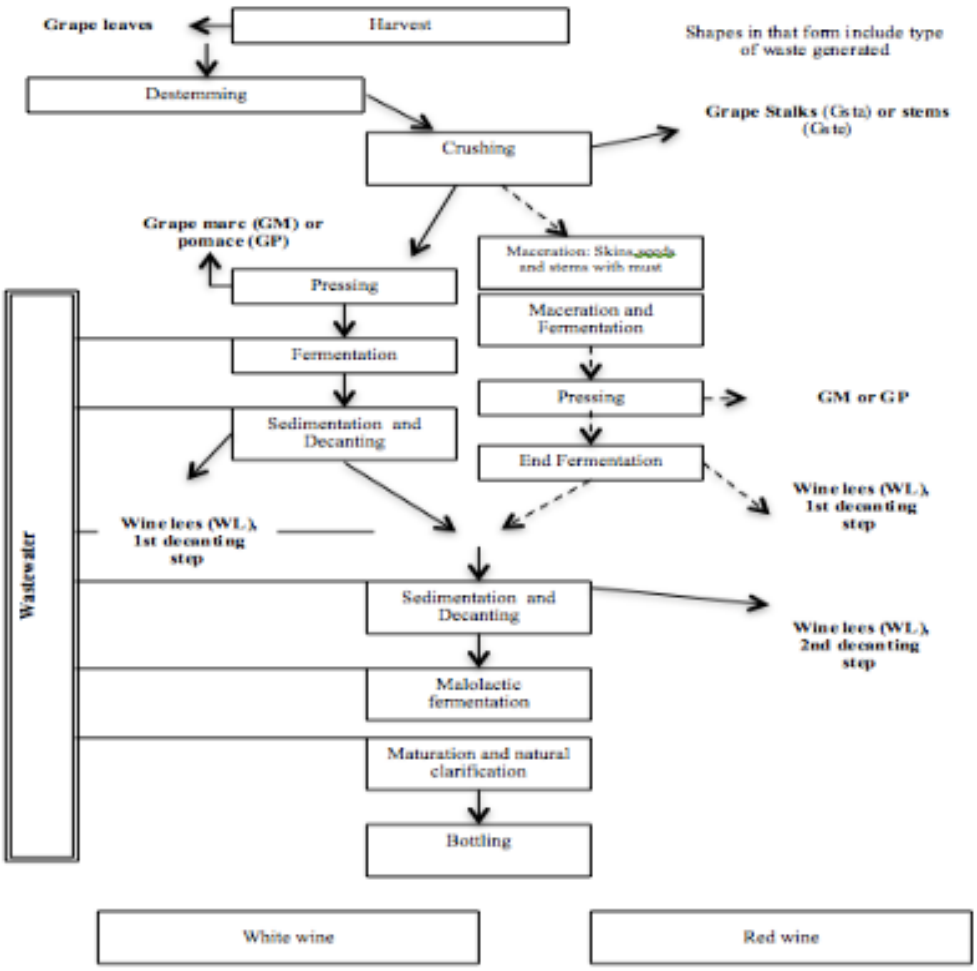


Figure 3.1: Schematic diagram of the vinification process of white and red wine production highlighting solid and liquid waste and by-products generation in relevant steps

3.4.1 Grape stalks or stems

Grape stalks or stems are obtained after the separation of stems and prunings from grapes. This solid by-product is nowadays obtained from machines that destem and simultaneously crush (breaks the skins to pour out the juice) the clusters (Figure 3.2).

Figure 3.2: Destemming process: separation of stalks or stems mechanically and manually (www.aubaimema.com/en/about-vinification.html (image on the left side), www.smith-haut-lafitte.com/en/smith-haut-lafitte-bio-precision.html (image on the right side), accessed 09/2015)

Lignocellulosic biomass, such as that formed from grape stalk is a rich source source of C5 and C6 sugars, namely glucose, xylose, mannose, galactose and arabinose. These monosaccharides derived via combined thermochemical and enzymatic treatment of lignocellulosic biomass leading to hemicellulose and cellulose hydrolysates could be converted into value-added chemicals and polymers via fermentation. In integrated usage of grape stalks, sugars could be used as fermentation media to carry out sequential lactic acid and xylitol production by *Lactobacillus rhamnosus* and *Debaryomyces hansenii*, respectively (Rivas *et al.*, 2007).

Hydroxycinnamic (mostly ferulic and p-coumaric) and hydroxybenzoic (mostly gallic) acids, are the main antioxidant chemical compounds present in grape stalks. In biorefineries yielding sugar-rich hydrolysates, the refining of grape stalks should begin with the extraction of antioxidants followed by combine thermochemical and enzymatic pre-treatment of grape stalks. Max *et al.* (2009) proposed a sequential treatment process involving pre-hydrolysis and alkaline hydrolysis. Pre-hydrolysis refers to acid hydrolysis

pretreatment so as to solubilize hemicellulose fraction followed by alkali hydrolysis for delignification, yielding at the end of lignin hydrolysis phenolic acids including ferulic and coumaric acids. These antioxidant-rich extracts could find applications in food, pharmaceutical and cosmetic sectors (Ou and Kwok, 2004).

3.4.2 Grape marc or pomace or pressed cake

"Grape marc" is the residue formed after the pressing of fresh grapes, whether or not fermented (EC 491/2009). "Exchausted grape marc" is the residue obtained from distilleries after the extraction of ethanol from grape marc, used for the production of spirituous liquors (e.g. distilled beverages). Grape marc contains hemicellulose as grape stalks. It has been used for the bioconversion of sugars to lactic acid (xylose and glucose could be used for lactic acid production by *Lactobacillus pentosus*) and production of intracellular biosurfactants or extracellular emulsifiers (Portilla-Rivera *et al.*, 2007; Portilla-Rivera *et al.*, 2010). Vatai *et al.* (2009) and Conde *et al.* (2011) used organic solvents, supercritical carbon dioxide and isothermal autohydrolysis for the extraction of phenolics with andioxidant activity from grape marc. Another economical and environmentally friendly process regarding grape marc valorization has been proposed by Ping *et al* (2011) was the extaction of condensed tannins from grape pomace for adhesive applications. Diaz *et al.* (2009) studied the production of hydrolytic enzymes (e.g. xylanases, exo-polygalacturonase), through solid state fermentation of mixtures of grape pomace and citrus peels. Using of compost or vermicompost derived from wine marc as fertilizer has been also studied by Martinez *et al.* (2016) and Paradelo *et al.* (2010).

3.4.3 Wastewater

Wastewater in wineries is mainly produced through washing operations during the crushing and pressing of grapes and rinsing of the mechanical equipment used for the winemaking process (Pettruccioli *et al.*, 2000). Wastewater volumes and pollution loads varies over the working period of the vinification process (e.g. racking, bottling) and the technologies followed for the production of different oenological products such as white, red and special wines. Washing operations are carried out several times in many different

winemaking periods in: 1) vintage (washing and disinfection of materials), 2) grape reception (destemmers, hoppers, conveyors, floors, crushers), 3) fermentation and clarification (tanks, vats, floors).

3.4.4 Wine lees

Wine lees are waste streams formed during the winemaking process containing high quantities of suspended solids. This waste stream should be recycled, valorized or treated before disposal, since it can cause environmental problems. According to the European Council Regulation (EC) no 491/2009 on the common organisation of agricultural markets, wine lees, known also as dregs (EC Regulation no 337/79) are defined as "the residue that accumulates in the bottom of vessels or recipients containing wine after *fermentation*, during *storage* or after *authorised treatment*, as well as the residue obtained after *filtration* or *centrifugation*" (Figure 3.1).

Wine lees in literature are also mentioned as dregs or "heavy" and "light" lees. "Heavy" wine lees could be defined as the sediment that precipitates from the wine, immediately, within the first 24 h after the completion of fermentation. This stream contains large particles (larger than 100 μm) such as grape derived particles, agglomerates of yeasts, tartrate salts and protein-polysaccharide-tannin complexes. "Light lees" refer to the residue precipitated from the winemaking process after the first 24 h that the fermentation has been successfully concluded. "Light lees" mainly contain smaller particles than "heavy lees" (1-25 μm) composed of yeasts, tartrate salts, protein-tannin complexes, and some polysaccharides. Both types of wine lees contain ethanol. Mostly, "heavy" wine lees refer to the first decanding step and "light lees" to the second decanting step (Naziri *et al.*, 2012).

Generally, wine lees represent 2 – 6% of the total volume of wine produced. More specifically, after the first drawing lees represent the 4 – 6% (v/v) of the wine produced, while this percentage is decreased reaching a value of 0.5 – 1.5% (v/v) after the second drawing (Bai *et al.*, 2008). One main feature that is very interesting in chemical concentration of wine lees is the high presence of nitrogen compounds contained. The sediment that accumulates at the bottom of the containers, as mentioned before, consists mainly of: 1) yeast cells propagated during fermentation, 2) precipitated tartrate salts, 3)

proteins, 4) pectins and 5) alcohol usually proportional to the amount contained in the produced wine (Pérez-Serradilla *et al.*, 2011). It has to be highlighted that the conentration of individual components of lees (Table 2.1) vary significantly depending on the chemical composition of the wine and the vegetation period among other factors. For this reason, the composition of wine lees is highly affected by the winemaking technology. According to Bustamante *et al.* (2005), wine lees are characterized by: 1) pH values ranging from 3 to 6, 2) chemical oxygen demand (COD) values higher than 30,000 mg/L, 3) organic matter content in the range of 900 and 35,000 mg/L, 4) potassium concentrations higher than 2,500 mg/L, and 5) phenolic components in quantities higher than 1000 mg/L. Table 3.1 presents indicative composition of wine lees.

Table 3.1: Indicative composition of dried wine lees

Components	Percentage (%)
Moisture	9.26
Potassium hydrogen tartrate	18.03
Calcium tartrate	1.04
Crude proteins	33.81
Oil	1.10
Ash	5.40
Fibers	2.11

3.5 Valorisation of wine lees

The food industry is nowadays forced to integrate technologies for waste valorization leading to a more eco-friendly industrial production. Within this concept, wineries have started to evaluate technologies for waste minimization or valorisation. In many cases, waste prevention is not feasible so it is of utmost importance innovative procedures to be designed, developed and implemented so as to recycle, reuse and recover valueable components from winery waste streams including wine lees. This approach is consistent with the last few years trend of increasing demand of bio-based products derived from waste streams, which are nowadays considered as industrial feedstocks of the

sustainable bio-economy era. Valorization of wine lees can minimize operating costs of wine production and minimize the costs for treatment and disposal of wine lees. Besides economic benefits, the wineries will also benefit by reducing the environmental impact of winemaking promoting sustainable development.

It is crucial to mention that all of the potential uses of wine lees, as raw materials for the production of different products, depend on: 1) the market demand and value of the obtained products, 2) the management strategy of the waste adopted by the winery. Various literature-cited studies and commercial applications have focused on the valorization of wine lees taking advantage of the rich chemical composition of wine lees. Some of these applications are presented below:

Wine lees for the production of biocontrol agents against pathogens

Bai *et al.* (2008) evaluated the production of biocontrol agents against plant diseases via solid-state fermentation of *Trichoderma viride* WEBL0703 using wine lees as substrate. It has been stated that this strain was capable of producing chitinase, β-glucanase and pectinase with yields equal to 47.8 U/g, 8.32 U/g and 9.83 U/g, respectively. Chitinase and β-glucanase can hydrolyze pathogens (fungal) cell wall (Markovich and Kononova, 2003) and pectinase can evoke plant disease resistance (Bai *et al.*, 2004).

Wine lees in soil conditioning

The use of vinification lees and grape marc as soil conditioner seems to be a possible alternative, under circumstances such as the stabilization of organic matter and minimization of environmental effects of this type of waste. Some research has been done in using wine lees as growing media for plants. Although wine residues might potentially be used for soil conditioning or fertilizing, the implementation of non-decomposed wastes or non-stabilized compost to soil may lead to immobilization of plant nutrients and exert phytotoxicity effects (Butler *et al.*, 2001). Phytotoxic effects have been noted in germination of cress (*Lepidium sativum* L) (Moldes *et al.*, 2007). Paradelo *et al.* (2010) investigated the optimal conditions for obtaining plant substrates. It is claimed that this

could be achieved by composting 1:1 mixtures of hydrolyzed grape marc and vinification lees in the presence of 5 g $CaCO_3$ per 100 g of hydrolyzed grape marc.

Value-added products extracted from wine lees

Wine lees are nowadays used for potable alcohol production (EC no 479/2008). According to Greek national regulation (Joint Ministerial Decision, no 50910/2727, 2003), wine lees must be collected in containers and delivered to suitable authority having the respective permission for transferring to a distillery for the commercial production of ethanol. It has been estimated that from 100 kg of wine lees, 4 - 8 L of ethanol can be recycled (ca. 95 %, v/v). The recovered ethanol from wine lees is used as solvent, combustible or additive to distilled beverages depending on purity and alcohol content of the final product (Braga *et al.*, 2002).

Calcium tartrate could reach values equal to 100 - 150 kg per t wine lees (Braga *et al.*, 2002). Due to the high quantities produced worldwide, wine lees became an ideal raw material for commercial production of tartaric acid (Versari *et al.*, 2001; Braga *et al.*, 2002). Industrial production of tartaric acid from wine lees is briefly based in the following process:

1) The potassium bitartrate contained in wine lees is converted to calcium tartate by the addition of $Ca(OH)_2$, $CaCO_3$ or CaCl.

2) The calcium tartrate reacts with an aqueous solution of sulfuric acid yielding tartaric acid

3) Decolorization of tartaric acid solution with activated carbon is carried out

4) Production of tartaric acid is achieved by crystallization.

Nowadays, tartaric acid is used as an additive for the correction of acidity in wines (Boulton *et al.*, 1995) and in many other products, such as candies, bakery goods like cookies, and beverages like sodas. It is a popular alternative to the widely used citric and phosphoric acids in food and beverage industries (Salgado *et al.*, 2010). Tartaric acid offers relative microbial stability to food products. Spoilage bacteria and yeasts can hardly metabolize and degrade tartaric acid. So, addition of tartaric acid decreases the need for

additional chemical or thermal preservation. Furthermore, tartaric acid is used in various applications, such as textile coloring, galvanizing and mirror production (Boulton *et al.*, 1995).

Rivas *et al.* (2006) have optimized an integral process for valorization of wine lees that was further improved by Salgado *et al.* (2010), which includes the extraction of tartaric acid with a high degree of purity and reducing the cost of evaporation.

An important aspect of wine lees valorization is its utilization as nutrient-rich fermentation supplement. This approach has been investigated in some literature-cited publications. Bustos *et al.* (2004) used various lees (from red or white wines, distilled or untreated, from the first or second decanting step) as nutrient supplement for lactic acid production (105.5 g/L) using *Lactobacillus rhamnosus* cultivated on 100 - 110 g/L of initial glucose concentration as carbon source. Rivas *et al.* (2006) employed distilled wine lees that were used, after the removal of tartaric acid, as nutrient supplement for lactic acid production by *Lactobacillus pentosus* using hemicellulosic vine shoot hydrolysates as carbon source, obtaining acceptable sugar to lactic acid conversion yield (0.7 g/g). Salgado *et al.* (2010) proposed a process where tartaric acid was recovered and the remaining wine lees were used as fermentation medium for xylitol production (33.4 g/L). Pérez-Bibbins *et al.* (2015) employed wine lees (liquid, solid and whole fraction) as nitrogen source for the production of xylitol by *Debaryomyces hansenii*, using commercial xylose as carbon source.

Phenolic compounds have been mainly studies in grape skins, seeds and solid waste fractions. As a consequence, the vast majority of commercial polyphenol-rich extracts are produced by grape stems or stalks and marc or pomace. However, some studeis have focussed on the evalutation of wine lees as a rich source of phenolic compounds (Pérez-Serradilla *et al.*, 2008). Phenolic compounds have been obtained using microwave-assisted extraction (Perez-Serradilla and Luque de Castro, 2011) or supercritical fluids (Wu *et al.*, 2009). The presence of low molecular weight phenolic compounds (released free flavonol aglycones and pyranoanthocyanins) having functional and bioactive properties were recently studied by Barcia *et al.* (2014).

One of the main constituents of wine lees is yeast cells that have been evaluated for different applications. Yeasts have been used as supplement in fish food and animal feed.

Lara-Flores *et al.* (2003) evaluated yeast as probiotic (at 0.1 %) on growth performance of Nile Tilapia. Besides whole yeast cells, (1,3)-β-D-glucans can be recovered from spent yeast contained in wine lees. It has been estimated that 20 % of the total dry weight of yeast is cell wall, while around 30 – 60 % of this amount is (1,3)-β-D-glucans. The (1,3)-β-D-glucans are naturally occurring polysaccharides consisted of D-glucose monomers linked by (1,3)-β-glycosidic bonds derived from spent yeast. They have attracted attention over the years because of their bioactive and medicinal properties. These chemical compounds are characterized as immunomodulators, having beneficial anti-inflammatory or antimicrobial effects in human health (Vetvicka and Vancikova, 2010). Other health benefits include hepatoprotection, wound healing, weight loss, antidiabetic and cholesterol lowering functions (Zekovic *et al.*, 2005). Freimud *et al.* (2003) proposed a new method for the isolation of non-degrading (1,3)-β-D-glucans.

Mannoproteins could be also extracted from spent yeast cells contained in wine lees. Yeast mannoproteins are highly glycosylated proteins containing over 90 % sugars, mainly mannose. Most of them act as structural components, giving the cell wall its active properties and being partially responsible for its permeability (Cid *et al.*, 1995). Mannoproteins act like polysaccharides rather than like proteins attributed that characteristic to their high sugar concentration. In *Saccharomyces cerevisiae*, the glycan portion of mannoproteins is mainly composed of mannose, with some neutral oligosaccharides that contain N-acetylglucosamine and acidic sugars containing mannosylphosphate (Klis *et al.*, 2002). Mannoproteins have good emulsifying and stabilizing effects *in vitro* when assayed at various pH values and salt concentrations commonly applied in the food industry to formulate or preserve foods (Araujo *et al.*, 2014).

The studies presented above do not take advantage of the full potential of wine lees valorization as most of the products presented above could be produced in an integrated biorefinery. Therefore, this study has focussed on the evaluation of the fractionation of wine lees for the production of four major products including the production of a nutrient-rich fermentation supplement used subsequently for the production of poly(3-hydroxybutyrate).

CHAPTER 4

POLYHYDROXYALKANOATES

4.1 Reasons towards replacement of petroleum-derived plastics

Petroleum-derived plastics have dominated the market for over 50 years due to their low cost and versatile end-uses. Petroleum-derived plastics offer their wide range of applications to some characteristics such as: 1) durability in harsh conditions, especially towards chemical and microbial decomposition and degradation, 2) long life spam, 3) low weight, and 4) low cost.

Global plastic production increased from 1.5 million tonnes in 1950 to 299 million tonnes in 2013-2014 (Plastics Europe, 2014/2015). This significant increase in plastic production has caused serious environmental concerns, since disposal of petroleum-derived plastics increase land, water and air pollution. In addition, the largest proportion of plastic debris is discarded in landfills, becoming an excellent breeding location for mosquitoes aggravating environmental pollution (Flechter, 1993).

However, minimizing the consumption of plastic products is very difficult. Bearing in mind that plastics generated from petroleum are not renewable and biodegradable materials, a sustainable replacement is needed. Inevitably, finding alternatives to plastics, having the same properties being simultaneously biodegradable is a matter of high importance in an attept to cover plastic demand (46.3 million tonnes consumed in 2013 in packaging, agriculture, building and construction, automotive, furniture, electrical and electronics etc. (PlasticsEurope, 2014/2015)) and protect the environment. Thus, replacement of non-biodegradable by biodegradable plastics is of major interest both to decision-makers and to plastic industry (Song *et al*., 1999).

4.2 Biodegradable and bio-based plastics

According to the ASTM standard D-7991 (2015) definition of ‘‘biodegradable’’ is indicated as ‘‘capable of undergoing decomposition into carbon dioxide, methane, water, inorganic compounds, and biomass’’ (www.astm.org accessed 12/2015).

The difference between the definition of "bio-based" and "biodegradable" plastics should be pinpointed. "Bio-based" polymers include "biodegradable polymers" as they are defined as those polymers that have biomass or renewable origin. For instance, poly-(butylene succinate) (PBS) and poly-caprolactone (PCL) could be produced using petroleum-derived monomers but many microorganisms have the ability to degrade them. On the other hand, poly(lactic acid) (PLA), starch and cellulose blends as well as PHB are synthesized from renewable resources. Furthermore, polyethylene (PE) and Nylon 11 (NY11) have the potential to be produced from biomass or renewable resources, but they are not biodegradable (Tokiwa *et al.*, 2009).

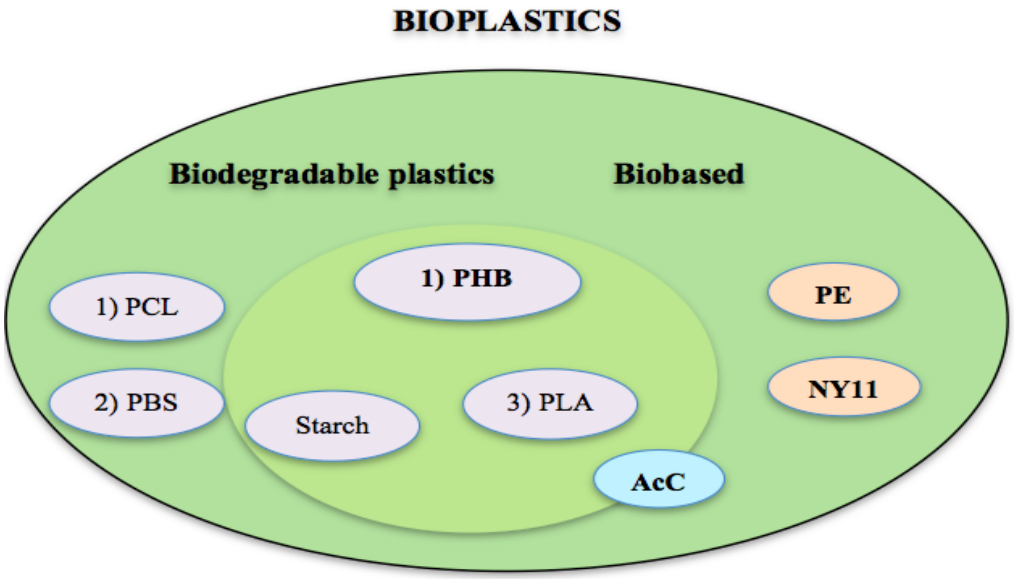


Figure 4.1: Bioplastics comprised of biodegradable and biobased polymers **(**PCL: polycaplonactone, PBS: poly(butylene succinate), PHB: poly(3-hydroxybutyrate), PLA: polylactide, AcC: Acetyl cellulose, PE: Polyethylene, NY11: Nylon 11)

Polyhydroxyalkanoates (PHAs) is a family of natural polymers that are biodegredable. These aliphatic polyesters can be synthesized from renewable resources and their polymerization takes place under mild process conditions, with minimal environmental impact (Philip *et al.*, 2007). Studies dealing with polymer-degrading microorganisms, revealed that PHA degraders are widely distributed in different ecosystems. Comparing the polymers presented in Figure 4.1 with PLA, it was clearly demonstrated that the rate of biodegradation was in the following order:

PHB=PCL>PBS>PLA (Pranamuda *et al.*, 1997; Nishida and Tokiwa., 1993). Due to the fact that biodegradability comprises a key factor for the further commercialization of PHAs, it will be further analyzed in section 4.4.1.

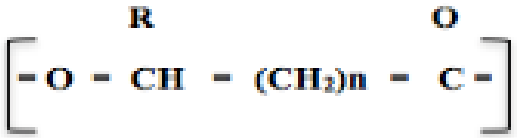
Simultaneously there are also bio-based polymers, which can either be biodegradable or not. As such, acetyl-cellulose (AcC) can be biodegradable or not depending on the degree of acetylation. Those having low-degree are easily biodegraded but there are also others with high degree of substitution ratios, which cannot be degraded by microorganisms (Puls *et al.*, 2011).

4.3 Polyhydroxyalkanoate - a family of biodegradable polyesters

4.3.1 Structure, classification and historical progress of PHAs

PHAs are thermoplastic or elastomeric polymers (poly-esters), consisted of R-hydroxy-alkanoic acids linked by ester bonds, biosynthesized by a wide range of gram positive and gram negative bacteria as carbon and energy storage compounds (Lee *et al.*, 1995; Anderson and Dawes, 1990).

PHAs are synthesized in the presence of excess carbon source and limiting concentration of another essential nutrient, such as N, P, S, O, or Mg (Koller *et al.*, 2008). In general, unbalanced nutrient supply triggers the accumulation of discrete insoluble granules of PHAs in order to store excess carbon and energy. The size of the chain length can vary in number depending on the microbial strain and the fermentation media used (Anderson and Dawes, 1990). PHAs have the general structure presented in Figure 4.2. Most of the members of the PHAs family contain 3-hydroxy acids which are optically active. Most of the monomers are in the R-configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase (Sudesh *et al.*, 2000).



n=1	R=hydrogen	poly(3-hydroxypropionate)
	R=methyl	poly(3-hydroxybutyrate)
	R=ethyl	poly(3-hydroxyvalerate)
	R=propyl	poly(3-hydroxyhexanoate)
	R=pentyl	poly(3-hydroxyoctanoate)
	R=nonyl	poly(3-hydroxydodecanoate)
n=2	R=hydrogen	poly(4-hydroxybutyrate)
	R=methyl	poly(4-hydroxyvalerate)
n=3	R=hydrogen	poly(5-hydroxyvalerate)
	R=methyl	poly(5-hydroxyhexanate)
n=4	R=hexyl	poly(6-hydrododecanoate)

Figure 4.2: General structure of PHA (Francis, 2011)

Polyhydroxyalkanoates can be divided into three groups depending on the carbon chain length of the monomeric units:

(a) Short-Chain-Length (*scl*-PHA), consisted of 3 to 5 carbon atoms

(b) Medium-Chain-Length (*mcl*-PHA), consisted of 6 to 14 carbon atoms and

(c) Long-Chain-Length (*lc1*-PHA), consisted of 17 to 18 carbon atoms.

Scl-PHAs have similar properties to conventional thermoplastics, while *mcl*-PHAs are regarded as elastomers or rubbers (Madison and Huisman., 1999). PHA can be either homopolymers or heteropolymers. Homopolymers are consisted of one type of monomer units and heteropolymers (copolymers) contain more than one type of monomer units. The most common *scl*-PHA homopolymers, are poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyvalerate (PHV), having 3-hydroxybutyrate and 3-hydroxyvalerate as the respective monomers. Some well known copolymers are: poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(HB-*co*-HV), P(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HHx), P(3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB), P(3-

hydroxyhexanoate-*co*-3-hydroxyoctanoate) P(3HHx-*co*-3HO) and P(3-hydroxybutyrate-*co*-3-hydroxyoctanoate) P(3HB-*co*-3HO) (Braunegg *et al.*, 1998; Madison and Huisman, 1999; Kobayashi *et al.*, 2005). Beijerinck (1888) was the first who described the presence of PHA in bacterial cells. Back then, scientists who first tried to investigate these polymers described them as "lipids" (Braunegg *et al.*, 1998). The historical review of intracellular PHAs dates back to 1920s, when the French microbiologist Maurice Lemoigne discovered a polyester, called poly(3-hydroxybutyrate) forming intracellular granules in the Gram-positive bacterium *Bacillus megaterium* (Lemoigne, 1926). In the 1950s, it was reported that the PHB accumulated by strains belonging to the genus *Bacillus* funtion as an intracellular carbon and energy reserve. Forsyth *et al.* (1958) observed that the occurrence of PHB is a common phenomenon in Gram negative aerobic bacteria. Wallen and Rohwedder (1974) reported the presence of polymers other than 3-hydroxybutyrate in chloroform activated sludge. It was also noted that the observed inclusions was a copolymer consisted of 3-hydroxyvalerate and of 3-hydroxyexanoate units.

Findlay and White (1983) reported the presence of 3HB and 3HV in marine sediments using capillary gas chromatography as the prevalent chemical compounds among 11 other HAs. De Smet *et al.* (1983) while studying the accumulation of PHA in *Pseudomonas oleovorans* cultivated on n-octane found out that the polymer inclusions were based principally of 3-hydroxyoctanoate and of 3-hydroxyhexanoate units in lower quantities. The oil crisis boosted the quest for the commercial production of alternative plastics. Meanwhile, the British Imperial Chemistry Industry, named ICI, produced the copolymer P(3HB-*co*-3HV) under the trade name Biopol® (ICI/Zeneca BioProducts, Bellingham, UK). By 1999, it was clear that these storage polymers were not only synthesized by Gram-negative bacteria but also by Gram positive aerobic bacterial strains (cyanobacteria), anaerobic photosynthetic bacteria (non-sulfur and sulfur purple bacteria) and archae (Anderson and Dawes, 1990; Rehm and Steinbüchel, 1999). Scientific interest has also focussed towards the investigation and understanding of PHA biosynthesis at genetic level (biosynthetic pathways, production in transgenic plants as alternative potential producers of PHAs) along with genetic manipulation in an attempt to efficiently achieve the production of tailor made biopolymers (Peoples and Sinskey, 1989; Poirier *et al.*, 1992).

4.3.2 Microorganisms and carbon sources leading to PHA production

More than 300 different microorganisms are capable of synthesizng PHAs, such as *Alcaligenes eutrophus* or *Ralstonia eutropha* (currently known as *Cupriavidus necator*), *Alcaligenes latus, Azotobacter vinelandii, Pseudomonas oleovorans,* several strains of *methylotrops, recombinant E. coli* and *Klebsiella aerogenes* (Table 4.1). All these strains have the potential to be cultivated and accumulate PHAs in varying productivities, using different carbon sources. Apart from wild-type and recombinant bacterial strains, PHAs can be produced by recombinant eukaryotes, via metabolic transformation of various carbon sources (Sudesh *et al.*, 2000; Anderson and Dawes, 1990).

Table 4.1: Organisms shown to produce PHAs (Bastioli, 2005)

Organisms
Gram-positive bacteria
Actinomycetes, Bacillus, Caryophanon, Corynebacterium, Clostridium, Micrococcus, Microcystis, Nocardia, Rhodococcus, Staphylococcus, Streptomyces
Gram-negative bacteria
Acinetobacter, Alcaligenes, Aphanocapsa, Aphanothece, Aquaspirillum, Asticcaulus, Azospirillum, Azomonas, Azotobacter, Beggiatoa, Beijerinckia, Beneckea, Caulobacter, Chloroflexus, Chlorogloea, Chromatium, Chromobacterium, Derxia, Ectothiorhodospira, Escherichia, Ferrobacillus, Gloeothece, Haemophilus, Haloferax, Halobacterium, Hyphomicrobium, Lamprocystis, Lampropaedia, Leptothrix, Methanomonas, Methylosinus, Methylobacterium Methylocystis, Methylomicrobium Methylomonas, Methylovibrio, Microcoleus, Moraxella, Mycoplana, Nitrobacter, Nitrococcus, Oceanospirillum, Paracoccus, Photobacterium, Protomonas, Pseudomonas, Rhizobium, Rhodobacter, Rhodopseudomonas, Rhodospirillum, Sphaerotilus, Spirillum, Spirulina, Stella, Syntrophomonas, Tetrahymena, Thiobacillus, Thiocapsa, Thiocystis, Thiodicotyon, Thiopedia, Thiosphaera, Vibrio, Xanthobacter, Zoogloea
Eukaryotes
Arabidopsis thaliana, Brassica napus, Gossypium hirsutum, Nicotiana tabacum, Pichia pastoris, Saccharornyces cerevisiae, Solanum tuberosum, Spodoptera frugiperda, Zea mays.

Various substrates such as renewable resources (glucose, sucrose, starch, cellulose, triacylglycerols), fossil resources (methane, mineral oil, lignite, hard coal), agro-industrial by-products and waste streams (e.g. molasses, whey, crude glycerol, rice bran), organic acids (propionic acid, 4-hydroxy-butyric acid, acetic acids), and carbon dioxide have been reported as raw material for the production of PHAs (Reddy *et al.*, 2003; Khanna and Srivastava 2005; Steinbüchel and Eversloh, 2003; Steinbüchel, 2005). Table 4.2 presents some of the microorganisms that have been reported as PHA producers to date, along with

their initial carbon source and the type of polymer produced each time. PHAs production from wastes and by-product streams generated by the agro-industrial and chemical sector will be further discussed.

Table 4.2: Overview of bacterial strains and carbon substrates for PHAs synthesis

Bacterial Strains	Carbon source	PHAs	Reference
Cupriavidus necator	Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil, glycerol	PHB, copolymers	Kim *et al.* 1995; Kichise *et al.* 1999; Taguchi *et al.* 2003; Kahar *et al.* 2004; Khanna and Srivastava, 2005; Volova and Kalacheva 2005; Volova *et al.*, 2005
Cupriavidus necator H16	Hydrogen, carbon dioxide	PHB	Pohlmann *et al.*, 2006.
Aeromonas hydrophila	Lauric acid, oleic acid	*mcl*-PHAs	Lee *et al.*, 2000; Han *et al.*, 2004
Alcaligenes latus	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	Wong *et al.*, 2004
Bacillus cereus	Glucose, e-caprolactone, sugarbeet Molasses	PHB, terpolymer	Yilmaz and Beyatli, 2005; Valappil *et al.*, 2007
Bacillus spp.	Glucose, alkanoates, soy molasses	PHB, P(3HB-*co*-3HV), copolymers	Full *et al.*, 2006; Shamala *et al.*, 2003
Burkholderia cepacia	Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugarbeet molasses	PHB, P(3HB-*co*-3HV)	Keenan *et al.*, 2004; Nakas *et al.*, 2004
Escherichia coli mutants	Glucose, glycerol, palm oil, ethanol, sucrose, molasses	PHB	Mahishi *et al.*, 2003; Kahar *et al.*, 2005; Park *et al.*, 2005; Nikel *et al.*, 2006
Halomonas boliviensis	Starch hydolysate, maltose, maltotetraose and maltohexaose	PHB	Quillaguaman *et al.*, 2005
Haloferax mediterranei	Vinasse	P(3HB-*co*-3HV)	Bhattacharyya *et al.*, 2012

4.3.3 Metabolic pathway of poly(3-hydroxybutyrate) production

The most studied member of the PHA family is PHB. *Cupriavidus necator* is the most intensively studied microorganism for PHB production due to its potential to yield high productivity, yield and final PHB concentration (Ashby *et al.*, 2004). The biosynthetic pathway of PHB consists of three enzymatic reactions catalyzed by three different enzymes. The three enzymes along with their encoding genes that are involved in PHB production are shown in Table 4.3.

Table 4.3: Enzymes and encoding genes along with their function in biosynthetic pathway of PHB

Gene	Enzyme	Function
phaA	β-kethothiolase	Condense two acetyl-CoA molecules to form acetoacetyl-CoA
phaB	Acetoacetyl-CoA reductase	Catalyze the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, this enzyme is NADPH- dependent
phaC	PHA synthase	Catalyze the polymerisation of (R)-3-hydroxy-butyryl-CoA monomers

Bacteria throughout their metabolic activities produce acetyl-coenzyme-A (acetyl-CoA), which is converted into PHB. The process of PHB biosynthesis involves the following steps, when glucose or glycerol are employed as carbon sources:

1) The process starts with the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA by β-ketothiolase, encoded by phaA gene.

2) The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase, encoded by phaB gene.

3) Finally, (R)-3-hydroxybutyryl-CoA monomers are polymerised by PHA synthase, to formulate PHB.

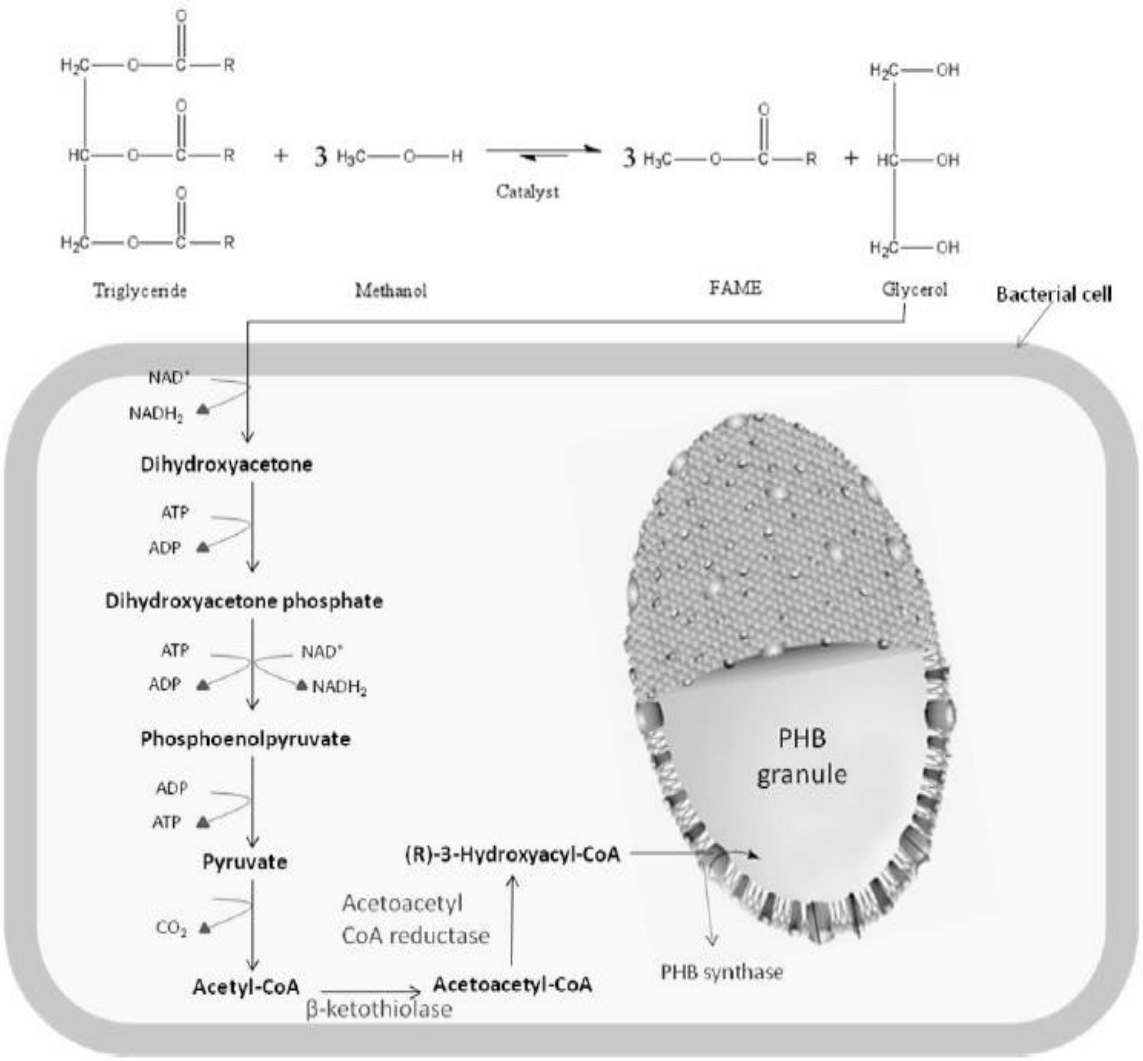


Figure 4.3: Biochemical pathway involved in bacterial production of PHB from glycerol

PHA synthase is the key enzyme for PHB biosynthesis. The consumption of glucose or glycerol by *Cupriavidus necator* leads to the production of PHB or other short-chain-length monomers (Suriyamongkol *et al.* 2007). All three enzymes are located in the cytosol of the cell, where PHB accumulation takes place (Anderson and Dawes, 1990). Owning to similarities existing in metabolic pathways (Figure 4.3), PHB biosynthetic pathway from glycerol is similar to the one followed when glucose is consumed. In the case of PHB synthesis from glycerol, bacteria first convert glycerol to dihydroxyacetone that is further converted to acetyl-CoA through various biochemical conversions. Acetyl-CoA is then converted into PHB by the action of β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase.

4.3.4 Commercialization of PHA products

Nowadays many polyhydroxyalkanoates have been explored in potential commercial applications, however, only a few members of the PHA family have been commercialized. Large scale PHA production has been involved in the case of: 1) PHB by Chemie Linz AG Austria; 2) P(3HB-*co*-3HV) by ICI in the UK and TianAn in China; 3) P(3HB-co-4HB) by Tianjin Green Bio-Science; and 4) P(3HB-*co*-3HHx) by the joint efforts of Tsinghua University, KAIST and P&G. Thus, there is much scientific research that should be carried out to enhance large-scale production of PHA. Up to date, 24 companies are known to participate in organizing and operating R&D department on PHA production and trading PHAs. Some of these companies ceased PHA production mainly due to the low prices of petroleum (Chen *et al.*, 2009).

The production process of PHA polymers is aiming to encourage sustainable development and environmental protection, through the usage of renewable resources thus leading to refuced emmisions of CO_2. Within this prospect, the overall interest in large scale production of PHAs has increased despite the high commercial prices of marketed polyhydroxyalkanoates (higher than 3 $/Kg). Apart from that, the high cost of petroleum and the fluctuation of its price have enhanced the research towards the industrial production of PHAs.

In 1996, Zeneca sold its Biopol business to Monsanto. Monsanto followed the work started by Zeneca in producing PHAs from genetically-modified crops. At the end of 1999, the Biopol production line was ceased. Metabolix bought Biopol rights for production, selling and expertise in 2001. In 2007, Metabolix inc. and Archer Daniels Midland co. (ADM) formed a joint venture producing PHAs under the trade name Mirel®.

Metabolix inc. utilizes genetically-modified crops as feedstocks for PHA production and in 2010 announced the completion of field trials of genetically engineering of tobacco into PHA biobased polymers. PHAs Metabolix products are: PHA Latex Barrier Coating for paper and cardboard, Mirel® Micropowders for microbeads in cosmetic and personal care and Mirel® effective polymeric modifier for PLA (www.adm.com, accessed 10/2015).

So far, it seems that Boston-based Metabolix industry shares the longest-running production of PHAs owning more than 500 licensed patents and having been awarded

several times for its contribution to protect the environment. In 2007, Meredian Inc. purchased P&G's PHA technology.

Figure 4.4: Meredian PHA packages (www.mhgbio.com, accessed 10/2015)

From 2007 to 2015, Meredian Inc. has become one of the most well-known manufacturer of PHAs, under the trade name Nodax^{TM}. Nodax (having been approved by FDA for food contact and as a harmless waste) has several applications especially for single-use in food service and medical items, packaging, personal care and household products.

Table 4.4 presents the major companies producing PHA the type and the usage of each product obtained by each one, the production scale and the duration that each enterprise markets PHA.

Table 4.4: Major PHA producers

Company	PHAs	Production scale	Period	Applications	Reference
Biomers, Germany	PHB	Unknown	1990s to present	Packaging & drug delivery	www.biomer.de/IndexE.html, accessed 8/2015
Metabolix, USA	Several PHA	Unknown	1980s to present	Packaging	www.metabolix.com/products/biopolymers/all, accessed 10/2015
Bio-on, Italy	PHA (unclear)	10,000	2007 to present	Raw materials	www.bioplastics.org/en/component/customproperties/tag?tagId=5, accessed 10/2015
Tianan, China	PHBV	2,000	1990 to present	Raw materials	www.helianpolymers.com/category/products/, accessed 10/2015
Meredian, USA	Medium chain length PHAs	10,000	2007 to present	Raw materials (food containers, bottles,e.t.c)	www.mhgbio.com, accessed 10/2015
Tianjin Green Bioscience	P3HB4HB	10,000	2004 to present	Raw materials and packaging	www.tjgreenbio.com/en/newsText.aspx?nid=99, accessed 10/2015
Shandong Ecomann Technology Co.	Several PHA	Unknown	2008 to present	PHA biopellets aquarium filter media (marine), & PHA plastic microbeads as abrasives in facial and body scrubs	www.ecomann.com/doc/product.html, accessed 10/2015
Biocycle, Brasil	PHBV	100	1992 to present	Raw materials of quick use	www.biocycle.com.br/site.htm, accessed 11/2015

4.3.5 Strategies and projections for worldwide PHA market

High cost of oil and awareness of environmental problems involved with plastic manufacturing have enlarged the need of partial or total replacement of plastic materials. Governments gradually develop positive attitude toward green policies. As a consequence, the rising demand for biodegradable polymers has attracted companies to invest on PHA research and production (Table 4.4).

The European Union's competitiveness strategy Europe 2020 (European Commission, Europe 2020) intends to promote sustainable development for the protection

of the environment, reduction of greenhouse gas emmissions and promotion of the bioplastic industry. Within this context, the world market of PHA is expected to flourish in the following years.

In 2013, the PHA market in Europe reached a value of $21.8 million. It has been estimated that the annual growth rate of the European market is 23.5% and that by the end of 2018 the revenues from PHAs selling commodities will reach $62.6 million.

The current share in Europe of the global PHA market is 41.7% and it is projected to increase to 43.7 % by the end of 2018. In 2013, China recorded the largest revenue from PHA products ($ 3.9 million) and it is estimated to triple by 2018 ($11.8 million), accounting to 32.1 % of global PHA market. (www.grandviewresearch.com/industry-analysis/pha-polyhydroxyalkanoates-market, accessed 11/2015).

Despite the versatility in the end-uses of PHAs and the environmental beningness, the industrial production of PHAs and the global market is still in its "infancy". Thus, significant steps are still required to enhance large scale production of PHAs.

4.4 Properties of PHA

The average molecular mass (Mn) and polydispersity index (Mw/Mn) of PHA produced by bacteria has been estimated up to 4×10^6 Da and approximately 2, respectively (Agus *et al.*, 2006). Mn depends on the type of the microorganism used and the growth conditions. The material characteristics of these biopolymers are similar to conventional plastics such as polypropylene. The densities of crystalline and amorphous PHB are 1.26 and 1.18 g/cm^3, respectively (Marchessault and Yu, 2004; Tsz-Chun *et al.*, 2005).

PHAs are biopolymers having 60 - 80 % crystallinity. In the bacterial cell, their form is in amorphous, water-soluble inclusions. It is only at the time that cells are disrupted, to extract the biopolyester, when polymer crystallisation occurs. A kinetic nucleation mechanism might explain that reaction based on the hypothesis that when cells are disrupted, granules coalesce and at that time rapid heterogeneous nucleation takes place (Sudesh *et al.*, 2000).

As far as other mechanical properties are considered, the members of PHA family do not present similar characteristics. Depending on their monomer composition, PHAs

can vary from hard crystalline to elastic. For instance, *mcl*-PHAs are semi-crystalline elastomers. Most of them have low melting point and tensile strength. As a concequence, they exhibit high elongation to break. The glass transition temperature of PHB is almost 180^{o}C. Mechanical properties like Young's modulus and tensile strength are close to that of polypropylene, while extension to break is lower. PHB is stiffer and becomes brittle upon storage for several days in ambient conditions. De Koning *et al* (1993) stated that PHB becomes brittle, upon storage, after crystallization takes place followed by melting. This second crystallization entails the reorganization of lamellar crystals, causing amorfous chains between crystals.

P(3HB-*co*-3HV) copolymers have almost the same degree of crystallinity as homopolymer (50 - 70 %). Copolymers of 3HB and 3HV are isodimorphic, meaning that they exhibit a minimum melting point at 30 mol % 3HV content. As the molar fraction of 3HV units increases, the copolymer becomes more flexible (decrease in Young's modulus). The increase of 3HV molar ratio corresponds to an increase in elongation to break. A decrease in melting temperature of P(3HB-*co*-3HV) copolymers without affecting degradation temperature is a critical factor to enable further thermal processing of copolymers without thermal degradation.

Consequently, it is possible to manipulate thermal and mechanical properties of P(3HB-*co*-3HV) copolymers by regulating the 3HV ratio during the fermentation process. Incorporation of other units than 3HV, improve material properties of copolymers (Bugnicourt *et al.*, 2014) (Table 4.5).

4.4.1 Biodegradation of PHA

The main characteristic of PHA biopolymers is their biodegradabability in aerobic and anaerobic environments in small periods of time, without formation of any toxic compounds (Figure 4.5).

Many microorganisms, in their natural habitat are able to degrade PHA aerobically to water and carbon dioxide or anaerobically to methane by the action of PHA hydrolases and PHA depolymerases (Jendrossek *et al.*, 1996). Microorganisms including bacteria and fungi excrete extracellular PHA depolymerases that hydrolyze the water insoluble macromolecules to water soluble oligomers or monomers. Then, the degraded oligomers or

monomers are transported through cell wall to be further metabolised as carbon and energy sources. The enviromental conditions as well as the chemical synthesis of PHA affect the activities of hydrolytic enzymes (Numata *et al.*, 2009). Several aerobic and anaerobic PHA-degrading microorganisms have been found and isolated in soil, seawater, lakewater and sludge (*Pseudomonas, Aspergillus, Streptomyces, Alcaligenes, Comamonas* species) (Tokiwa *et al.*, 2009).

Table 4.5: Comparison of PHAs with conventional plastic (Doi *et al.*, 1990; Bugnicourt *et al.*, 2014)

Polymer	Glass transition temperature (T_g)	Melting temperature (T_m)	Tensile strenght (Mpa)	Elongation to break (%)
P(3HB)	4	180	43	5
P (3HB-co-9 mol% 3HV)	3	162	37	35
P(3HB-co-20 mol% 3HV)	-1	145	20	50
P(3HB-co-16 mol% 4HB)	-7	150	26	444
P(3HB-co-10 mol% 3HHx)	-1	127	21	400
Polypropylene	-10	176	38	400
Low-density polyethylene	-30	130	10	620

Figure 4.5: Biodegradation of PHA bottles within 2 months (www.serdp-estcp.org/Program-Areas/Weapons-Systems-and-Platforms/Waste-Reduction-and-Treatment-in-DoD-Operations/WP-1478, accessed 12/2005)

4.5 Production of PHA

The PHA production process involves a series of unit operations beginning from biomass growth, polymer accumulation, cell harvesting, polymer extraction and purification. All these steps affect the final polymer productivity and quality.

4.5.1 Fermentation strategies for PHA production

Several factors should be considered prior to designing fermentation processes for PHA production. These parameters include: 1) the carbon and nitrogen source, 2) the appropriate selection of bacterial strain, 3) the presence of macro- and micronutrients, and 4) cultivation conditions, including temperature and optimum pH for bacterial growth. Despite the high number of microorganisms that have been identified as potential producers of PHAs, most of them demonstrate low capability of PHAs accumulation entailing low volumetric productivities.

Several PHA production strategies have been described, including batch, fed-batch and continuous processes. In industrial production of PHAs, batch or fed-batch fermentations are mainly used depending on the microorganism employed. In one stage cultivation, the microorganisms used have the ability to grow and accumulate PHAs during bacterial growth and therefore the application of batch mode of operation is feasible (Jung *et al.*, 2001). When microorganisms need nutrient limitation for PHA biosynthesis, a two stage fed-batch cultivation process is employed (Figure 4.6). In the first stage, cell growth takes place, so as to ensure satisfactory biomass production, using a nutrient-complete fermentation medium. Then, the bacterial cells are subjected to nutrient limitation in order to induce the initiation of the PHA accumulation phase. Throughout the nutrient limitation stage, cells are no longer able to grow and their bacterial concentration remains constant. At this stage, cells begin to enlarge and increase their size due to the intracellular accumulation of PHAs as storage products. Fed-batch fermenations are practical and often provide the optimum choice for large scale production of PHAs (Sudesh and Abe, 2010).

Continuous cultivation is another strategy for PHA production as higher productivities can be attained overcoming potential growth inhibition due to the use of high carbon source concentrations at the beginning of batch or fed-batch processes (Koller

et al., 2010). It has been employed for production of both *scl*-PHAs and *mcl*-PHAs. Nonetheless, it is impractical at large scale production (Mothes and Ackermann, 2005).

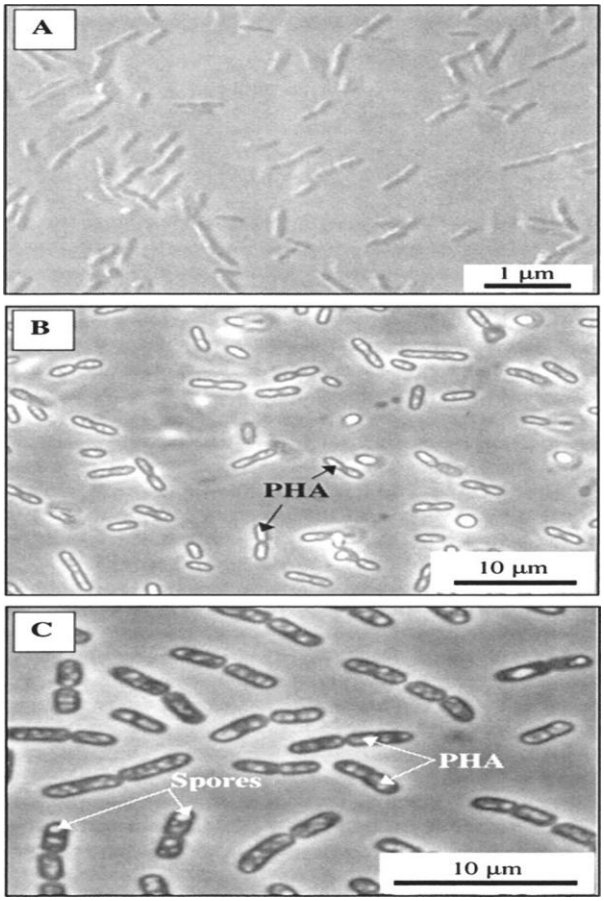


Figure 4.6: Polyhydroxyalkanoate inclusions in microorganisms, serving as storage materials in a) growth phase, b) accumulating phase, and c) end of accumulation phase (Sudesh and Abe, 2010)

4.5.2 Downstream processes for recovering PHA

Downstream processing to recover the intracellular biopolymer is applied after the termination of the fermentation process.Various technologies have been applied and can be classified as follows:

1) Solvent extraction for solubilizing PHA granules: This is the most extensively used method for recovering PHA from cell debris owning to its simplicity and low duration. The mechanism for the extraction of the biopolymer involves two main steps. In

the first step, the permeability of cell membrane increases, permitting the release of the granules, and in the second step solubilization of PHA intracellular granules takes place. Chlorinated hydrocarbons (chloroform, 1,2-dichloroethane), some cyclic carbonates (ethylene carbonate or 1,2-propylene carbonate) and acetone are the most commonly used solvents for the extraction of PHAs. Precipitation of PHAs is mainly induced by methanol and ethanol (Ramsay *et al.*, 1990; Dong and Sun, 2000). Apart from the efficiency in recovery, this method holds other advanages since it does not decompose the polymer, bacterial endotoxins are removed and a polymer of high purity is achieved. However, solvent extraction is primarily followed in lab-scale studies, but large scale usage is inhibited mainly due to high operational and capital costs. Furthermore, environmental concerns associated with usage and emissions of volatile and toxic compounds in the environment, in case of accidents, raise major obstacles to large scale application of solvent extraction methodology (Han *et al.*, 1994).

2) Digestion of cellular materials surrounding PHA granules. Digestion involves solubilization of residual biomass. This extraction approach can be sub-categorized into chemical or enzymatic digestion.

Chemical digestion involves solubilization of non-PHA materials by the usage of chemicals like sodium hypochlorite (Choi and Lee, 1999) and surfactants (Hahn *et al.*, 1995) such as sodium dodecyl sulfate (SDS), Triton X-100 and palmotoyl carnitine. Due to the low purity achieved using sodium hypochlorite or surfactants, a combination using both was developed showing better performance and rapid recovery of PHA (Dong and Sun, 2000).

Recovery processes via enzymatic digestion is a rather complex process, since solubilization of cell materials involves heat pre-treatment, enzymatic hydrolysis and surfactant washing (Holmes *et al.*, 1990). Various types of enzymes, mainly proteases, have been used for trigerring cell lysis. The main disadvantages of this methodology are the high cost of enzymes. However, considering the high specificity of enzymes, high purity of PHAs can be obtained.

3) Mechanical disruption. Disruption of cell walls with high pressure homogenization takes place under high pressure through an adjustable valve. Combination of mechanical disruption and chemical digestion has showed good performance regarding

the PHA purity achieved. Ghatnekar *et al.* (2002) using 5% SDS to *Methylobacterium sp* V49 biomass as a pretreatment step and then hogenization at 400 Kg/cm^2 achieved PHA recovery of 95 and 98% after two cycles. Mechanical disruption of cells is influenced by many parameters, such as the type of cells, state of growth, the possibility of thermal degradation of the product, and interference with cellular components.

4) Supercritical fluids. Supercritical carbon dioxide ($scCO_2$) is the most widely used method among supercritical fluids. This PHA extraction method ows its popularity to low flammability, low toxicity and moderate critical temperature and pressure (Couto *et al.*, 2009). It can be considered as an enviromentally friendly approach among PHA extraction methods. Khosravi-Darani *et al* (2004) achieved 81 % recovery of PHA from wet cells of *Cupriavidus necator* by incorporating 1% (v/v) toluene as modifier and two times $scCO_2$ release, under pressure of 200 bar and 30 ^oC. To improve process economics trying to avoid costly freeze drying, NaOH (0.4 v/v) was employed as a pretreatment step.

Many other methodologies, such as self flotation of cell debris, increase of fragility of cell walls during accumulation, gamma irradiation of cells, have been employed. The efficiency of PHA downstream separation and purification affect significantly the PHA production cost.

4.6 Applications of PHA

PHA films have been used as packaging films mainly in bags, containers and paper coatings, disposable items, such as razors, utensils, diapers, feminine hygiene products, cosmetic containers-shampoo bottles and cups. They have also been used for the production of flushables, non-wovens, binders, flexible packaging, thermoformed articles, synthetic paper and medical devices. PHAs have found also applications in food packaging and the production of beverage bottles, paper coated milk cartons due to their gas barrier properties (Clarinval and Halleux, 2005).

Apart from the food sector and every-day bulk commodities, PHA have also served agriculture domain, as coatings for urea fertilizers in rice fields and as co-mixtures (PHA-pellets) with insecticides for manipulating release of the latter (Chen *et al.*, 2005)

Lastly, PHAs are attractive materials for biomedical applications, due to their origin, biocompatibility, biodegradability, lack of toxicity and ability to support cell growth and adhesion. For instance, cardiovascular products, such as pericardial patches to prevent post-surgical adhesions between the heart and the sternum, artery augmentation, atrial septal defect repair, vascular grafts and heart valves (Valappil *et al.*, 2006) have been proved of having great performance. Additionally, their usage is expanding in dental and maxillofacial treatment (guiding tissue and bone regeneration), drug delivery (tablets, implants, microcarriers), orthopaedic and urology procedures and wound management (sutures, dusting powders, dressings) (Phillip *et al.*, 2007).

4.7 Sustainable development through PHA production

The interest in PHAs and specifically PHB is constantly increasing due to their physical properties and their complete biodegradation under various conditions by different microorganisms within a period of one year (Anderson and Dawes 1990). On the contrary, huge volumes of plastic waste material discarged in the environment are hardly decomposed. For instance, plastic bottles need more than 450 years to decompose (www.plasticsindustry.org, accessed 12/2015).

Industrial production of PHAs is impeded by high production costs. Factors leading to relatively high costs of PHAs include high cost of substrate, low polymer concentration, yield, and productivity as well as costs associated with downstream separation and purification. Among these, carbon source is the most significant parameter for the total production cost of PHB. According to Choi and Lee (1999), 40-48% of the total production costs are ascribed to raw materials, in which carbon source could account for 70-80% of the total expenditure. Finding ways to substitute costly substrates with inexpensive raw materials, such as agro-industrial and chemical industry derived by-products or wastes, will not only reduce PHA production costs but will also promote sustainability.

4.7.1 Utilization of agro-industrial waste and by-product streams for PHA production

A wide variety of substrates, such as whey from dairy and cheese industries (Koller *et al.*, 2007; 2008; Nikel *et al.*, 2006), lipid-rich waste streams from slaughterhouses and

edible oils (Majid *et al.*,1999; Verlinden *et al.*, 2011), sugar wastes (Albuquerque *et al.* 2007; Solaiman *et al.* 2006; Solaiman *et al.*, 2006; Yu *et al.*, 2008), wastes from crop refining such as starch or rich starch residues, bran and lignocellulosic waste (Chanprateep *et al.*, 2010; González-García *et al.*, 2011; Xu *et al.*, 2010; Koutinas *et al* 2007; Huang *et al.*, 2006) have been reported as potential carbon substrates for PHA production (Arcos-Hernandez *et al.*, 2013; Sudesh *et al.*, 2011). Table 4.6 presents literature-cited studies on the production of PHAs using various renewable resources.

Table 4.6: Overview of waste streams producing PHAs

Strain (%)	PHA feedstock	PHA type	Biomass (g/l)	PHA (%)	References
Dairy and cheese whey-wastes					
Recombinant *Escherichia coli*	Whey	PHB	96.1	72.9	Nikel *et al.*, 2006
Cupriavidus necator DSM 545	Whey permeate	PHB	8.5	18.0	Povolo *et al.*, 2009
Thermus thermophilus HB8	Whey	PHA	1.6	35.6	Pantazaki *et al.*, 2009
Waste oils					
Pseudomonas aeruginosa 42A2	Waste cooking oil	*mcl*-PHA	1.5	29	Fernandez *et al.*, 2005
Cupriavidus necator	Waste frying Oil	PHB	1.2	38	Verlinden *et al.*, 2011
Pseudomonas putida	From digested ensiled grass	*mcl*-PHA	1.56	39	Cerrone *et al.*, 2014
Sugar industry residues					
Bacillus sp. COLI/A6	Sugarcane molasses	PHB	3.3	55	Santimano *et al.*, 2009
Recombinant Escherichia coli	Sugarcane molasses	PHB	4.0	75.7	Saranya and Shenbagarathai, 2011
Ralstonia eutropha	Bagasse hydrolyzate	PHB	6.0	65	Yu *et al.*, 2008
Mixed culture	Sugarcane molasses	P(3HB-*co*-3HV)	1	30	Albuquerque *et al.*., 2007
Crop residues					
Aeromonas sp. KC007-R1	Raw starch	PHB	0.6	33	Chien and Ho, 2008

Ralstonia Eutropha	Potato starch Residues	PHB	94	55	Haas *et al.*, 2008
Halomonas boliviensis LC1	Wheat bran Hydrolysate	PHB	1	34	Van-Thuok *et al.*, 2008
Cupriavidus necator	Wheat based biorefinery	PHB	15.8	44	Xu *et al.*, 2010
Cupriavidus necator	Hydrolyzed wheat	PHB	29.9	58	Koutinas *et al.*, 2007a

4.7.2 Crude glycerol for PHB production

New uses for glycerol have been the subject of research to alleviate a market glut of this commodity and to leverage the economics of biodiesel production. One potential use of glycerol is in industrial fermentation processes, where it can be employed as a substrate for microbial growth and the biosynthesis of microbial products. The utilization of crude glycerol is of immense importance to alleviate its disposal problem on top of producing value added products (Raveendran *et al.*, 2011). Utilization of crude glycerol for biopolymer production could be an alternative choice to decrease production cost of biodiesel and assist in managing the vast quantiteies of crude glycerol produced.

Mothes *et al.* (2007) studied the effect of salt-contaminants included in crude glycerol, deriving from rapeseed biodiesel production, during PHB fermentation production process. The bacteria used were *Paracoccus denitrificans* and *Cupriavidus necator* JMP 134, grown in a mineral medium, and in case of *P. denitrificans* the medium was supplemented with yeast extract. The above strains were able to accumulate PHB from crude glycerol to a content of 65 % of total cell dry mass. When using crude glycerol containing 5.5 % NaCl, a reduced PHB content of 48 % was observed at a bacterial dry mass of 50 g/L. Furthermore the PHB yield coefficient was reduced, due to the synthesis of by-products or as a result of the increased energy requirements for osmoregulation. The effect of glycerol contaminated with K_2SO_4 on PHB production was less pronounced.

Cavalheiro *et al.* (2009) used pure glycerol and waste glycerol from biodiesel industry as primary carbon source for cell growth and PHB synthesis. *Cupriavidus necator* DSM 545 was cultivated in basal medium supplemented with 1 g/L yeast extract. On pure glycerol, productivities between 0.6 g/L/h and 1.5 g/L/h were attained. The maximum cell

dry weight was 82.5 g/L with a PHB content of 62 %. When crude glyderol was used, 68.8 g/L of total dry weight with a PHB accumulation of 38 % resulting in a final productivity of 0.84 g/L/h was obtained. By decreasing the biomass concentration at which accumulation was triggered, a productivity of 1.1 g/L/h was achieved.

Zhu *et al.* (2010) investigated the potential production of PHA from crude glycerol, as sole carbon source in varying concentrations of 3 - 9% (v/v) and mixtures of crude glycerol and xylose using *Burkholderia cepacia* ATCC 17759. In shake flask fermentations, better results were achieved using crude glycerol of 3% (v/v) along with minerals and xylose as culture media and after 96 5.9 g/L biomass, 4.9 g/L PHA and 80 % PHB content were achieved. Fermentations in a 200 L scale bioreactor led to the production of 23.6 g/L of total dry wight and 7.4 g/L of PHB after 120 h.

Cavalheiro *et al.* (2012) cultivated *Cupriavidus necator* in crude glycerol and yeast extract for the production of short-chain polyhydroxyalkanoate co-polymers, such as poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-co-4HB) and terpolymers poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-4HB-*co*-3HV). Incorporation of 4HB monomers was promoted by γ-butyrolactone (GBL). Propionic acid, a precursor of 4HB accumulation, increased the 4HB molar ratio 2-fold, but also acted as 3HV precursor, yielding the terpolymer P(3HB-*co*-4HB-*co*-3HV). Maximum production of P(3HB-*co*-4HB) was achieved with γ-butyrolactone supplementation leading to PHA content of 36.1% (w/w) and 0.17 g/L/h volumetric productivity. In the case of P(3HB-*co*-4HB-*co*-3HV), the maximum PHA content achieved was 36.9% (w/w) with a volumetric productivity of 0.25 g/L/h.

Ashby *et al.* (2011) investigated PHB production by the cultivation of *Pseudomonas oleovorans* in batch mode, using 1 % (w/v) crude glycerol, 1 % (v/v) methanol and mineral salts as fermentation media. Maximum biomass production was 2.85 g/L corresponding to 1.06 g/L PHB after 72 h. Sathianachiyar and Devaraj (2013) studied the effect of crude glycerol under nitrogen and phosphate limitation, using microbial isolates from rhizospere soil of *Jatropha curcas* for the production of PHB.

A literature cited overview on PHA production from crude glycerol, including the type of microorganism and the feedstock employed along with details of fermentation and productivity, are presented in Table 4.7.

Table 4.7: Overview of PHA production using crude glycerol as fermentation feedstock

Strain	Feedstock	PHA type	Total dry weight (g/L)	PHA (g/L)	PHA content (%)	Reference
Osmophilic wild-type strain under characterisation	Crude Glycerol (CG), Meat and bone meal			5.9		Koller *et al.*, 2005
P. denitrificans and *C. necator*	CG, Yeast extract	PHB	50		48.65	Mothes *et al.*, 2007
C. necator	CG, Yeast Extract (YE)	PHB	68.8		38.5	Cavalheiro *et al.*, 2009
Zobellella denitrificans	CG, NaCl	PHB	4.78	4.16	87	Ibrahim *et al.*, 2009
Burkoholderia cepacia	CG, Xylose	PHB	23.6	7.4	87	Zhu *et al.*, 2010
Mixed microbial consortia	CG, ammonium chloride	PHB		0.17	67	Dobroth *et al., 2011*
Pseudomonas oleovorans	CG, YE	PHB	2.85	1.06		Ashby *et al.*, 2011
C. necator	CG, YE, γ-butyrolactone	P(3HB-co-4HV)		10.9	36.1	Cavalheiro *et al.*, 2012
	CG, YE, propionic acid	P(3HB-co-4HV-co-4HV)		16.7	36.9	
C. necator	CG, rapeseed meal hydrolysates	P(3HB-co-4HV)		10.9	55.6	García *et al.*, 2013
Halomonas sp SA8	CG, Peptone from potato, meat extract		7.25	3.91	54	Castro *et al*, 2014

4.8 Conclusions and future perspectives

Based on the literature review presented in this chapter, it is obvious that PHA constitute a promising alternative as substitutes to conventional petroleum derived plastics. Recently, significant research work has been performed in the formulation of PHB with additives and blends overcoming the intrinsic brittleness. These advances will give impetus to PHB bioplastics to enter market outlets based on thermal amd mechanical properties. Still, limitations exist such as the high cost of commercial PHA production, low availability and the use of refined feedstocks primarily used as food. One way of increasing availability and minimizing the production cost could be the use of low cost carbon sources, wastes and by-products derived from agriculture, food and chemical industrial sectors. Crude glycerol is abundant and holds great potential for PHA production. Research and novel procedures should be carried out focusing on producing PHAs from renewable resources targeting the integration in existing manufacturing lines. In thit way, waste managment issues could be resolved, promote sustainability and enhance the formulation of value added products under the prospect of developing a bio-based economy.

CHAPTER 5

SCOPE OF PHD THESIS

The aim of this study was to evaluate the potential of wine lees as renewable resource for biorefinery development. The implementation of the biorefinery concept is based on the development of sustainable processes that fractionate a renewable resource into value-added products, with the lowest possible environmental impact. Thus, the potential of wine lees as renewable resource for biorefinery development was investigated for the production of four major products, namely potable ethanol, tartrate salts, antioxidant-rich fraction and nutrient-rich supplement for fermentation processes.

The fractionation process was initially developed in Chapter 6. This chapter also describes the optimization study carried out for the production of nutrient-rich supplements from wine lees. The production of several value-added products from the original resource is essential in order to enhance the profitability of the whole process including PHB production that is a biopolymer of low market value. The PHB production cost is high and the only way to increase the profitability of a process producing PHB is the development of a biorefinery concept focusing on the fractionation of a renewable resource into different co-products.

PHB production has been evaluated in Chapters 8 and 9 using various renewable resources. Chapter 8 focussed on the production of PHB from flour-based waste hydrolysates, rapeseed meal hydrolysates and wine lees hydrolysates. Flour based waste streams were supplied by a confectionary industry as either out-of-date products returned from the market or side streams produced at the industrial production line. The rapeseed meal employed was produced as by-product stream from a biodiesel production plant after the extraction of oil from rapeseeds. The wine lees derived hydrolysates were produced according to the methodology developed in Chapter 6. The main target of this chapter was to evaluate and compare different renewable resources derived as side streams from different industrial sectors for the production of PHB. The main conclusion was that wine lees hydrolysates was an efficient nutrient supplement for PHB production. The production of PHB in Chapter 8 was evaluated suing both bacterial strains of *Cupriavidus necator* DSMZ 545 and DSMZ 7237.

Chapter 9 focussed on the optimization of PHB production in shake flask and fed-batch bioreactor cultures using the bacterial strain *C. necator* DSMZ 7237 cultivated on crude glycerol and wine lees derived hydrolysates. The crude glycerol used in this sudy was produced as by-product stream from a biodiesel production plant. The results showed that it is feasible to use crude glycerol and wine lees derived hydrolysates for the efficient production of PHB.

Chapter 10 focussed on process design and techno-economic evaluation of the biorefinery concept presented in Chapter 6 using wine lees as raw material leading to the production of yeast cells, alcohol, antioxidants and tartrate salts. A sensitivity analysis was carried out in order to assess the effect of plant capacities and the minimum selling price of antioxidant-rich extracts on the cost-competitiveness of the whole process.

The successful utilisation of wine lees for the development of a novel biorefinery and the production of PHB, using also crude glycerol as carbon source, sets an example of potential synergies between different industrial sectors leading to the production of bio-based chemicals and polymers in the bio-economy era. Within this context, restructuring conventional industrial sectors (e.g. wineries, biodiesel production from oilseeds) could lead to the development of novel biorefineries incorporating novel value chains, leading to the production of several value-added products.

CHAPTER 6

MATERIALS AND METHODS

6.1 Preservation and inoculum preparation for fungal strains

Solid state fermentations (SSF) for the production of crude enzyme consortia (mainly proteases) were carried out using an industrial strain of *Aspergillus oryzae* that was provided by Professor Colin Webb (University of Manchester, UK). The strain was originally isolated and purified by Wang *et al.* (2005) from a soy sauce industry (Amoy Food Ltd., Hong Kong). The fungal strain was maintained in slopes at 4 °C containing 3 % (w/v) rapeseed meal, 2 % (w/v) bran-rich wheat flour milling by-products and 2 % (w/v) agar (Sigma-Aldrich). The protocols for storage and sporulation for inoculum preparation in the case of *A. oryzae* have been described by Wang *et al.* (2005). The fungal spores were transferred and cultivated in 250 mL Erlenmeyer flasks using 50 mL of the same solid medium described above. The incubation temperature and duration used in both slopes and Erlenmeyer flasks were 30 °C for 72 hours (Koutinas *et al.*, 2001, 2005, 2007b).

SSF were also carried out for the production of predominantly amylolytic enzymes as well as proteolytic enzymes with the fungal strain *Aspergillus awamori* provided by Professor Colin Webb (University of Manchester, UK). The fungal strain was maintained in slopes at 4 °C containing 5 % (w/v) bran-rich wheat flour milling by-products and 2 % (w/v) agar. The protocols for storage and sporulation for inoculum preparation in the case of *A. awamori* have been described by Koutinas *et al.* (2001). The fungal spores were transferred and cultivated in 250 mL Erlenmeyer flasks using 50 mL of the same solid medium described above. The incubation temperature and duration used in both slopes and Erlenmeyer flasks were 30 °C for 96 hours.

Inocula for solid state fermentations, when either *A. oryzae* or *A. awamori* was used, were prepared by adding 50 mL of sterilized tap water on the surface of the solid culture in the Erlenmeyer flasks followed by scratching the surface of the fungal solid culture with a wire loop. The creation of a fungal spore suspension was facilitated by the addition of some glass beads (4 mm diameter) and some drops of Tween 80.

6.2 Preservation and inoculum preparation for bacterial strains

PHA production was carried out by the bacterial strains *Cupriavidus necator* DSM 545 and *C. necator* DSM 7237. All bacterial strains were received from the culture collection in lyophilised form. Rehydration and cultivation of bacterial cells was carried out using a medium containing 5 g/L peptone (Fluka) and 3 g/L meat extract (Himedia). Inocula preparation for shake flask and bioreactor cultures was carried out using bacterial stock cultures stored at 4 °C in petri dishes. The solid medium contained the same nutrients mentioned above supplemented with 2% (w/v) agar (Sigma-Aldrich). Bacterial cultures were also preserved in cryovials at -80 °C using aqueous solutions of 50% pure glycerol.

The preparation of inoculum for submerged fermentations for PHA production by both *Cupriavidus necator* strains was carried out in 250 mL Erlenmeyer flasks containing a liquid medium with 10 g/L commercial glucose or glycerol (Sigma-Aldrich), 10 g/L yeast extract (Himedia) and 5 g/L peptone (Fluka). Inoculum preparation was carried out at 30 °C and 180 rpm in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) using 50 mL broth volume. The duration of incubation was 12 h for both bacterial strains. In shake flask cultures, 1 mL of inoculum was aseptically transferred in the fermentation broth. A 10% (v/v) inoculum was used in bioreactor cultures.

6.3 Raw materials used in bioprocess and biorefinery development

The wine lees used in this study were provided by the winery Ampelou Techni Theodoros Stavrakis (Tyrnavos, Greece). Four wine lees were originally obtained from the red wine making process of Xinomauro and Merlot grape varieties, and the white wine making process of Malagouzia and Asyrtiko grape varieties. Figure 6.1 presents the initial wine lees and the liquid fraction obtained after centrifugation when the Malagouzia and Merlot derived wine lees were used. The wine lees were employed for biorefinery development and the production of nutrient-rich fermentation supplements used in this study in bacterial fermentations for the production of PHB.

Figure 6.1: White and red wine lees obtained from wine making processes using Malagouzia and Merlot wine varieties. The liquid fractions obtained after centrifugation are also presented.

The rapeseed meal was provided by Professor Colin Webb (University of Manchester, UK). Rapeseed meal was produced as by-product from biodiesel production derived after the extraction of rapeseed oil. Since rapeseed meal is a by-product displaying high contents of protein, carbohydrates and minerals its potential as media for fermentation processes was investigated. The composition of rapeseed meal used in this study is presented in Table 6.1 that was taken from Wang *et al.* (2010).

Table 6.1: Composition of rapeseed meal used in this study (Wang *et al.*, 2010)

Components	Content (%)
Moisture	10.59%
Total nitrogen (mg/g, db)	62.19
Protein (%, 6.25xTN, db)	38.87%
Oil (%, db)	2.45%
Ash (%, db)	7.48%
FAN (mg/g, db)	0.6504
Total phosphorus (mg/g, db)	19.19
Inorganic phosphorus (mg/g, db)	0.8064

The crude glycerol used in this study was provided by P. N. Pettas S.A. biodiesel producing industry (Patras, Greece) as by-product of the biodiesel production process using sunflower oil as raw material. The composition of grude glycerol is presented in Table 6.2 that was taken from Kachrimanidou *et al.* (2014). The original crude glycerol had a purity of 91% (w/w) and was derived from sunflower oil transesterification with methanol using NaOH as catalyst. This crude glycerol was pretreated via decanting using separation funnels leading to the separation of the upper phase containing non-polar compounds, not miscible with glycerol. The composition of the crude glycerol obtained after decanting is presented in Table 6.2.

Table 6.2: Composition of crude glycerol before and after treatment via decanting (Kachrimanidou *et al.* 2014)

Component	Crude glycerol composition	Crude glycerol composition 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

Bran-rich wheat milling by-products were used for the cultivation of *A. oryzae* and *A. awamori*. The starch, protein, phosphorus and moisture content of wheat milling by-products used in this study were 12%, 20%, 1.1% and 9.7% (w/w), respectively, as was taken from Tsakona *et al.* 2014).

Flour-rich waste streams were supplied by Jotis S.A., a Greek confectionery industry that produces a wide range of confectionery products and food for infants. The starch, protein and moisture content of flour-rich waste streams used in this study were 84.8%, 7.3% and 5% (w/w), respectively.

6.4 Autolysis of yeast cells contained in wine lees

The aim of these series of experiments was to evaluate the potential of autolysis as a natural process for the lysis of yeast cells contained in wine lees leading to the production of a nutrient supplement similar to commercial yeast extract. The wine lees employed were derived from the winemaking processes of Malagouzia, Asyrtiko, Xinomauro and Merlot grape varieties.

The method followed to carry out yeast cell autolysis was based on the methodology followed by Charpentier and Feuillat (1993) and Charpentier and Freyssinet (1989). In order to investigate the feasibility of autolysis of yeast cells in the wine lees used in this study, the effect of pH and temperature was evaluated. Five different pH values (3, 4, 5, 6, 7) and five different temperatures (30 °C, 40 °C, 45 °C, 50 °C, 55 °C) were evaluated. The pH value was adjusted to the aforementioned values using 5 M NaOH or 10% (v/v) H_2SO_4 in each type of wine lees. The pH value was measured throughout each experiment and it was verified that remained constant. An initial wine lees concentration of 50 g/L (on a dry basis) was used in each experiment. The autolytic reactions were carried out in 200 mL Duran bottles and the solution was agitated with magnetic stirrers. The Duran bottles were placed in waterbaths at the aforementioned temperatures. At random intervals, samples (2 mL) were taken and the solids separated from the liquid phase via centrifugation (10 min, 9000 rpm, 4 °C). Free amino nitrogen (FAN) and inorganic phosphorus (IP) concentrations were determined in the liquid phase.

6.5 Solid state fermentation of *A. oryzae* and *A. awamory*

SSF with *A. oryzae* was carried out using rapeseed meal as the sole solid substrate in 250 mL Erlenmeyer flasks at 30 °C according to the methodology described by Koutinas *et al.* (2001). SSF with *A. awamori* were carried out using bran-rich wheat flour milling by-products as the sole solid substrate in 250 mL Erlenmeyer flasks at 30 °C according to the methodology described by Koutinas *et al.* (2007). Each Duran bottle contained 5 g of solid substrate that was sterilized at 121°C for 30 min. Sterilised tap water (121 °C, 15 min) was used for the creation of fungal spore suspension and for setting the moisture content at 65% (w/w) at the beginning of the fermentation. The inoculation with spore suspension took place under aseptic conditions in a laminar flow cabinet (Holten-

TL2448). The spore suspension was formulated after the addition of 50 mL of sterilised tap water, some glass beads (4 mm diameter) and some drops of Tween 80 at the surface of the fungal solid culture. The fungal suspension was formed by vigorous shaking and by scratching the surface of the fungal solid culture with a wire loop. The concentration of fungal spores was 10^7 - 10^8 spores/mL. Owing to the fact that the moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities, the optimum moisture level must be ensured in order to optimise the SSF of any substrate (Baysal *et al.*, 2003).

In the case of *A. oryzae*, fermented solids produced after 72 h SSF duration were suspended in different liquid streams in order to extract the crude enzymes produced during SSF to be subsequently used for hydrolysis of rapeseed meal and wine lees solid fractions. In the case of *A. awamori*, fermented solids produced after 96 h SSF duration were suspended in different liquid streams in order to extract the crude enzymes produced during SSF to be subsequently used for hydrolysis of flour-rich waste streams. The liquid streams used were sterilized tab water, the liquid fraction of wine lees and the free of alcohol liquid fraction of wine lees. The suspension of solids was macerated using a kitchen blender, enhancing the extraction of enzymes to the surrounding medium.

6.6 Flour-based and rapeseed meal hydrolysis

The aim of these sets of experiments was to produce glucose and FAN rich media derived from the hydrolysis of flour-based and rapeseed meal, respectively. The produced hydrolysates were used as fermentation feedstocks for the production of PHB by *C. necator* DSZM 545 and DSMZ 7237. The fungal strain *A. awamory* used in this study can produce mainly amylolytic as well as other enzymes (*i.e.* proteases, phytases) when cultivated on wheat milling by-product streams. The fungal strain *A. oryzae* is a prolific producer of proteolytic enzymes as well as other enzymes (e.g. phytases, phosphatases) when cultivated on rapeseed meal (Wang *et al.*, 2010).

Enzyme-rich aqueous extract produced according to the methodology presented in the previous section were filtered to remove the solids. The liquid fraction containing crude enzymes produced by *A. awamori* were mixed with various concentrations of flour-rich waste streams in order to produce glucose-rich hydrolysates (Koutinas *et al.*, 2005). Figure

6.2 presents a common hydrolysate produced from flour-based waste streams. The liquid fraction containing crude enzymes produced by *A. oryzae* were mixed with various concentrations of rapeseed meal in order to produce nitrogen-rich hydrolysates (Wang *et al.*, 2010). All hydrolysis reactions were carried out in 1 L Duran bottles containing 500 mL of solid suspensions. The hydrolysis temperatures used was 55 °C in the case of flour-rich waste streams and 50 °C in the case of rapeseed meal. Samples of 2 mL were taken at random intervals and mixed with 5% trichloroacetic acid (1:1 ratio) in order to deactivate the enzymes and terminate the hydrolysis of solids. Determination of glucose and FAN was carried out in the case of hydrolysis of flour-based waste streams. Determination of FAN and IP was carried out in the case of rapeseed meal hydrolyses.

Figure 6.2: Hydrolysate derived from flour-based waste streams

6.7 Hydrolysis of yeast cells contained in wine lees

The hydrolysis of the yeast cell contained in wine lees using crude enzymes produced via SSF of *A. oryzae* have been carried out using different liquid and solid fractions. The liquid fractions used for the extraction of enzymes from SSF were: a) sterilized tap water, b) the liquid fraction of wine lees, and c) free of ethanol liquid fraction produced after distillation. The solid fractions used were: a) whole wine lees solid fraction, b) wine lees solid fraction free of tartrate salts, and c) wine lees solid fraction free of both tartrate salts and phenolic compounds. The final sets of experiments focused on the optimization of temperature, pH, initial wine lees concentration and initial proteolytic activity during enzymatic hydrolysis.

6.7.1 Production of wine lees hydrolysates using whole solid fraction of wine lees

In this set of experiments, different liquid fractions were used for the extraction of crude enzymes from SSF as mentioned above. The fermented solids were suspended in sterilized tap water, the liquid fraction of wine lees, or free of ethanol liquid fraction produced after distillation. The solids were subsequently macerated using a kitchen blender followed by vacuum filtration in order to obtain the crude enzyme consortia extract. Subsequently, the enzyme-rich extract was used to carry out wine lees hydrolysis. Initially, the whole solid fraction of wine lees was used. The enzyme-rich extract had 6.8 or 12 U/ml of initial proteolytic activity at the beginning of hydrolysis. The experiments were carried out in 500 mL Duran bottles containing 100 g/L wine lees (on a dry basis, db). The pH value was not controlled and the Duran bottles were placed in a water bath at 55 °C. At random intervals, samples (2 mL) were taken and immediately diluted (1:2) with trichloroacetic acid (5%, w/v) ensuring that the enzymatic reaction was terminated. The samples were centrifuged (10 min, 9000×g, 4 °C) and the liquid fraction was used for the analysis of FAN and IP. Hydrolyses experiments, as well as subsequent analyses, were performed in triplicates. For every hydrolysis experiment performed, a simultaneous control experiment was carried out under the same conditions using the same liquid and solid fractions without any enzyme addition.

6.7.2 Production of wine lees hydrolysates using a solid fraction free of tartrate salts

In the next set of experiments, the hydrolysis of the wine lees solid fraction free of tartrate salts was evaluated. Five experiments were carried out at uncontrolled pH and five different temperatures (35 °C, 40 °C, 45 °C, 50 °C, 55 °C) using the liquid fraction of wine lees that was free of ethanol obtained after distillation. This liquid stream was used for the extraction of crude enzymes from the SSF solids of *A. oryzae*. As for the solid fraction of wine lees, the tartrate salts had been removed prior to hydrolysis. The initial concentration of suspended solids was 100 g/L (db). The enzyme-rich extract had 6.8 or 12 U/ml of initial proteolytic activity at the beginning of hydrolysis. It should be stressed that for comparative reasons the same experiments were carried out using the solid fraction of wine lees without extraction of tartrate salts. The sampling procedure and analysis has been described in section 6.7.1.

The separation of tartrate salts from the wine lees solid fraction was carried out based on the methodology described by Versari *et al.* (2001), Salgado *et al.* (2010) and Rivas *et al.* (2006). The dried solid fraction of wine lees obtained after centrifugation was dissolved in 3.15 L H_2O per kg containing 0.361 L HCl per kg and agitated with a magnetic stirrer for 10 minutes. This procedure led to the formation of soluble tartaric acid. Following solubilisation of tartaric acid, the solid suspension was centrifuged (10 min, 9000×g, 4 °C) so as to remove the supernantant aqueous phase from the solid fraction. The solids obtained after centrifugation were used as the sole substrate to carry out hydrolylsis experiments (Figure 6.3).

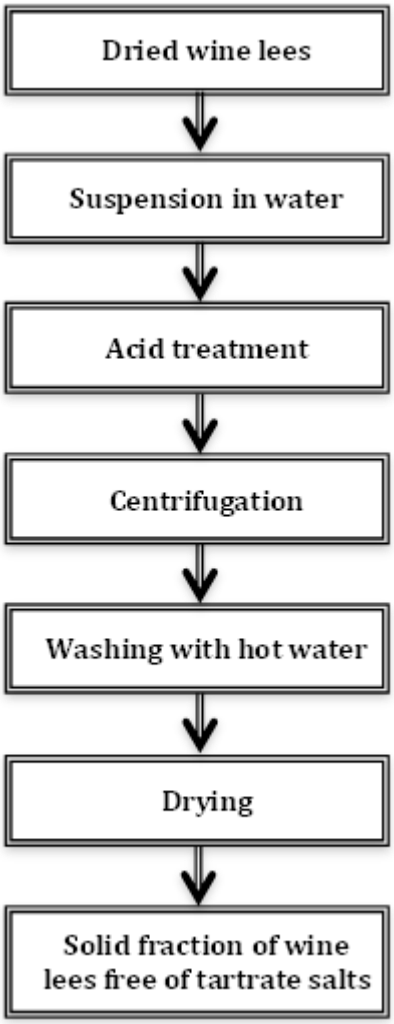


Figure 6.3: Process flowsheet of solubilization of tartrate salts with acid treatment so as to extract tartaric acid from the solid fraction of wine lees

The liquid fraction obtained via centrifugation was used for the recovery of calcium tartrate. Precipitation of tartrate salts was carried out using calcium carbonate (7.9 g per L of liquid) and calcium chloride (8.05 g per L of liquid). The precipitate was separated by centrifugation (12,000×g, 15 min, 10◦C) (Rivas *et al.*, 2006). The dry weight of the solid fraction obtained before and after HCl treatment was determined by drying samples at 105 ºC until constant weight was obtained.

6.7.3 Production of wine lees hydrolysates using a solid fraction free of tartrates and phenolics

The hydrolysis efficiency of yeast cells was subsequently evaluated using the solid fraction of wine lees after the extraction of both tartrate salts and phenolics. The solid fraction was initially treated with two different solvent systems to separate phenolic compounds. Phenolic compounds have been described as natural bioactive compounds present in winery waste streams including wine lees (Teixeira *et al.*, 2014). The aim of this work was to investigate the effect of phenolic compounds extraction on the hydrolysis of yeast cells and the production of a fermentation medium. Thus, optimization of phenolic extraction was not carried out as was not the main target of this study. The remaining solid fraction of wine lees after the separation of phenolics was subsequently treated with HCl solution as described in the previous section 6.7.2 in order to remove the tartrate salts.

Many solvent systems have been used for the extraction of phenolic compounds. Methanol proved to be 20% more effective than ethanol and 73% more effective than water in the extraction of anthocyanins (Metivier *et al.*, 1980). Phenolic compounds were extracted from wine lees using modified methodology reported by Chira *et al.* (2009). The extraction of phenolic compounds was carried out by mixing the solid fraction of wine lees with the following solvent systems:

A) initial treatment with acetone:H_2O (80:20, v/v) followed by treatment with methanol:H_2O (60:40, v/v)

B) ethanol:H_2O (70:30, v/v)

The treated solids were separated from the solvent system via centrifugation (15 min, 9,000×g, 10 ºC). These solid fractions were free of phenolic compounds. The second

solvent system of ethanol and water was employed in order to exploit the ethanol removed from the liquid fraction of wine lees. In this way, the ethanol contained in wine lees could be employed for the recovery of phenolic compounds without any need for using any commercial solvents.

The solid concentration used for the extraction of phenolics was 50 g wet wine lees (moisture content around 70%) mixed with 400 mL of each organic solvent system. The suspension was placed in 1000 mL Erlenmeyer flasks closed with rubber stoppers and wrapped in aluminium foil, followed by agitation in a rotary shaker (Shaker, ZHWY-211B Rocking Incubator) for 3 h with acetone:H_2O (80:20, v/v) solvent mixture and 2.5 h with methanol:H_2O (60:40, v/v) solvent mixture. The temperature and agitation used were 25 ^{0}C and 160 rpm.

The solid fraction resulted after the extraction of phenolics and tartrate salts was evaluated for hydrolysis efficiency. Five experiments were performed (as described in section 6.7.2) at five different temperatures (35 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC) and uncontrolled pH (the pH value was around 4). The experimental set-up was the same as the one employed in sections rest hydrolytic process followed was the same, as described in 6.7.1 and 6.7.2.

6.7.4 Optimisation of hydrolysis efficiency

The optimization of yeast cell hydrolysis using the wine lees solid fraction obtained after the extraction of phenolics and tartrate salts was carried out by determining the optimum values of the following parameters: 1) the duration of the process, 2) the temperature, 3) the pH value, 4) the initial activity of proteolytic enzymes, and 5) the initial concentration of the solid fraction of wine lees. The main objective was to optimize the yeast cell hydrolysis so as to produce a generic nutrient-rich fermentation medium. The ideal case would be to achieve disruption of yeast cell membrane followed by hydrolysis of the proteins and other macromolecules for the production of a medium with equivalent nutrient composition to yeast extracts as the ones produced from other industrial processes (Chae *et al.*, 2001).

The enzyme-rich extract produced by solid state fermentation of *A. oryzae* (as described in section 6.5) was added in 1 L Duran bottles contained varying quantities of pretreated wine lees derived solids depending on the experiment. The solids used were produced after the extraction of phenolics and tartrate salts as described in section 6.7.3. The Duran bottles were placed in a water bath (Figure 6.4) at varying temperatures depending on the experiment.

Figure 6.4: Enzymatic hydrolysis of wine lees derived solids

Twenty experiments were carried out in triplicate aiming to evaluate the effect of temperature (35 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC), pH value (4.0, 4.5, 5.0, 5.5, 6.0, 6.5), initial solid concentration (30 g/L, 50 g/L, 75 g/L, 100 g/L, 150 g/L, 200 g/L, 300 g/L, 400 g/L) and initial proteolytic activity (12 U/mL, 23.6 U/mL). Mixing of the suspension was achieved with magnetic stirrers. Samples were collected at random intervals and the solids were separated via centrifugation (10 min, 9000×g). The supernatant was used for the analysis of free amino nitrogen and inorganic phosphorus. Hydrolysis yield was expressed as the percentage of total Kjeldahl nitrogen (TKN) to FAN conversion.

6.8 Production of fermentation media for *C. necator* bioconversions

After the end of hydrolysis of wine lees derived solids, rapeseed meal and flour-based waste streams, the remaining solids were removed by vacuum filtration. The filtrate was filter-sterilized using a 0.2 μm filter unit (Polycap TM AS, Whatman Ltd.). Figure 6.5

presents the wine lees hydrolysate obtained after filtration that was subsequently evaluated as fermentation feedstock for poly(3-hydroxybutyrate) production.

Figure 6.5: Wine lees hydrolysate obtained after vacuum filtration and filter sterilization

The pH value of all hydrolysates was adjusted to the optimum range (6.7 - 6.9) for *C. necator* growth by adding 5 M NaOH. The optimum range of pH for *C. necator* growth is in the range of 6.7 - 7.0 (Aramvash *et al.*, 2015).

6.9 Bacterial fermentations using renewable resources

6.9.1 Flour-based media as sole fermentation feedstock for PHB production

Flour-based hydrolysates were evaluated as the sole nutrient source for PHB production by *C. necator* DSM 545 and *C. necator* DSM 7237. Four shake flask fermentations were carried in 250 mL Erlenmeyer flasks in order to evaluate PHB production. The effect of different initial glucose (10 g/L, 20 g/L, 25 g/L, 30 g/L) and FAN (43, 80, 120, 140 mg/L) concentrations were investigated. The production of hydrolysates from flour-rich waste streams has been described in sections 6.5 and 6.6.

Flasks containing 50 mL of the fermentation feedstock were aseptically inoculated with 1 mL of 14 h pre-culture of *C. necator* DSZM 545 cells or *C. necator* DSZM 7237 cells and incubated in a rotary shaker at 30 °C and 180 rpm agitation. Samples of 1 mL were taken at random intervals that were centrifuged (9000×g, 10 min) in order to separate the liquid from the solid fraction. The sediment was washed with distilled water, centrifuged again and the remaining solids were suspended in acetone and transferred in

pre-weighed 14 mL McCartney universal bottles for the determination of total dry weight, PHB concentration and residual cell mass (RCM). The supernatant was used for the determination of glucose and free amino nitrogen concentrations. All fermentations were performed in triplicate.

6.9.2 PHB production using flour-based and nitrogen rich hydrolysates

Mixtures of flour based and rapeseed meal hydrolysates were evaluated as fermentation media for PHB production using the bacterial strain *C. necator* DSMZ 545 and *C. necator* DSMZ 7237**.** The aim of this set of experiments was to evaluate the effect of rapeseed meal as nitrogen rich source for PHB production. Four shake flask fermentations were carried out in the case of *C. necator* DSMZ 545 in order to evaluate the effect of different initial FAN concentrations (150 mg/L, 250 mg/L, 340 mg/L, 450 mg/L), while the initial glucose concentration was constant (20 g/L). Five shake flask fermentations were carried in the case of *C. necator* DSMZ 7237 using different initial FAN concentrations (150 mg/L, 212 mg/L, 396 mg/L, 501 mg/L and 598 mg/L), while the initial glucose concentration of 25 g/L was constant in all fermentations. The flasks were aseptically inoculated with 1 mL of 14 h preculture and incubated in a rotary shaker at 30 ^{o}C and 180 rpm agitation speed. Samples of 1 mL were taken at random intervals. A similar methodology as the one presented in section 6.9.1 was followed.

Mixtures of flour based and wine lees hydrolysates were also evaluated for the production of PHB by *C. necator* DSMZ 545 and *C. necator* DSMZ 7237. Nine experiments were carried out in the case of *C. necator* DSM 545 in order to study the effect of different initial FAN (100 g/L, 200 g/L and 300 g/L) and glucose (10 g/L, 15 g/L, 20 g/L) concentrations on PHB production. Nine experiments were also carried out in the case of *C. necator* DSMZ 7237 in order to study the effect of different initial glucose (30 g/L, 23 g/L and 15 g/L) and FAN (433.23 g/L, 295.42 g/L, 156.78 g/L) concentrations on PHB production. Wine lees hydrolysates were produced via enzymatic hydrolyses of 25 and 50 g/L (on a dry basis) initial solid concentration of wine lees derived solids. Flasks were aseptically inoculated with 1 mL of 14 h pre-culture and incubated in a rotary shaker at 30 ^{o}C and 180 rpm. Samples of 1 mL were taken at random intervals. A similar methodology as the one presented in section 6.9.1 was followed.

6.9.3 Evaluation of the initial glucose and FAN concentrations

The optimum initial glucose/FAN ratio in fermentation feedstocks containing flour based and wine lees hydrolysates were evaluated. The aim of this set of experiment was investigate the potential to maximize PHB production. The bacterial strain employed was *C. necator* DSMZ 545. Three experiments were conducted in 250 mL Erlenmeyer flasks (50 mL broth volume) evaluating the effect of different initial FAN concentration (40 mg/L, 60 mg/L, 80 mg/L and 100 mg/L), whereas the initial glucose concentration was constant at 20 g/L in all fermentations. Flasks were aseptically inoculated with 1 mL of 14 h pre-culture and incubated in a rotary shaker at 30 °C and 180 rpm agitation. Samples of 1 mL were taken in random intervals.

6.10 PHB production using crude glycerol and wine lees hydrolysates

6.10.1 Shake flask fermentations

Wine lees hydrolysates and crude glycerol were evaluated as sole renewable resources for PHB production. The bacterial strain *C. necator* DSMZ 7237 was used for the production of PHB employing the aforementioned crude renewable resources as fermentation feedstocks. Shake flask fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of fermentation broth. The initial concentration of crude glycerol was 25 g/L and wine lees hydrolysates with an initial FAN concentration of 100, 200, 300, 400 and 500 mg/L were used as crude nutrient-rich supplement. The wine lees hydrolysates used in all fermentations were produced after hydrolysis of wine lees initial solid concentration of 50 and 100 g/L at 40 °C, using initial proteolytic activity of 6.4 U/mL and 12 U/mL. Bacterial fermentations were inoculated by adding 1 mL of pre-culture medium and then were incubated for 12 h at 30 °C and 180 rpm using a rotary shaker. The initial pH value was in the range of 6.7-6.9. A similar methodology as the one presented in section 6.9.1 was subsequently followed.

6.10.2 Fed-batch bioreactor cultures

C. necator DSMZ 7237 bacterial strain was used for fed-batch bioreactor fermentations using crude glycerol and wine lees hydrolysates as fermentation feedstocks. The initial crude glycerol was around 25 g/L and the initial FAN concentration was varied (300, 500, 700, 950, 1100 mg/L). The wine lees hydrolysates used in all fermentations were produced after hydrolysis of wine lees solids (50-100 g/L) at 40 °C, using initial proteolytic activity of 6.4 U/mL. The suspension contained crude enzymes produced via SSF of *A. oryzae*.

Bioreactor fermentations with *C. necator* 7237 were performed in 1-L bioreactor (New Brunswick Scientific Co, USA, Figure 6.6) using a 10% (v/v) pre-culture medium as inoculum. A 12 h pre-culture in shake flasks was used as inoculum with an optical density ranging from 1.8 to 2 at 600 nm. Fermentation temperature was maintained at 30 °C, the aeration rate was maintained at a flow rate of 1 vvm and the pH value was regulated in the range of 6.7–6.9 using 5 M NaOH and 10% (v/v) H_2SO_4 solutions. The agitation speed was controlled in the range of 200-500 rpm in order to control the dissolved oxygen (DO) concentration in the bioreactor at 20% of saturation. Concentrated crude glycerol (75% v/v) solution was used as feeding media in fed-batch bioreactor fermentations, while the trace element solution contained: 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L $FeCl_3 \cdot 6H_2O$, 0.02 g/L $ZnSO_4$, 0.06 g/L $MnSO_4$, and 0.15 g/L $CaCl_2 \cdot 2H_2O$. All bacterial fermentations were carried out in duplicate.

Figure 6.6: The lab-scale bioreactor used in the fermentations for PHB production (New Brunswick Scientific Co, USA)

In each fermentation, the pH probe was calibrated prior to sterilization of the vessel assembly. The crude glycerol and the bioreactor including the pH and DO probes were sterilized for 30 min at 121 °C. The wine lees derived hydrolysates were filter-sterilized, using a 0.2 μm filter unit (Polycap TM AS, Whatman Ltd.) and then were aseptically transferred in the bioreactor. The wine lees derived hydrolysates that were used in all fermentations were produced after hydrolysis of wine lees solids (50–100 g/L) at 40 °C, using initial proteolytic activity of 6.4 U/mL or 12 U/mL depending on the required initial FAN needed in each fermentation. The temperature was controlled using a heating jacket that was placed around the vessel.

Samples of 2 mL were taken at random intervals. The supernatant of the fermentation samples (obtained after centrifugation at 3000×g for 10 min) was analyzed for FAN, inorganic phosphorus, and glycerol determination. The sediment was washed with distilled water, centrifuged again and the remaining solids were suspended in acetone and transferred in pre-weighed 14 mL McCartney universal bottles for the determination of residual cell mass (RCM) and PHB concentration.

6.10.3 Fed-batch bioreactor cultures with supplementation of trace elements

The bacterial strain *C. necator* DSZM 7237 was used in bioreactor cultures carried out using the conditions presented in the previous section with supplementation of trace elements. This set of experiments evaluated the potential to enhance the production of PHB by adding the trace elements that may be absent or in low concentrations in the crude glycerol and wine lees derived hydrolysates used in this study.

The addition of trace elements was carried out along with wine lees derived hydrolysates with five different initial FAN concentrations (300, 500, 700, 950, 1100 mg/L). The initial crude glycerol concentration was around 25 g/L. The trace element solution used contained 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L $FeCl_3 \cdot 6H_2O$, 0.02 g/L $ZnSO_4$, 0.06 g/L $MnSO_4$, and 0.15 g/L $CaCl_2$ $2H_2O$. The trace element solution was sterilized at 121 °C for 15 min. The fermentation conditions and the bioreactor used as well as the sampling procedure and the analysis of fermentation parameters were the same as the ones described in section 6.10.2.

6.10.4 Fed-batch bioreactor cultures for the evaluation of the initial carbon to FAN ratio

Three fermentations were carried out in the bioreactor using the same conditions as the fermentations carried out in section 6.10.3 in order to evaluate the effect of the initial carbon to FAN ratio (8.6 g/g, 12.3 g/g, and 14 g/g) on the production of PHB. All fermentations were supplemented with mineral salts as described in section 6.10.3. The initial FAN concentration was 700 mg/L. The wine lees derived hydrolysates were produced via hydrolysis of 100 g/L wine lees derived solids with crude enzymes having initial proteolytic activity of 12 U/mL. The targeted carbon to FAN ratio was achieved by using initial glycerol concentrations of 15.3 g/L, 22 g/L and 25 g/L in the three fermentations. All fermentations were carried out under fed-batch mode and each fermentation was terminated when the PHB production stopped.

6.11 Analytical methods

6.11.1 Dry weight of wine lees

Wet samples of 100 mL of wine lees from malagouzia, asyrtiko, xinomauro and merlot varieties were weighted, then dried at 105 °C until contant weight and left to cool in a dessicator. The dried samples were weighted again and the dry weight was estimated according to the equation: Dry weight = (dry weight/wet weight)*100). The same procedure was followed in every step of the wine lees fractionation so as to determine the mass balances.

6.11.2 Estimation of fresh matter of solid fraction of wine lees after centrifugation

Fresh matter represents the moisture content of solid fraction resulted after centrifugation. Preweighted solids of 10 g were dried at 90 °C and the fresh matter was then calculated by the equation: Moisture content (%) = (wet weight-dry weight)/wet weight) x 100).

6.11.3 Protease activity

The proteolytic activity of the enzymes produced from *A. oryzae* during the SSF of wheat bran was studied. A quantity of 2.5 g of fermented solids were macerated in a kitchen blender after being suspended in 50 ml of phosphate buffer (pH 6). Then solids were removed through centrifugation (4 ^{0}C, 9000 rpm, 20 min) and 5 mL of the enzymatic extract were utilised in enzymatic assay after mixing with 5 mL of 7.5 g/L casein. 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 7.5 g/L of casein 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 under the defined conditions.

6.11.4 Total Kjeldahl Nitrogen

Total Kjeldahl Nitrogen (TKN) concentration was measured using a KjeltekTM 8100 distillation Unit (Foss, Denmark). The method is based on the total conversion of the initial forms of nitrogen in ammonium salts. Homogenized and dry wine lees of 0.5 g were accurately weighted (accurancy of 4 decimals) into a digestion tube. The samples were digested in 25 ml of sulfuric acid (H_2SO_4) in order to convert the nitrogen of protein to $(NH_4)_2SO_4$ at a boiling point elevated by the addition of K_2SO_4 with a Cu catalyst to enhance the reaction time (kjeltab tablets containing 3.5 g K_2SO_4 and Cu_2SO_4). Also, blank sample were prepared and placed in sample digestion apparatus. The digestion was carried out at 420 ^{0}C for 1h. After digestion the tubes were cooled down and then were placed in the distillation unit, where ammonium salts are converted to ammonia (NH_3) by automatic addition of 80ml H_2O and 50 ml (40%) NaOH in the tubes. NH_3 is distilled into a boric acid solution by alkaline steam distillation and quantified titrimetrically with standard acid solution (0.1 N HCl).

Total nitrogen was calculated according to the equation: Total Kjeldahl Nitrogen (%) = ((V_{sample} - V_{blank}) x N x 14.007 / mg of sample) x 100), where V_{sample} = ml of 0.1N HCl consumpted for the sample, V_{blank} = ml of 0.1N HCl consumpted for the blank, N =

normality of HCl. In order to estimate the protein content of wine lees, Total Kjeldahl Nitrogen was multiplied by 6.25, given the fact that proteins contain 16% nitrogen (100/16=6.25).

6.11.5 Free amino nitrogen determination

The free amino nitrogen (FAN) concentration in raw materials, autolysates, hydrolysates 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. Determination of FAN is detected at 570 nm (Kolakowski, 2005; Sun *et al.*, 2006).

For the colorimetric quantification of free amino nitrogen of amino acids and petides the preparation of the following solutions were employed:

1) Glycine stock solution: This solution was used to prepare the standard curve. In particular, 0.1072 g of glycine was dissolved in deionised water in a volumetric bottle of 100 mL.

2) Glycine standard solution: a final concentration of FAN 4 mg/L was prepared by adding 1 mL of the glycine stock solution in deionized water until 50 mL final volume.

3) Staining-Color Reagent: For the preparation of 1L of the reagent 49.71g Na_2HPO_4 $2H_2O$ were mixed with 5 g ninhydrin, 3 g of fructose and approximately 60 g K_2PO_4 until the pH adjusted to 6.6 - 6.8. The solution stored in dark place at 4 ^{0}C.

4) Dilution reagent: 2 g of KIO_3 (Potassium iodate) were dissolved in 500 mL of deionized water and subsequently 384 mL of absolute ethanol (99%) were added. Then, the solution transferred into 1000 mL graduated cylinder and filled with water up to 1000 mL total volume. The solution stored in dark place at 4 ^{0}C.

The methodology followed for the FAN analysis was based on the protocol published by Lie (1973): In a test tube 1 mL of diluted sample was added and then mixed

with 0.5 mL color reagent followed by vortex. A blank sample (control) was prepared by the same procedure just mentioned, but 1 mL of deionized water was added in the test tube instead of sample. Then, the tubes were transferred to a water bath (100 ^{0}C) for 16 min exactly and immediately cooled down in a bowl with ice and water H_2O (20 ^{0}C) for 20 min exactly. In each test tube 2.5 mL dilution reagent were added followed by vortexing for 20 sec exactly. Thereafter the absorbance was read in spectrophotometer at 570 nm (U-2000, Spectrophotometer, Hitachi) against blank sample. All the samples were carried out in duplicate.

The results were expressed using reference curves, which were prepared by diluting standard glycine solution so as to achieve concentrations of 0.3, 0.5, 1, 1.5, 2, 2.5 mg / L FAN. Measuring the absorbance of the known concentrations at 570 nm the equation of the calibration curve was: y = ((5.484 x (ABS mean)) + 0.002) x dilution), (R^2=0.999), where y axis depicts concentration of FAN, in mg/L.

6.11.6 Inorganic Phosphorus determination

Inorganic phosphorus (IP) in samples was assayed by the ammonium molybdate spectrophotometric method (Harland and Harland, 1980). Phosphorus reacts with perchloric acid to produce orthophosphate, which under acidic conditions reacts with ammonium molybdate yielding a phosphomolybdate. The phosphomolybdate is subsequently reduced from ascorbic acid resulting in the molybdenum blue. The concentration of inorganic phosphorus is proportional to the intensity of the blue color (King, 1932; Allen, 1940).

Before starting the colorimetric analysis for the determination of IP in the samples the following solutions were prepared:

1) Standard inorganic phosphorus solution: 0.0561 g K_2HPO_4 was dissolved in 1000 mL of deionised water so as to achieve a final concentration of 10 mg/L IP. The reagent was diluted in order to obtain known concentrations of IP ranged from 1 to 10 mg/L, which were used for the preparation of the calibration curve.

2) 60% v/v percloric acid: via dilution of 70 % perchloric acid ($HClO_4$) (ACS reagent, Sigma-Aldrich).

3) 1% (w/w) ascorbic acid ($C_6H_80_6$) (Sigma-Aldrich).

4) 5% (w/v) ammonium molybdate ($(NH_4)_2MoO_4$, Sigma Aldrich).

The standard procedure for the IP analysis includes firstly the appropriate dilution of the sample with deionized water up to 5 mL final volume into glass test tubes. Then, 0.4 mL of 60% (v/v) perchloric acid was added following vortex for 10 sec. Subsequently, 0.3 mL of freshly prepared ascorbic acid (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 poured into the tubes and 20 sec vortexing was employed. The tubes were left for 10 min, allowing the colour development and then absorbance was read at 730 nm using a spectrophotometer (U-2000, Spectrophotometer, Hitachi). Also, a blank sample (control) was prepared replacing the sample with 5 mL of deionized water. All the samples were carried out in duplicate. Standard equation used for IP determination was: y = ((4.4346 x (ABS mean) - 0.2872) x dilution), (R^2=0.999), where y axis depicts concentration of IP, in mg/L.

6.11.7 Analysis via High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC, Waters 600E) equipped with an Aminex HPX-87H (300 mm x 7.8 mm, Bio Rad, CA, USA) column, coupled to a differential refractometer (RI Waters 410) was used for the determination of glycerol, glucose, ethanol, tartaric acid, acetic acid, malic acid and succinic acid. Operating conditions were: a mobile phase of 0.005 M H_2SO_4 and a flow rate of 0.6 mL/min was set. The sample volume was 40 μL and the column temperature was 65 °C. Before injection samples were diluted to appropriate concentration with deionised water and filtered through a 0.2 μm membrane filter.

Quantification of unknown concentrations of glucose, glycerol, ethanol, tartaric acid, acetic acid and succinic acid in samples was performed by estimating the area at the retention times of known concentrations of glucose, glycerol, ethanol, tartaric acid, acetic acid and succinic acid. Standard solutions of known concentrations of glycerol, glucose, ethanol, tartaric acid, succinic acid and acetic acid were prepared giving the following equations:

1) Glycerol: y = ((410^{-6} x +0.0016) x dillution), where x=integration of the peak formed at 13min and y=concentration of glycerol, in g/L, R^2 = 0.99

2) Glucose: y = ((410^{-6} x + 0.0141) x dillution), where x = integration of the peak formed at 9.2 min and y = concentration of glucose in g/L

3) Ethanol: y = ((7 10^{-6} x + 0.0712) x dillution), where x = integration of the peak formed at 21.2 min and y = concentration of ethanol in g/L, R^2 = 0.99

4) Acetic acid: y = ((4.23 10^{-6} x- 0.003) x dillution), where x = integration of the peak formed at 14.8 min and y = concentration of acetic acid in g/ L, R^2 = 0.99

5) Tartaric acid: y = ((5 10^{-6} x- 0.0879) x dillution), where x = integration of the peak formed at 11.5 min and y = concentration of tartaric acid in g/L, R^2 = 0.99

6) Succinic acid: y = ((3.27 10^{-6} x - 0.0771) x dillution), where x = integration of the peak formed at 14.8 min and y = concentration of tartaric acid in g/L, R^2 = 0.99

6.11.8 Total dry weight analysis of microbial mass

The fermentation samples were centrifuged (3000×g for 10 min) and the supernatant was collected for the analysis of FAN, IP and glycerol or glucose concentration. The sediment was washed with distilled water, centrifuged again and the remaining solids were suspended in acetone and transferred in pre-weighed 14 mL McCartney universal bottles for the determination of residual cell mass (RCM) and PHB. The total dry weight (TDW) was analysed by drying the sediment from each fermentation sample at 50 °C until constant weight (Figure 6.13). The RCM was determined by subtracting the PHB concentration measured by GC from the TDW. All analyses were performed in triplicate and results were expressed in g/L.

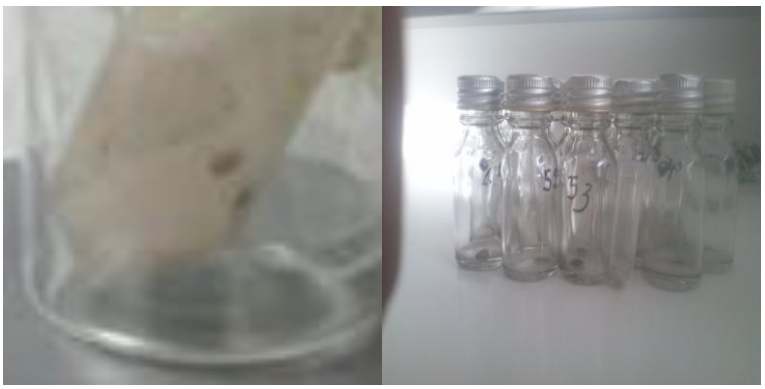


Figure 6.7: Fermentation samples containing the sediment after drying until constant weight and before pretreatment for PHB analysis

6.11.9 Determination of PHB concentration

The PHB concentration was determined by the method described by Riis and Mai (1988). In particular, 4 mL of 1,2-dichloroethane:acidified propanol (HCl:n-propanol 1:4) mixture solution at 1:1 ratio (v/v) were added to McCartney bottles contained dry biomass cells. The samples were placed in a water bath at 100 °C for 2 hours. After the samples were cooled 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. Benzoic acid (99.6%, Acrōr Organics) was used as internal standard at a concentration of 200 mg/L.

A gas chromatographic analyzer (GC Fisons 8060) equipped with a Flame Ionization Detector (FID) and a Chrompack column (60mx0.25mm, film thickness 0.25 μm, J&W Scientific) was used for PHB concentration analysis. Helium was used as carrier gas at a flow rate of 2 mL/min. Oven initial temperature was set at 120 ^{0}C for 1 min, followed by a temperature ramp of 10 ^{0}C/min to 200 ^{0}C, held constant for 5 min, and then increased to 220 ^{0}C at 10 ^{0}C/min with a final isothermal period of 3 min. The injector temperature was 240 ^{0}C and detector 230 ^{0}C. The appearance of the PHB monomer in the chromatogram was confirmed based on the retention time of the respective monomer from commercial PHB standard (Sigma). The quantification of PHB in samples was performed using a standard PHB curve developed by integrating peaks at known concentrations of PHB.

CHAPTER 7

BIOREFINERY DEVELOPMENT BASED ON WINE LEES

7.1 Aim of novel biorefinery strategy exploiting wine lees

Bearing in mind that the development of any sustainable strategy for grape cultivation and wine production should take into account the reuse and valorisation of by-product and waste streams, the aim of this study was to evaluate the potential of wine lees as renewable resource for biorefinery development. The implementation of the biorefinery concept (Chapter 1) is based on the development of sustainable processes that convert biomass into value added products, with the lowest environmental impact. Thus, the potential of wine lees as renewable resource for biorefinery development was investigated through the initial extraction of value added products followed by the production of a generic fermentation feedstock.

Wine lees, were kindly provided by the winery Ampelou Techni Theodoros Stavrakis (Tyrnavos, Greece) and quantitative and qualitative analysis was carried out in order to evaluate the nutrient content. In this way, the wine lees were evaluated as nutrient-rich medium in comparison to commercial nutrient supplements (e.g. yeast extract). The release of nutrients from wine lees was attempted either via autolysis of yeast cells or via enzymatic hydrolysis using crude enzymes produced via solid state fermentation. This chapter presents the experimental results on the production of a nutrient-rich feedstock from wine lees**.**

7.2 Characterisation of wine lees

The wine lees were initially processed via centrifugation for the separation of a liquid and a solid fraction. Table 7.1 presents the dry weight, moisture, total Kjedahl nitrogen, protein, tartrate salts and inorganic phosphorus content in the solid fraction of four wine lees produced from four wine production processes.

Table 7.1: Composition of solid fraction of wine lees originated from different grape varieties

Origin of WL - grape variety	Dry weight of original wine lees[1] (%, w/v)	Moisture content of solids after centrifugation (%, fm[2])	Total Kjeldahl nitrogen (TKN) (mg/g, db[3])	Protein (%, 6.25×TKN, db)	Tartrate salts (%, w/w)	Inorganic phosphorus (%, w/w)
Malagouzia	17.5	63	25.3	15.8	15.3	0.25
Asyrtiko	21.9	60	13.6	8.5	14.2	0.32
Xinomauro	24.6	53.5	22.4	14	21.2	0.56
Merlot	31.4	69.4	24.8	15.5	1.36	0.50

[1] represents dry weight of 100 mL wine lees
[2] fresh matter, regarded as the moisture of the solids separated from the liquid layer by centrifugation of wine lees
[3] dry basis, standing for dry weight content of solid fraction of wine lees

As can be observed in Table 7.1, the solid phase of wine lees contains a significant protein content. The high protein content determined in the solid fraction of wine lees is mainly attributed to the high protein content of yeasts cells. Assuming that the protein content in yeast cells is approximately 40 - 50% (Csonka, 1935), it could be deducted that the solid fraction of lees contains a significant amount of yeasts cells. According to Table 7.1, the protein content was 15.8 %, 15.5 %, 14 %, and 8.5 % (on a dry basis) for wine lees derived from the winemaking varieties of Malagouzia, Merlot, Xinomauro, and Asyrtiko. The wine lees from Malagouzia and Merlot contained the highest protein content. The dry weight is higher when using lees from the red winemaking (xinomauro, merlot) technology rather than the white wine making (malagouzia, ayrtiko). The highest concentration of tartrate salts was determined in Xinomauro derived wine lees followed by Merlot, whereas the lowest concentrations were observed in Malagouzia and Asyrtiko. The vast majority of tartaric acid existing in wine grapes and in wine products is in the form of potassium salts (potassium hydrogen tartrate). All wine lees presented in Table 7.1 contained inorganic phosphorus in the range varying from 0.25 to 0.56 %.

The high protein and phosphorus content of the solid fraction of wine lees indicates that it could be used as a nutrient supplement in fermentation processes. The nutrient content of wine lees is variable depending on the racking process, decanting time and the wine making processing step in which lees are retrieved (Chapter 3). In this study, the wine lees used were derived from the third decanting step.

Table 7.2 presents the glucose, glycerol, ethanol, organic acid (malic, tartaric, succinic and acetic acids) and free amino nitrogen content in the liquid fraction of wine lees. Glucose was detected in all tested varieties of liquid fraction of wine lees, but in low concentrations, especially in wine lees derived from Merlot and Malagouzia varieties. The low sugar concentration was anticipated as the sugar content has been converted into ethanol during fermentation. The highest glycerol concentration was determined in Merlot wine lees (9.7 g/L), whereas the lowest was observed in Xinomauro derived wine lees (1.8 g/L). Besides ethanol, glycerol is a major metabolic product in alcoholic fermentation. The usual glycerol concentration in wine ranges from 4 to 9 g/L (Radler and Schülz, 1982). Sweetness is the main contribution of glycerol to sensory characteristics at levels commonly found in wines (Noble and Bursick, 1984). The concentration of malic, acetic and succinic acids were rather low in all wine lees presented in Table 7.2. Acetic acid was present in low concentrations in lees obtained from red winemaking. In general, acetic acid bacteria (*Gluconobacter oxydans, Acetobacter pasteurianus, and Acetobacter aceti*) are present at all stages of winemaking, from the grape harvest to vinification. In the red wine production technology, must is left in contact with grape skins in order to improve the aroma and allow coloring agents and tannins to diffuse in must. This practice increases the possibility of finding acetic acid in wine lees produced during production of red wines (Joyeaux *et al.*, 1984).

Table 7.2: Composition of liquid fraction of wine lees originated from different grape varieties, produced after centrifugation of initial wine lees

Origin of wine lees	Glucose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	FAN (mg/L)
Malagouzia	1.2	8.0	103.1	3.9	1.0	0.1	0.1	152.7
Asyrtiko	3.3	8.2	89.9	5.3	1.1	-	0.3	556.9
Xinomauro	2.6	1.8	88.5	2.4	0.3	0.1	0.9	264.3
Merlot	1.3	9.7	108.2	4.1	-	1.3	0.8	161.3

Tartaric acid was determined in the liquid fraction of wine lees in low quantities, owning to the fact that it had been precipitated in the solid fraction of wine lees in the form of corresponding salts, such as potassium bitartrate and calcium tartrate.

It is worth noting that the free amino nitrogen (FAN) of the liquid fraction of wine lees was reasonably high, especially in the case of Asyrtiko derived wine lees (556.9 mg/L). Such a high concentration of FAN could be adequate in various fermentation processes as it is equivalent to around 11 g/L of yeast extract that usually contains 50 mg FAN per g. The high FAN content indicates that yeast cell autolysis may have occurred. Gómez *et al.* (2004) reported that lees derived from Sherry and other wines contained 15.1% protein, 4.1% sugars and 24.6% tartrate salts.

7.3 Autolysis of wine lees

Autolysis experiments were carried out so as to evaluate if wine lees autolysates could be used as nutrient supplements for microbial fermentations, without any further treatment of wine lees (such as separation of liquid from solid fraction and extraction of tartrates and ethanol).

The cell wall of yeast cells (Figure 7.1) is mainly comprised of glucan, mannan, chitin and proteins (Vuković and Mrša., 1995). During autolysis, the cell wall is gradually degraded through the hydrolysis of glucan and chitin fibres along with mannoproteins (Figure 7.1), a task performed by the lytic enzymes secreted by the yeast cells. The high content of nitrogenous compounds in wine is considered a measure of autolysis (Sato *et al.*, 1997). Autolysis is a natural process resulting in the hydrolysis of macromolecules of yeast cells releasing directly digestible nutrients in the surrounding liquid (Pueyo *et al.*, 2000). In fact, the autolysis process can be divided into two stages: 1) degradation of the cell wall (Figure 7.1), and 2) degradation of cellular consituents, which predominantly include the hydrolysis of proteinaceous substances also known as proteolysis.

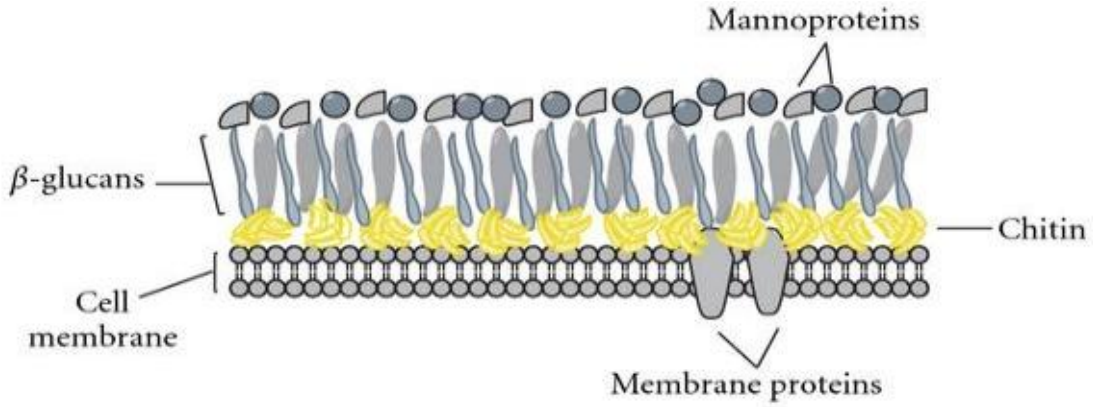


Figure 7.1: Cell wall of yeast cells (Tresseder, 2015)

In order to investigate the feasibility of autolysis of yeast cells in the wine lees used in this study, the effect of pH and temperature was evaluated. Five different pH values (3, 4, 5, 6, 7) and five different temperatures (30 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC) were evaluated.

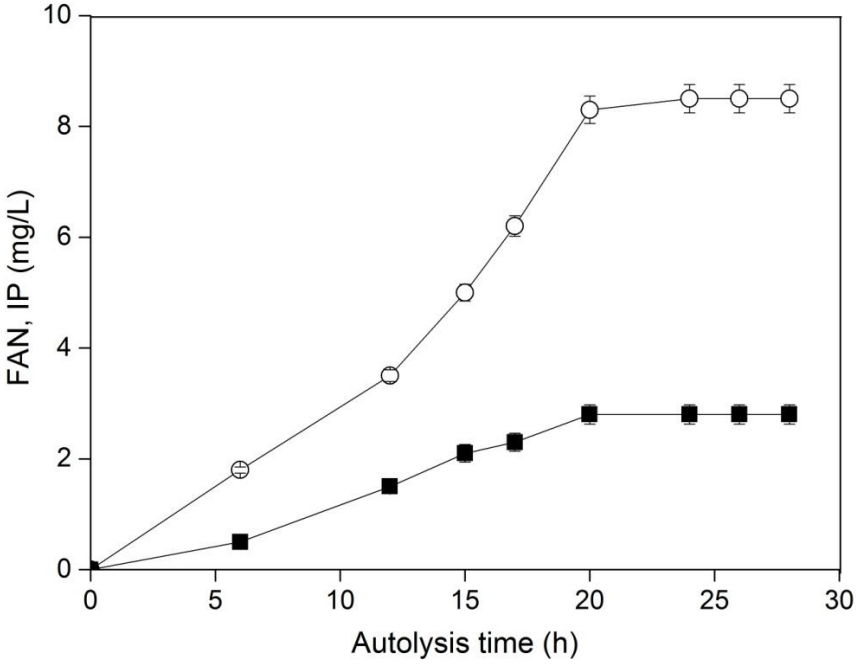


Figure 7.2: Production of FAN (○) and IP (■) during autolysis experiments of the yeast cells contained in Merlot wine lees at pH value of 4 and 50 ºC (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 7.2 presents the production of free amino nitrogen (FAN) and inorganic phosphorus (IP) during autolysis carried out at a pH value of 4 and 50 °C. The FAN and IP production during autolysis was rather low in all tested pH values and temperatures indicating that autolysis of wine lees is not feasible. This means that in the wine lees used in this study the yeast cells were partially or totally inactivated during storage of wine lees.

7.4 Hydrolytic potential of crude enzymes produced via solid state fermentation

In autolysis experiments, yeast cell lysis was not feasible. For this reason, the production of nutrient rich supplement was evaluated using crude enzymes produced via solid state fermentation.

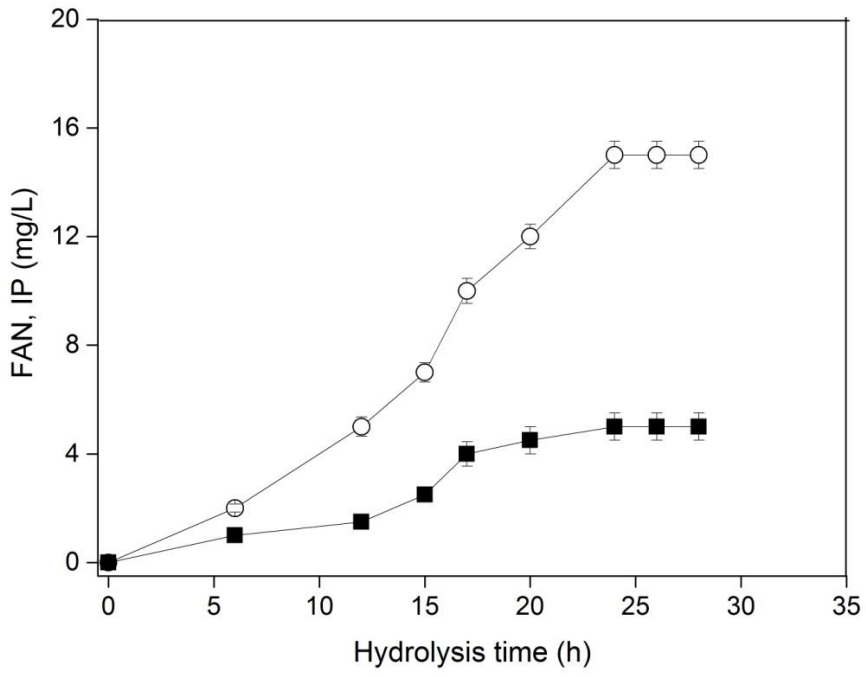


Figure 7.3: Production of FAN (○) and IP (■) throughout Merlot wine lees hydrolysis (at pH value of 4 and 55 °C) using crude enzymes produced via SSF of *A oryzae* that were extracted with sterilized tap water (data presented are the mean values of three replicates and error bars represent their respective standard deviation)

A series of experiments was carried out using crude enzymes (mainly proteases) produced via solid state fermentation of *Aspergillus oryzae* cultivated on wheat bran.

These enzymes could hydrolyse all macromolecules (e.g. proteins) in wine lees into directly assimilable nutrients. The production of crude enzymes (mainly proteases and phosphatases) has been described in section 6.4. The wine lees used in this study was the one produced from Merlot wine production. Besides yeast cells, wine lees contain ethanol, tartrate salts and various antioxidants. The production of nutrient rich supplements was initially investigated without extraction of any wine lees component. In the experiment presented in Figure 7.3, the solid fraction of wine lees (100 g/L, on a dry basis) was mixed with an aqueous suspension of crude enzymes produced via solid state fermentation. The extraction of enzymes was carried out with sterilized tap water. The aim of this experiment was to investigate whether hydrolysis on the untreated solid fraction of wine lees was feasible. Although the production of FAN (15 mg/L) and IP (5 mg/L) was slightly higher than the one observed in autolysis experiments (Figure 7.2), these concentrations are not sufficient to be considered as nutrient supplements for fermentation processes.

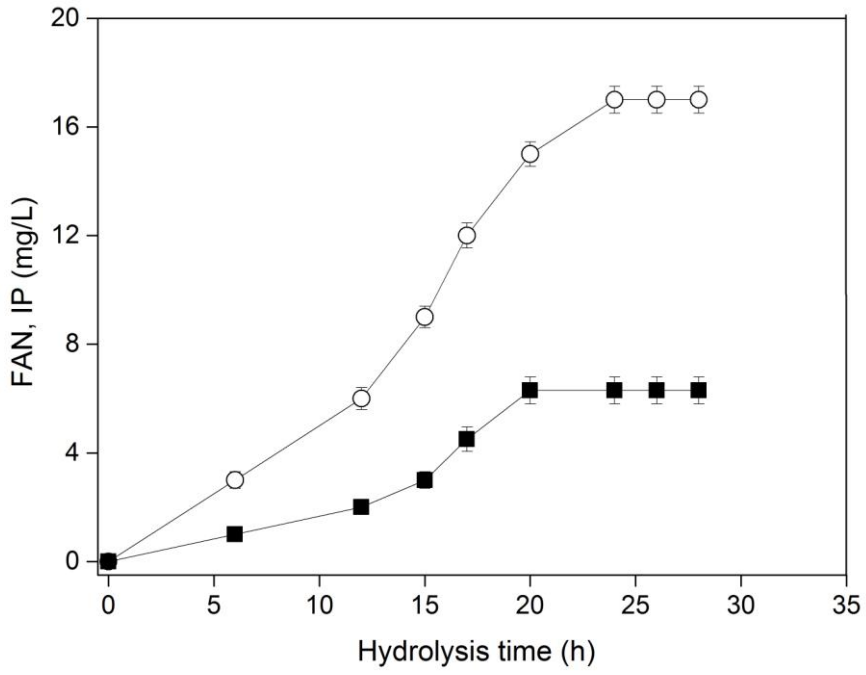


Figure 7.4: Production of FAN (○) and IP (■) during hydrolysis of Merlot wine lees at pH value of 4 and 55 °C carried out by the action of crude enzymes produced via SSF of *A. oryzae*, using the liquid fraction of wine lees (untreated) for suspending crude enzymes (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

In the experiment presented in Figure 7.4, the extraction of enzymes was carried out with the liquid fraction of wine lees derived after the centrifugation of wine lees. This enzyme-rich solution was mixed with the solid fraction of wine lees (100 g/L, on a dry basis). The aim of this experiment was to avoid using tap water for the extraction of enzymes and to explore the utilization of both liquid and solid wine lees fraction in hydrolytic experiments. The FAN (15.6 mg/L) and IP (5.73 mg/L) concentration obtained were almost the same as in experiment presented in Figure 7.3, indicating that ethanol or other wine lees component inhibited the hydrolytic action of crude enzymes. These experiments were carried out in the same pH and temperature conditions as the ones used in the previous experiment.

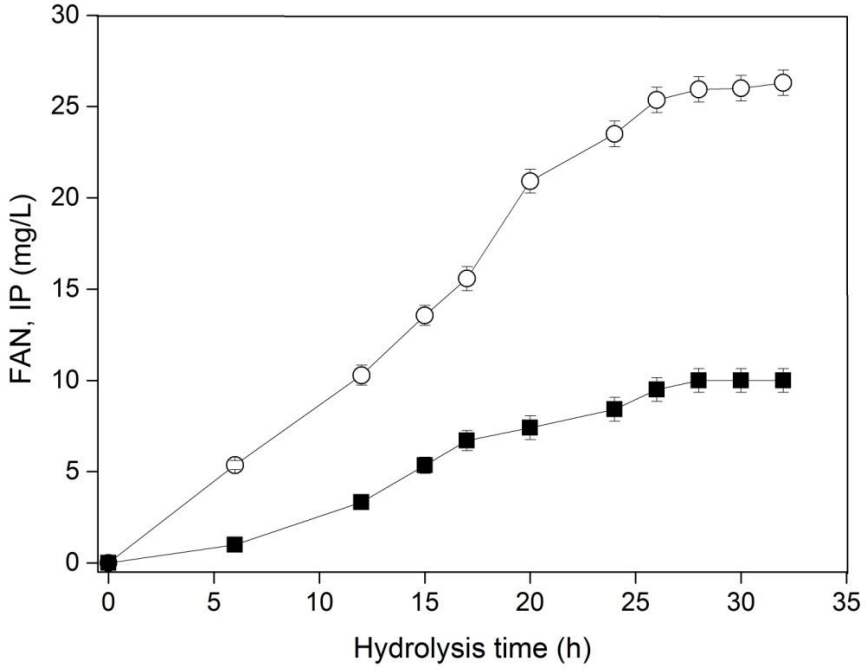


Figure 7.5: Production of FAN (○) and IP (■) throughout hydrolysis of wine lees at pH value of 4 and 55 ºC, using a free of ethanol liquid medium originated from wine lees for the extraction of crude enzymes derived from SSF of *A. oryzae* (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

The previous experiments indicated that one or more of the major components present in wine lees may have inhibited enzymatic hydrolysis. For this reason, in

subsequent experiments the major components were sequentially removed. Ethanol was the first component that was removed from the liquid fraction via distillation. In the experiment presented in Figure 7.5, the solid fraction of wine lees (100 g/L, on a dry basis) was mixed with a free of alcohol enzyme-rich suspension that was produced via extraction of SSF solids with the alcohol-free liquid fraction from wine lees.

The aim of this experiment was to explore whether hydrolysis on the whole fraction of wine lees was feasible, after the extraction of ethanol. The FAN (25.36 mg/L) and IP (9.41 mg/L) concentrations produced in this experiment was slightly higher than the respective concentrations achieved without ethanol removal. This experiment indicates that the high alcohol concentration in wine lees may hinder the efficiency of hydrolysis by inactivating crude enzymes.

7.5 Optimisation of hydrolysis after the removal of ethanol from wine lees

The previous experiment indicated that the removal of ethanol improves the hydrolysis of yeast cells. Therefore, the set of experiments that is presented in Figure 7.6 shows the production of FAN and IP achieved under natural (uncontrolled) pH and five different temperatures (35, 40, 45, 50, 55 °C). In all experiments, Merlot wine lees (100 g/L, on dry basis) were used. A free of alcohol liquid fraction produced via centrifugation of wine lees was used for extracting crude enzymes produced via SSF.As it can be seen in Figure 7.6, the maximum FAN (48.56 mg/L) and IP (18.72 mg/L) production were achieved at 40 °C. Above 45 °C, lower FAN and IP production was achieved via enzymatic hydrolysis, implying that the optimum temperature of crude enzymes is approximately 40 °C. The optimum temperature observed for maximal FAN production was different than the optimum temperature reported in literature-cited publications that evaluated enzyme-rich extracts from the same strain of *A. oryzae* for the hydrolysis of protein from different raw materials (Koutinas *et al.*, 2006; Wang *et al.*, 2005). The optimum temperature for the hydrolysis of gluten was around 50 °C (Koutinas *et al.*, 2006). This difference may be attributed to the complex nature of enzymes produced by *A. oryzae* and the different raw materials used.

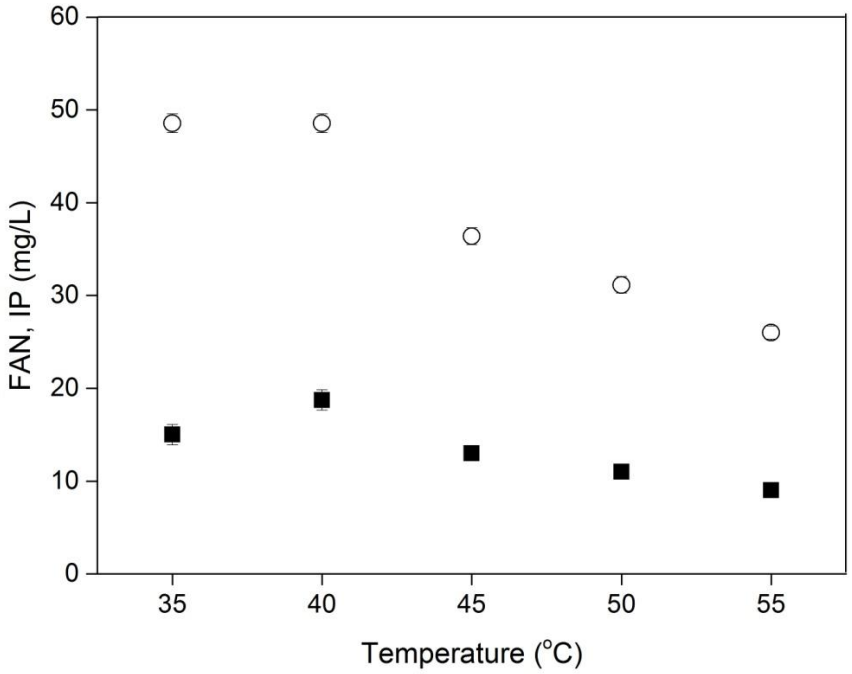


Figure 7.6: Maximum production of FAN (○) and IP (■) in different temperatures varying from 35 to 55 °C during hydrolyses experiments of wine lees (100 g/L, on dry basis), using a free of alcohol wine lees medium for suspending crude enzymes and untreated solid fraction of wine lees (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 7.7 presents FAN and IP production during wine lees hydrolysis at pH value of 4 and 40 °C using a free of alcohol liquid fraction for the extraction of crude enzymes produced via SSF of *A. oryzae*. As it can be seen, FAN production is increasing constantly up to 32 h and then it reaches a plateau. The IP concentration is also gradually increased up to 32 h to its maximum value of 18.7 mg/L. The results obtained after the extraction of ethanol from the liquid fraction indicate than the removal of other major components in wine lees, apart from ethanol, such as tartrate salts and antioxidants, may improve the enzymatic hydrolysis of yeast cells contained in wine lees.

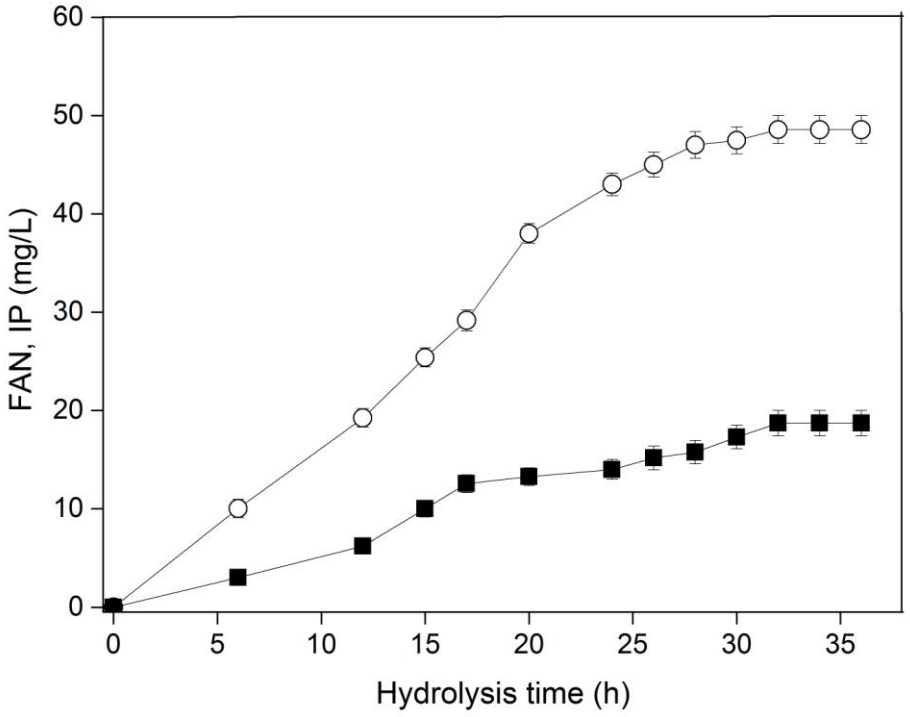


Figure 7.7: FAN (○) and IP (■) production during wine lees hydrolyses using a free of alcohol liquid fraction of wine lees for extracting crude enzymes produced via SSF of *A. oryzae*. This experiment was carried out at a pH value of 4 and 40 °C (experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

7.6 Optimisation of hydrolysis after the removal of tartrate salts

Figure 7.8 presents the FAN and IP production from a set of five hydrolysis experiments carried out after the extraction of ethanol from the liquid fraction of wine lees and tartrate salts from the solid fraction of wine lees (following the method presented in section 6.6.3). The same range of temperatures was used during hydrolysis (35, 40, 45, 50 and 55 °C) with uncontrolled pH. The aim of these experiments was two-fold: 1) to explore the effect of the removal of both ethanol and tartrate salts on the efficiency of hydrolysis, and 2) to investigate which is the optimum temperature for crude enzymes, leading to maximization of FAN and IP production.

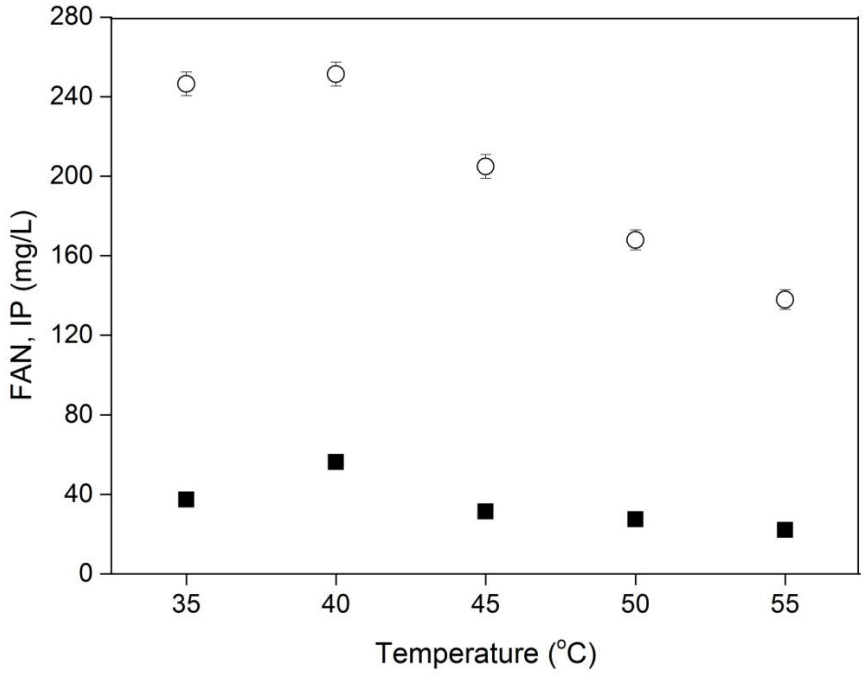


Figure 7.8: Maximum production of FAN (○) and IP (■) after 24 h of hydrolyses of 100 g/L wine lees (on dry basis) at varying temperatures and uncontrolled pH conditions. The tartrate salts and ethanol were removed from the solid and liquid fractions of wine lees, respectively (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

As it can been seen in Figure 7.8, the maximum FAN (250 mg/L) and IP (60 mg/L) concentrations were attained at 40 °C. Lower FAN concentrations were obtained when hydrolysis experiments were carried out at 45, 50 and 55 °C. However, at 35 °C, the maximum FAN concentration was slightly lower than the one achieved at 40 °C.

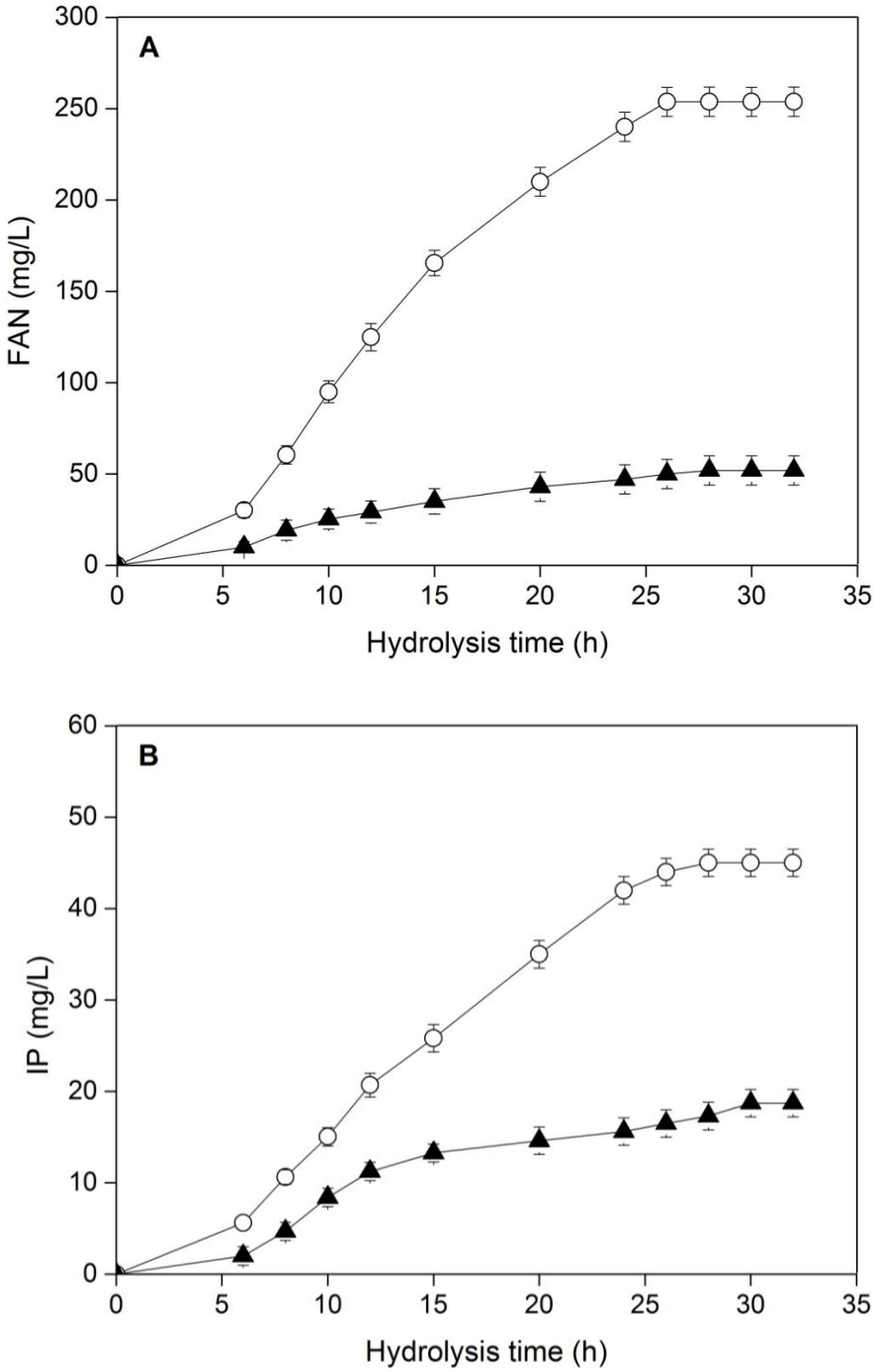


Figure 7.9: FAN (A) and IP (B) production in hydrolyses experiments carried out at 40 ºC using as medium: (▲) wine lees free of ethanol and (○) wine lees free of ethanol and tartrate salts. (The experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 7.9 presents the production of FAN and IP achieved in the hydrolysis experiment carried out at 40 ºC and uncontrolled pH. The results obtained during

hydrolysis of wine lees, where only ethanol was removed have been also presented for comparison purposes. It is obvious that both FAN and IP was higher when both tartrate salts and ethanol are removed from wine lees. The maximum FAN and IP production was 5-fold and 3-fold higher, respectively, when wine lees free of tartrate salts and ethanol were used.

7.7 Extraction of phenolic compounds and their influence on wine lees hydrolysis

In the previous sections, it has been demonstrated that ethanol and tartrate salts hindered hydrolysis of yeast cells (Figure 7.9). So, their extraction deemed necessary. Trying to increase hydrolysis yield and having observed that hydrolysis did not start immediately after the inoculation of crude enzyme consortia, the extraction of phenolic compounds was evaluated. The concentration of phenolic compounds in wine lees as well as other winery by-product streams is significant and, for this reason, it was considered that their removal could improve the production of a nutrient-rich supplement.

Five experiments were performed at different temperatures (35 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC) and uncontrolled pH (the procedure has been described in sections 6.6.3 and 6.6.4) in order to evaluate the potential inhibitory effect of phenolic compounds at different temperatures in the hydrolysis of wine lees. Figure 7.10 presents the maximum FAN and IP production at varying temperatures ranging from 35 to 55 ºC when wine lees were: A) free of ethanol, tartrate salts and phenolic compounds, and B) free of ethanol and tartrate salts.

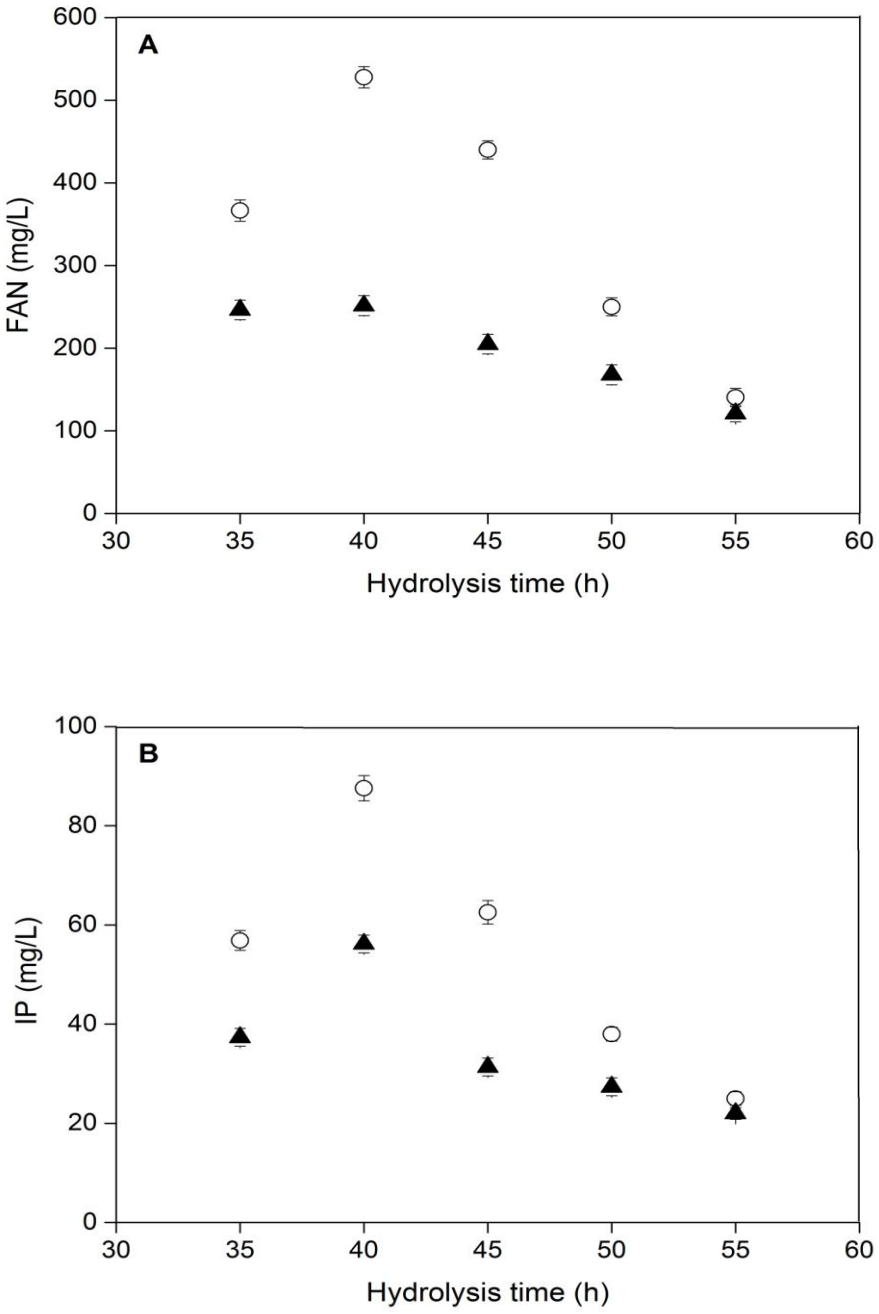


Figure 7.10: Maximum FAN and IP production, in wine lees hydrolysis carried out at different temperatures in two cases: 1) in wine lees free of ethanol and tartrates containing phenolic compounds (▲) and 2) in wine lees free of ethanol, tartrates and phenolics (○) (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

It is obvious that the maximum production of FAN (527.85 mg/L) and IP (87.5 mg/L) was achieved when phenolic cmpounds, besides ethanol and tartrate salts, had been

extracted from wine lees, at 40 °C hydrolysis temperature. More specifically, the maximum FAN concentration achieved (527.85 mg/L) was more than two times higher than the case that only ethanol and tartrate salts were removed (251.35 mg/L), whereas the TKN to FAN conversion yield was increased from 10.13% to 21.28%. Likewise, the IP concentration achieved (87.5 mg/L) was also higher than the IP concentration achieved when only ethanol and tartrate salts were removed (56.16 mg/L). The lowest FAN and IP production observed at 55 °C. In that case, the FAN and IP concentration were almost the same regardless whether the phenolic compounds were removed. It should be stressed out that the sequence of extraction of phenolic compounds and tartrates has been also evaluated. The hydrolysis experiments carried out by removing initially the phenolic compounds and then the tartrate salts had no effect on FAN and IP production. The results were the same as those presented in Fig. 7.10.

7.8 Development of wine lees biorefinery concept

Having evaluated the paramenters affecting the hydrolysis of wine lees, it was possible to develop a biorefinery concept where value-added products could be extracted from wine lees prior to the production of a nutrient-rich feedstock for fermentation processes. Figure 7.11 presents the processing steps that could be followed so as to produce a nitrogen-rich generic feedstock from merlot wine lees. The whole process begins with the fractionation of wine lees into a solid and a liquid fraction. Then, ethanol, antioxidants and tartrate salts were extracted. Subsequently, the residual solids that are enriched in yeast cells (residual solids 2), were lysed using crude enzyme-rich extracts from solid state fermentation of *Aspergillus oryzae* carried out on wheat milling by-products. The extraction of ethanol, tartaric acid and antioxidants, apart from improving hydrolysis efficiency, served also the biorefinery concept developed through the production of value added products, promoting the sustainable exploitation of winery by-products. Tartaric acid, antioxidants and ethanol have many uses and could open new diversified markets, since they could be sold as value added products, increasing the revenues of the process.

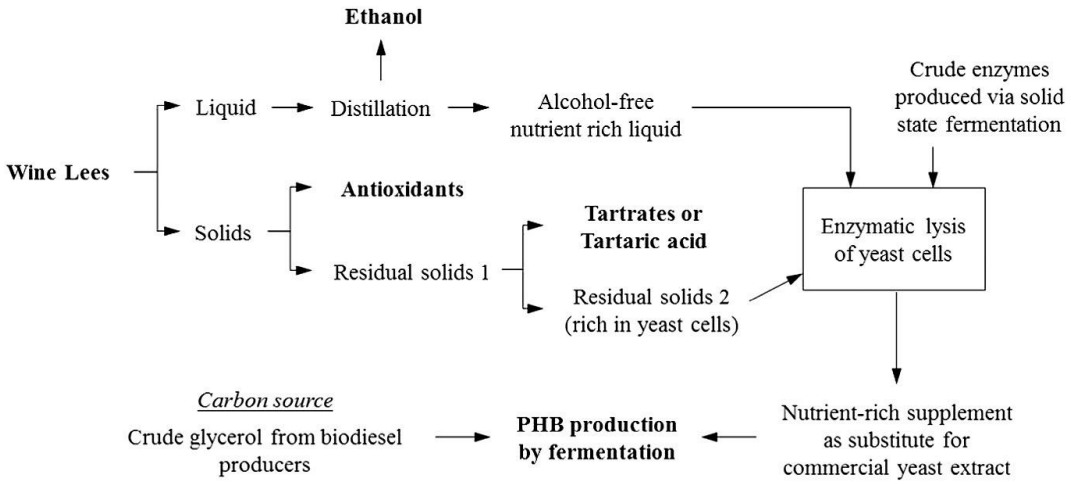


Figure 7.11: Process flow-sheet of wine lees-based biorefinery concept

Organic acids have traditionally been applied to a wide variety of foods and are currently the most commonly used food preservatives (Theron and Lues, 2011). In particular, tartaric acid alone or mixed with other organic acids exhibits significant antibacterial and antifungal properties (Eswaranandam *et al.*, 2004). Taste masking technologies in pharmaceuticals is another industrial field that tartaric acid finds application as a taste improver (Sohi *et al.*, 2004). Still, one of the most common tartaric acid applications is its usage as an acidity regulator (E334) in various foodstuffs (e.g. bakery, candies, jams, juices and the wine industry itself) (Council Directive No. 95/2/EC).

The high amounts of phenolic compounds that remain in winery by-products streams and the positive correlation between the antioxidant activity and the total phenolic compounds present in winery waste streams have already been previously reported (Alonso *et al.*, 2002). The presence of low molecular weight phenolic compounds (released free flavonol aglycones and pyranoanthocyanins), having functional and bioactive properties, was recently reported by Barcia *et al.* (2014).

Ethanol is an important product for either the food sector or as platform molecule with significant importance for sustainable chemical production (Sun and Wang, 2014).

7.9 Optimisation of hydrolysis process

Having achieved a novel biorefinery strategy leading to the production of a nutrient

rich hydrolysate and value-added products from wine lees, further optimization of: a) pH, b) temperature and initial proteolytic activity, c) intial solid concentration and d) time of hydrolysis, deemed necessary. The aim of these sets of experiments was to improve FAN and IP production so as to produce a generic feedstock that could be employed in microbial fermentations, substituting commercially available nutrient souces.

7.9.1 Effect of pH on wine lees hydrolysis

It is well known that the pH value is of great importance in enzymatic reactions, as it can affect their activity by changing the charges on amino acid residue, which is functional in substrate binding or catalysis (Sharma and Tripathi, 2013). The effect of the pH value on wine lees hydrolysis yield was evaluated by conducting experiments at pH values 4.5, 5, 5.5, 6 and 6.5 (Figure 7.12). As it can be seen in Figure 7.12, comparable TKN to FAN conversion yields were achieved at pH values in the range of 4.5 - 5.5, while hydrolysis yield was gradually decreased at pH values from 5.5 to 6.5. Additionally, the pH value of 5.5 led to the optimum wine lees hydrolysis, as an overall TKN to FAN conversion yield of 33% was obtained.

To the best of our knowledge, there is no literature cited results regarding wine lees hydrolysis, although some studies report the usage of commercial or crude enzymes that have been applied in hydrolyses of various products and by-products. Wang *et al.* (2010) carried out enzymatic hydrolysis of rapeseed meal using enzymes produced via SSF of *A oryzae*. In that study, the highest FAN production achieved when pH was uncontrolled.

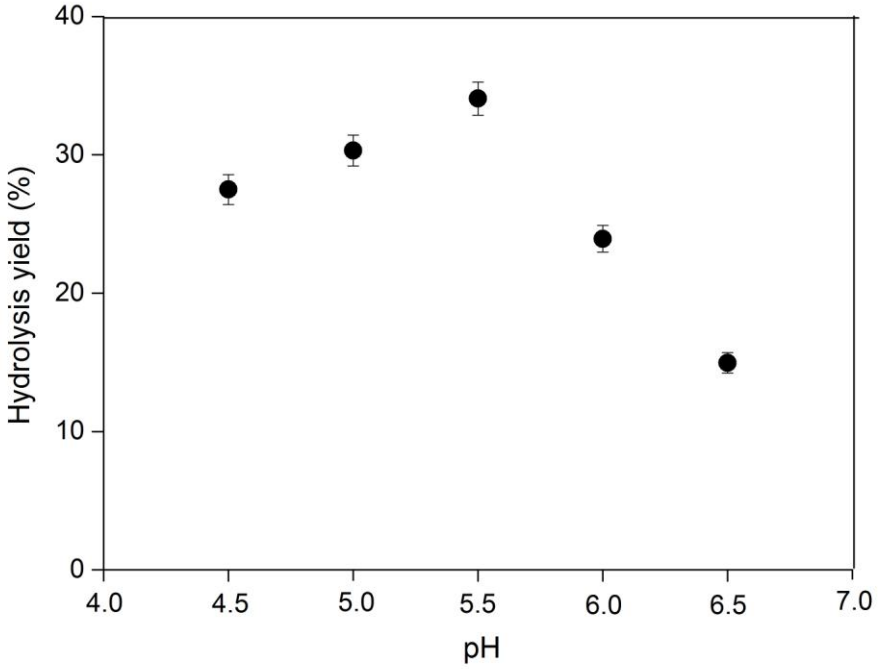


Figure 7.12: Effect of pH value on hydrolysis yield of wine lees (percentage of TKN to FAN conversion yield) after 24 h reaction at 40 °C using an initial solid concentration of 100 g/L and 12 U/mL of initial proteolytic activity (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

The differences observed in hydrolysis yields achieved at different pH values is expressed as the FAN released the protein content of wine lees. The proteases produced by fungal strains are usually a mixture of alkaline, neutral and acidic proteases. In the case of wine lees, the optimum temperature for the action of proteases produced by *A. oryzae* is 5.5 leading to a total Kjeldahl nitrogen to free amino nitrogen conversion yield of 0.32 g/g. Different pH values have been reported in literature-cited publications regarding the hydrolysis of proteins contained in crude renewable resources. Kumura *et al.* (2011) fractionated a crude enzyme complex produced by *A. oryzae* on a medium containing a whey protein isolate and revealed the presence of acid as well as alkaline proteases. Further investigation of the degradation profile of whey protein hydrolysis by these fungal proteases showed that a diversity of proteases emerged depending on the optimum pH value, temperature and cleavage sites of protein.

7.9.2 Effect of temperature and initial enzyme activity on wine lees hydrolysis

The effects of temperature (in the range of 35-55 °C) and initial enzyme activity (12 and 24 U/mL) on wine lees hydrolysis were simultaneously investigated conducting experiments using 100 g/L initial solid concentration and pH value of 5.5. The solids used were fractionated from wine lees after the removal of the liquid fraction and the extraction of antioxidants and tartrate salts. Figure 7.13 presents the results of the aforemented two sets of experiments.

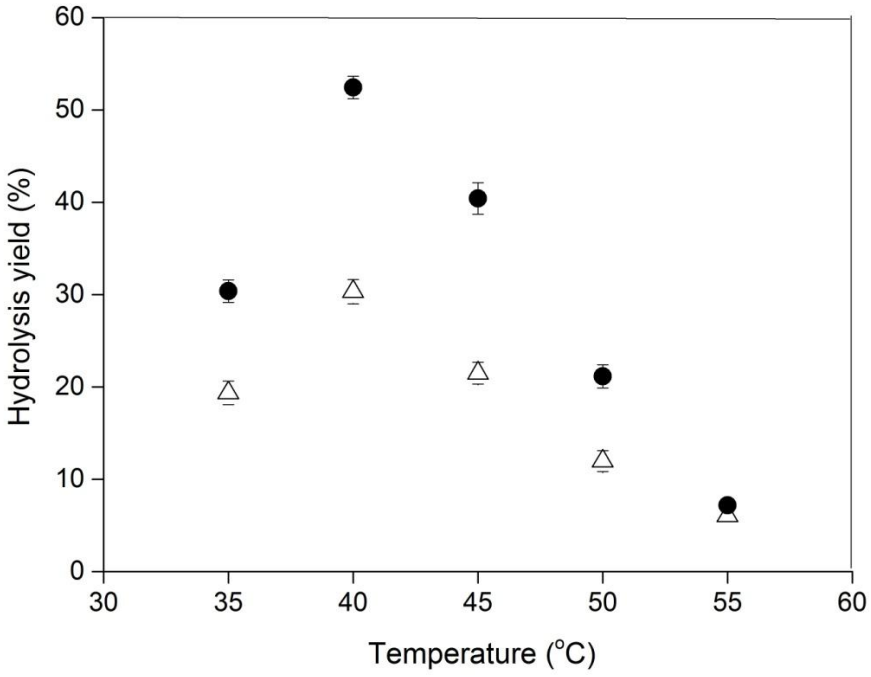


Figure 7.13: Effect of temperature (35-55 °C) and initial proteolytic activity 12 U/mL (△) and 24 U/mL (●) closed cirlces) on hydrolysis yield (percentage of TKN to FAN conversion) of wine lees using an initial solid concentration of 100 g/L after 24 h of reaction (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

As it is depicted in Fig. 7.13, the hydrolysis yield was increased to a maximum (0.524 g/g) at 40 °C and then abruptly declined with further increase of temperature, indicating that temperatures higher than 40 °C increase the deactivation of proteases leading to a significant decrease of FAN production. However, many differences regarding the optimum temperature of proteases are reported in the literature. This occurs because

hydrolysis of crude renewable resources is strongly dependent on the concerted action of various enzymes. Wang *et al.* (2009) reported that the maximum activity of proteolytic enzymes produced by the same strain of *A. oryzae* was observed at 55 °C. However, temperatures higher than 45 °C led to lower protein hydrolysis when crude enzymes from the same fungal strain of *A. oryzae* were used in sunflower meal hydrolysis experiments (Kachrimanidou *et al.*, 2013).

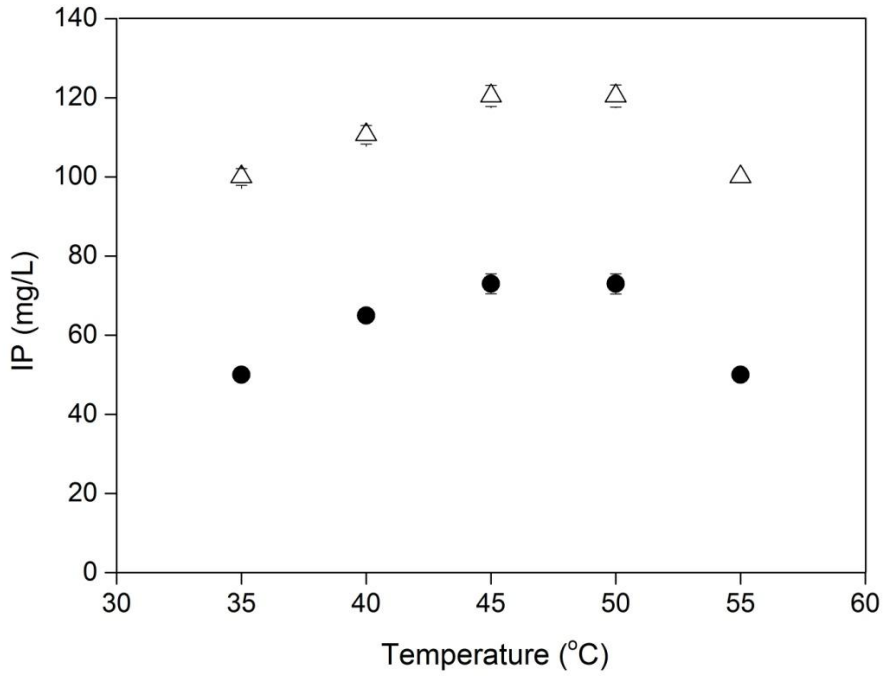


Figure 7.14: Effect of temperature (35-55 °C) and initial proteolytic activity (△) 12 U/mL and (●) 24 U/mL on IP production from wine lees using an initial solid concentration of 100 g/L after 24 h (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 7.14 presents maximum IP production throughout hydrolyses experiments carried out at varying temperature (in the range of 35 - 55 °C) and initial enzyme activity (12 and 24 U/mL) using 100 g/L initial solid wine lees concentration and pH value of 5.5. The aim was to investigate the effect of temperature and initial proteolytic activity on IP production. In Figure 7.14, it can be seen that IP production increased proportionaly when the initial enzyme activity was doubled. Another interesting observation is that the maximum IP production was approximately 120 mg/L achieved in hydrolyses experiments

carried out in temperature range between 40-50 ºC, especially 45 ºC and 50 ºC, when the enzyme activity was 24 U/mL, implying that this was the temperature optima for phosphatases.

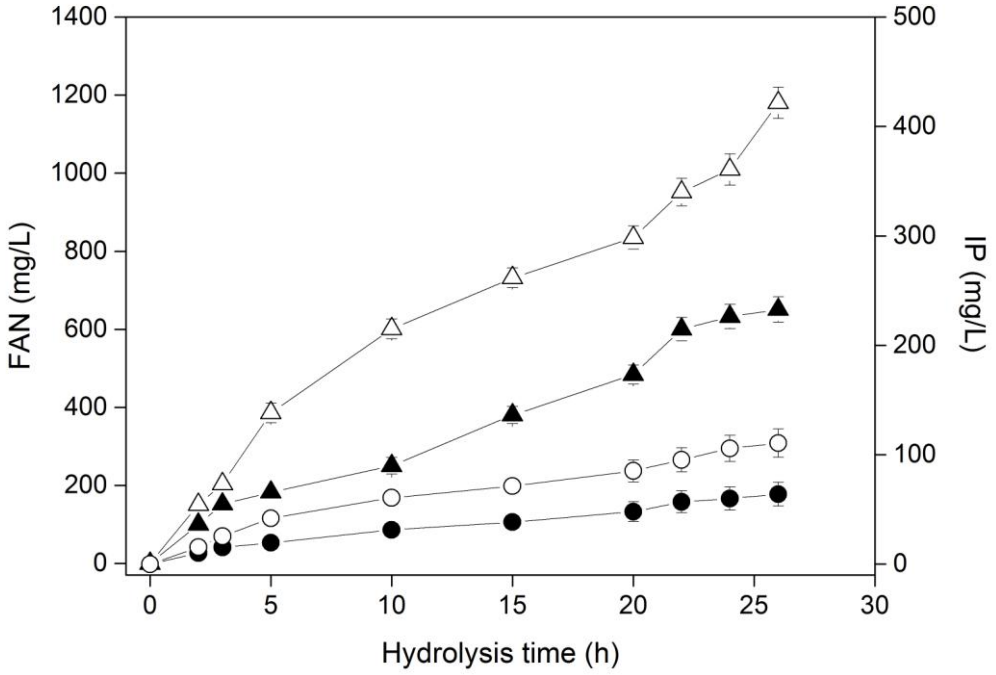


Figure 7.15: FAN and IP production after 24 h at 40 °C and initial proteolytic activities of 12 U/mL and 24 U/mL, using an initial solid concentration of 100 g/L. FAN production using crude enzymes with initial proteolytic activities of 12 U/mL (△) and 24 U/mL (▲); IP production using crude enzymes with initial proteolytic activity of 12 U/mL (○) and 24 U/mL (●) (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 7.15 presents FAN and IP production throughout 24 h wine lees hydrolysis, at 40^{o}C, using proteolytic enzymes with initial activity of 12 and 24 U/mL, for comparison reasons. The chosen temperature is the optimum one for FAN production and close to the optimum range for IP production. From the results presented in Fig 7.15, it can be observed that the maximum FAN and IP production was almost doubled when the initial proteolytic activity of crude enzymes used for wine lees hydrolysis was increased from 12 to 24 U/mL. The almost 1.2 g/L of final FAN concentration produced corresponds to almost 24 g/L of yeast extract concentration.

7.9.3 Effect of initial solid concentration on wine lees hydrolysis

The evaluation of the effect of initial solid concentration on wine lees hydrolysis was performed at a pH value of 5.5 and at 40 °C using an initial proteolytic activity of 12 U/mL. Seven experiments were carried out in order to evaluate the production of FAN and IP during wine lees hydrolyses, using different initial solid concentration (50, 75, 100, 150, 200, 300 and 400 g/L).

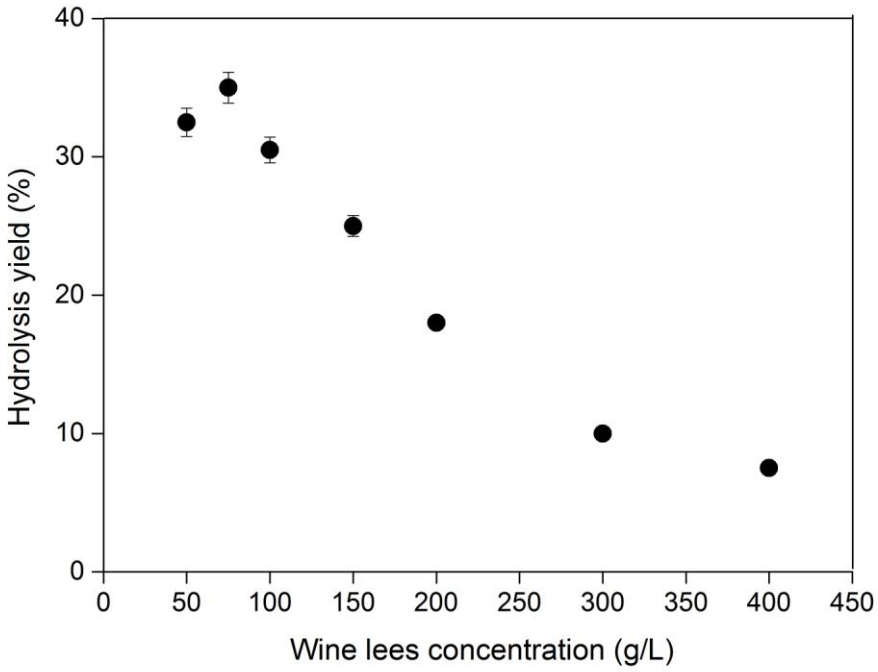


Figure 7.16: Effect of initial wine lees solid concentration (50 - 400 g/L) on hydrolysis yield (percentage of TKN to FAN conversion yield) after 24 h of reaction at 40 °C, pH value of 5.5 and 12 U/mL of initial proteolytic activity (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 7.16 shows that the hydrolysis yield was maintained above 30 % at initial solid concentrations up to 100 g/L, while it decreased significantly using initial solid concentrations higher than 100 g/L (e.g. 8% at 400 g/L initial solid concentration). Higher hydrolysis yield (43.4 %) has been observed in the case of 100 g/L defatted peanut flour hydrolysis by crude protease extract produced by a fungal strain of *A. oryzae* (Su *et al.*, 2011).

The maximum FAN concentration (almost 1400 mg/L as it is observed in Figure 7.17) achieved in this study was equivalent to around 28 g/L liquid medium of yeast extract, which contains around 50 mg FAN per g of yeast extract. Furthermore, the maximum inorganic phosphorus concentration attained in this study from wine lees was less than that produced from sunflower meal, according to a previous publication reporting the production of 230 mg/L IP from 90 g/L initial concentration of sunflower meal (Kachrimanidou *et al.*, 2013). Hydrolysis of rapeseed meal resulted in the production of a higher FAN concentration of 2087.2 mg/L, while the TKN to FAN conversion yield was 54% (Wang *et al.*, 2010). A higher conversion yield has been achieved in the present study (56.45 %). Bearing in mind that oilseed meals contain higher protein concentrations than wine lees, the production of higher FAN concentrations should be expected. The wine lees valorization strategy developed in this chapter proved that wine lees through the implementation of a sustainable process can lead to the production of a nutrient rich hydrolysate that could substitute for commercially available nitrogen sources such as yeast extract, peptone, casein, used to carry out microbial fermentations. According to literature cited publications, yeast cell lysis can be achieved using a mixture of various enzymes, which act synergistically in order to achieve efficient cell wall lysis (Salazar and Asenjo, 2007). Scott and Schekman (1980) reported the necessity for synergistic action of glucanases and proteases. The fungal strain of *A. oryzae* used in this study could produce a variety of enzymes, including proteases, phosphatases, lipases, glucanases, and amylases among others (Koutinas *et al.*, 2007a, 2007b, 2010; Salazar and Asenjo, 2007).

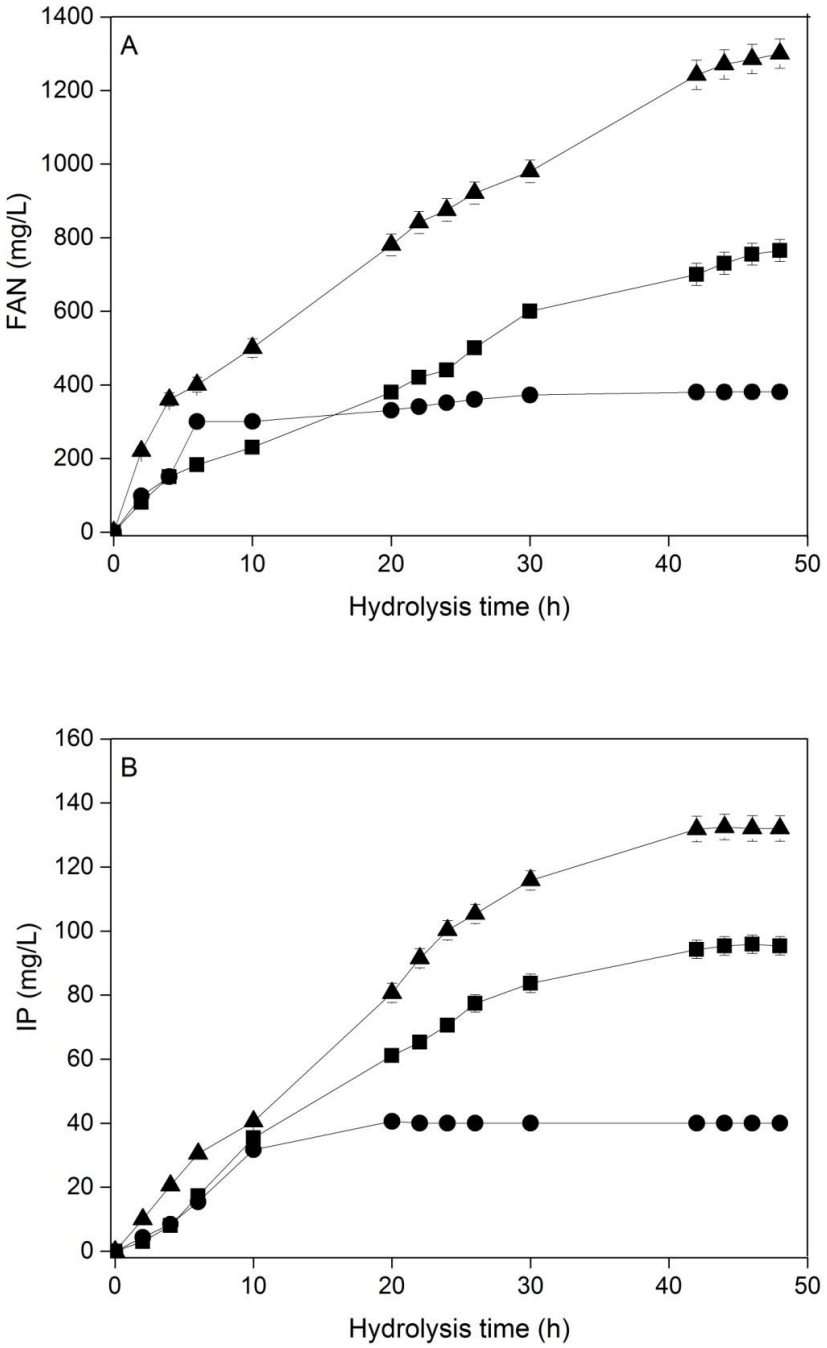


Figure 7.17: FAN (A) and IP (B) production during hydrolysis of 50 g/L and 100 g/L wine lees using 12 U/mL and 24 U/mL of initial proteolytic activity performed at 40 ºC and pH value of 5.5. FAN and IP production during hydrolysis of: (●) 50 g/L wine lees using 12 U/mL proteolytic activity; (■) 100 g/L wine lees using 12 U/mL proteolytic activity; (▲) 100 g/L wine lees using 24 U/mL of proteolytic activity (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

7.10 Material balances of wine lees biorefining

In the proposed wine lees biorefinery (Figure 7.18), the solid fraction separated via centrifugation was 31.4 g per 100 g of the initial wine lees (on dry basis). The solids were further treated with a 70% (v/v) aqueous ethanol solution for the extraction of an antioxidant-rich fraction. Tao *et al.* (2014) reported a total phenolic content of 59 mg of gallic acid equivalents per g of dry red wine lees, derived from a mixed variety of red wines (containing 30% Merlot), using an ultrasound-assisted extraction with an aqueous ethanol solution (42 - 44%, v/v). In this study, around 0.8 g dry weight of phenolic-rich extract was separated from 31.4 g of the solid fraction in wine lees.

It was estimated that the content of tartrate salts was approximately 6.5 g per 100 g of initial wine lees. The residual solid fraction that remained after the extraction of antioxidants (30.6 g dry weight) was acidified with HCl in order to solubilize the tartrate salts that were subsequently separated via treatment with $CaCO_3$ and $CaCl_2$ targeting the precipitation of calcium tartrate. This method was employed for precipitation of calcium tartrates from vinasses and resulted 2.21 g per 100 g of wet vinasses (12.2 g dry weight) as reported by Salgado *et al.* (2010).

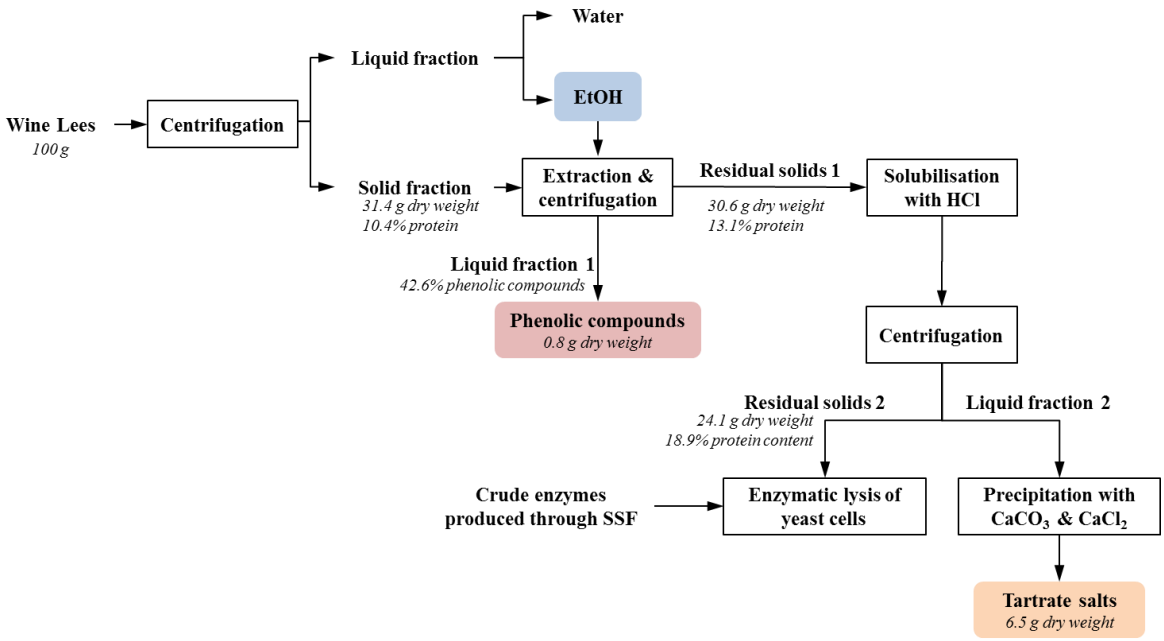


Figure 7.18: Process flow diagram of the wine lees refining concept

The residual solids (24.1 g dry weight derived from 100 g of wine lees) that remained after the extraction of antioxidants and tartrate salts contained 18.9 % protein. Assuming a 40-50 % protein content in yeast cells (Yamada and Sgarbieri, 2005) then around 8.2 g (dry weight) of yeast cells is contained in this solid stream. This solid stream has been used for the production of a nutrient-rich supplement via enzymatic hydrolysis as described in this chapter. A maximum FAN concentration of around 1400 mg/L was achieved corresponding to 28 g/L of yeast extract concentration.

7.11 Conclusions

The novel biorefinery concept developed in this chapter can be applied in wineries for wine lees valorisation leading to the production of value-added products with diversified market outlets. Besides ethanol, tartrate salts and antioxidant-rich fraction, the biorefinery concept leads to the production of a solid fraction enriched in yeast cells that could be used as substitute for commercial nutrient supplements in fermentation processes. In Chapter 9, the crude hydrolysate derived from wine lees has been evaluated as nutrient supplement for the production of PHB.

CHAPTER 8

POLY-(3-HYDROXYBUTYRATE) PRODUCTION USING VARIOUS RENEWABLE RESOURCES

8.1 Introduction

PHB production from pure commercially available carbon and nutrient sources (such as glucose, pure glycerol, yeast extract) may lead to high fermentation efficiency but it also leads to high production cost. The availability of waste and by-product streams from various agri-industrial resources could lead to the development of sustainable processes for the production of PHB. Within this frame, the utilization of 1) flour based by-products were evaluated as carbon source and 2) rapeseed meal and wine lees hydrolysates were evaluated as nitrogen supplements for PHB production. The bacterial strains *Cupriavidus necator* DSMZ 545 and DSMZ 7237 were evaluated for the production of PHB using the aforementioned crude renewable resources.

The flour based by-products used in this study were produced by a confectionery industry. They contained mainly starch as the sole carbon source with a content of 86.1 % (on a dry basis) with lower content of protein (7.3 %, on a dry basis), minerals and vitamins. This by-product stream comprised of either out-of-date products returned from the market or side streams produced at the industrial production line. The flour-rich by-product streams were enzymatically hydrolysed for the production of directly assimilable carbon sources and nutrients. The enzymatic hydrolysis was achieved using crude enzymes (mainly amylases and glucoamylases) produced via solid state fermentation of *A. awamory* that was cultivated in wheat milling by-products. The glucose-rich media produced via enzymatic hydrolysis of flour-rich by-products were evaluated as a nutrient rich carbon source for PHB production.

Rapeseed meal hydrolysates were also used in this study as nutrient supplements for PHB production. The rapeseed meal used in this study had a high protein content (38.8 %, on a dry basis) and it was produced in a biodiesel production plant after extraction of oil from rapeseeds using hexane as solvent. Rapeseed meal hydrolysates were produced via enzymatic hydrolysis using crude enzymes produced via solid state fermentation of *A.*

oryzae cultivated on rapeseed meal. Besides rapeseed hydrolysates, wine lees derived hydrolysates were also evaluated as nutrient supplements for PHB production in Chapter 7.

8.2 Utilisation of flour based by-products for PHB production using *Cupriavidus necator* DSM 545

Table 8.1 presents maximum concentrations of total dry weight (TDW), PHB, recidual cell mass (RCM) as well as PHB yield, productivity and content achieved in batch shake flask cultures using flour-based hydrolysates as the sole nutrient source for PHB production by *C. necator* DSM 545. The shake flasks fermentations were carried out using different initial glucose concentrations (10 g/L, 20 g/L, 25 g/L, 30 g/L). The hydrolysates of flour-rich by-products were produced using the two-stage bioprocess developed by Koutinas *et al.* (2001, 2004, 2005, 2007a,b). The crude enzymes were produced via solid state fermentation of *A. awamori* cultivated on wheat milling by-products. This fungal strain produces mainly glucoamylase and various enzymes, such as proteases and phytases, with lower enzymatic activities (Koutinas *et al.*, 2001, 2004, 2007a,b; Xu *et al.*, 2010; Du *et al.*, 2008). The initial FAN concentrations in the shake flask fermentations presented in Table 8.1 were 140 mg/L at the highest initial glucose concentration (30 g/L) and around 40 mg/L at the lowest intial glucose concentration (10 g/L). The same initial hydrolysate was used for the production of each fermentation medium that was derived via dilution with water.

Table 8.1: Microbial growth and PHB production achieved by *Cupriavidus necator* DSMZ 545 when cultivated on shake flask fermentations on hydrolysates from flour-rich by-products using different initial glucose and FAN concentrations (data presented are the mean values of three replicates)

Glucose (g/L)	FAN (mg/L)	Time (h)	TDW (g/L)	PHB (g/L)	RCM (g/L)	PHB yield (g/g)	PHB productivity (g/L/h)	PHB content (%)
10	43	42	1.4	0.48	0.92	0.048	0.011	34.3
20	80	49	4	1.2	2.8	0.06	0.024	30
25	120	55	4.7	1.4	3.3	0.056	0.025	29.8
30	140	58	3	0.7	2.3	0.023	0.012	23.3

As it can be easily seen in Table 8.1, increasing initial glucose concentration from 10 g/L to 25 g/L led to improved cell growth and PHB production. However, at 30 g/L of initial glucose concentration both TDW and PHB concentrations were decreased. Another interesting point was that increasing initial glucose concentrations from 10 to 25 g/L resulted to prolonged fermentation duration where the maximum TDW and PHB concentrations were observed. The highest total dry weight (4.7 g/L) and PHB concentration (1.4 g/L) were achieved when the initial glucose concentration was 25 g/L (Figure 8.1). PHB content and productivity reached maximum values of 34.3 % (w/w) and 0.025 g/L/h, when the initial glucose concentrations were 10 g/L and 25 g/L, respectively.

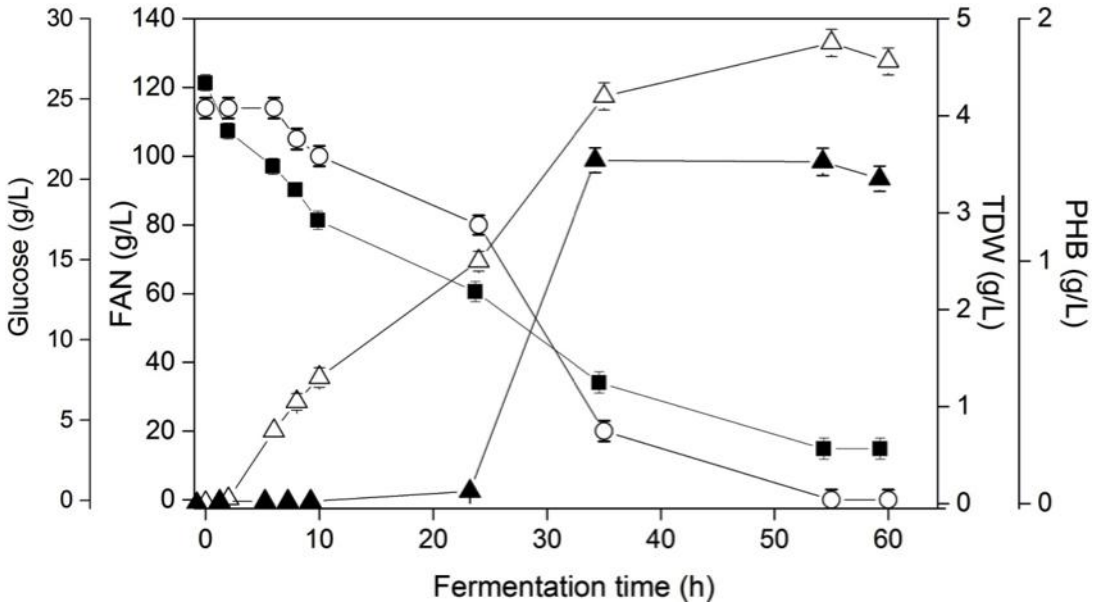


Figure 8.1: Consumption of FAN (○), glucose (■), as well as production of TDW (△), and PHB (▲), during shake flask fermentation using *C necator* DSZM 545 cultivated on flour based hydrolysates with initial glucose concentration of 25 g/L (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 8.1 shows that the production of PHB was triggered when FAN concentration was reduced to less than 80 mg/L demonstrating that another nutrient may have been exhausted prior to nitrogen depletion. Karbasi *et al.* (2012) studied the effect of different carbon sources, including pure glucose, using the same bacterial strain in shake flask cultures. They reported that higher PHB concentration (3.2 g/L), than the one

achieved in this study, was produced at 72 h indicating that different carbon sources, such as fructose, could enhance PHB production. Berezina (2012) reported the production of 3 g/L of TDW and a PHB content of 35% at 62 h in shake flask cultures when the same strain of *C necator* DSM 545 was cultivated in a medium containing commercial glucose and minerals. Berezina (2012) reported that mineral salts were of high importance for PHB production. Various nitrogen sources (e.g. yeast exract, peptone, casein, tryptone, ammonium chloride, ammonium sulphate) have been used in literature-cited publications for the production of PHB (Aramvash *et al.*, 2015). In this study, the nitrogen source was provided via protein hydrolysis that leads to the production of peptides and amino acids. Pure carbon sources generally lead to higher PHB production than crude renewable resources (Du *et al.*, 2004; Kim *et al.*, 1994; Zhang *et al.*, 2004). Bormann *et al.* (1998) stressed the need for further research on the development of microbial bioconversions utilising biomass-derived amino acids and/or oligopeptides since amino acids could lead to the production of PHB (Kumura *et al.*, 2011)

It can be deducted that hydrolysates of flour-based by-products (derived from the confectionary industry) could be used as media for PHB production by the bacterial strain *C. necator* DSMZ 545. However, the PHB production achieved is considered low. Thus, in order to enhance the production of PHB subsequent studies focused on the supplementation of flour-rich hydrolysates with nitrogen-rich hydrolysates (containing peptides and amino acids) from renewable resources (Koutinas *et al.*, 2007b).

8.3 Combination of flour-based and nitrogen-rich hydrolysates for PHB production by *C. necator* DSMZ 545

The renewable resources that were used as nutrient supplements together with hydrolysates from flour-based by-products were side streams from biodiesel production processes and wineries, namely rapeseed meal and wine lees. The production of hydrolysate from wine lees has been described in Chapter 7, whereas hydrolysate production from rapeseed meal has been described in sections 6.3 and 6.4. As previously mentioned, the commercialisation of PHB production is hindered by its production cost. Restructuring of fermentation strategies and substitution of traditional commercial protein hydrolysates (i.e. yeast extract, casein hydrolysates) with crude hydrolysates could lead to

reduction of production costs.

8.3.1 Rapeseed meal derived nutrient sources

Rapeseed meal hydrolysates were evaluated as nitrogen-rich supplements for PHB production, using glucose-rich streams (derived via hydrolysis of flour-based by-products) as carbon source. Rapeseed meal of 5 g/L, 15 g/L, 20 g/L and 30 g/L (on dry basis) initial solid concentration were hydrolyzed at 55 °C using crude enzyme consortia (mainly proteases among other enzymes such as phytases) produced via solid state fermentation of *A. oryzae* on a solid medium containing sunflower meal. Four shake flask fermentations (Table 8.2) were carried out with different initial FAN concentrations of 150 mg/L, 250 mg/L, 340 mg/L, and 450 mg/L and initial glucose concentration of 20 g/L. The TDW, RCM, PHB concentration, PHB content and yield presented in Table 8.2 were determined when glucose was completely consumed or when glucose consumption was prematurely stopped due to depletion of other nutrients (e.g. FAN).

Table 8.2: TDW, PHB and RCM production, as well as PHB content, yield and productivity achieved in shake flask fermentations of *Cupriavidus necator* DSZM 545 using flour-based and rapeseed meal hydrolysates as fermentation feedstock in varying initial FAN concentration (all values represent the mean of three replicates)

Glucose (g/L)	FAN (mg/L)	Time (h)	TDW (g/L)	PHB (g/L)	RCM (g/L)	PHB yield (g/g)	PHB productivity (g/L/h)	PHB content (%)
20	150	40	3.32	0.76	2.56	0.04	0.02	22.9
20	250	46	5.35	1.12	4.23	0.06	0.025	20.9
20	340	42	7.83	1.94	5.89	0.1	0.046	24.8
20	450	64	5.91	1.27	4.64	0.06	0.02	21.5

The highest total dry weight (7.83 g/L), RCM (5.89 g/L), PHB concentration (1.94 g/L), productivity (0.046 g/L/h), PHB content (24.8 %) and yield (around 0.1 g/g) were achieved when the initial FAN concentration was 275 mg/L (Table 8.2 and Figure 8.2). These results demonstrate that rapeseed meal hydrolysates provide nitrogen sources (e.g.

amino acids) for bacterial growth. However, the production of PHB was considered low. Figure 8.2 presents the profile change of glucose, FAN, TDW and PHB during shake flask fermentation using 275 mg/L initial FAN concentration.

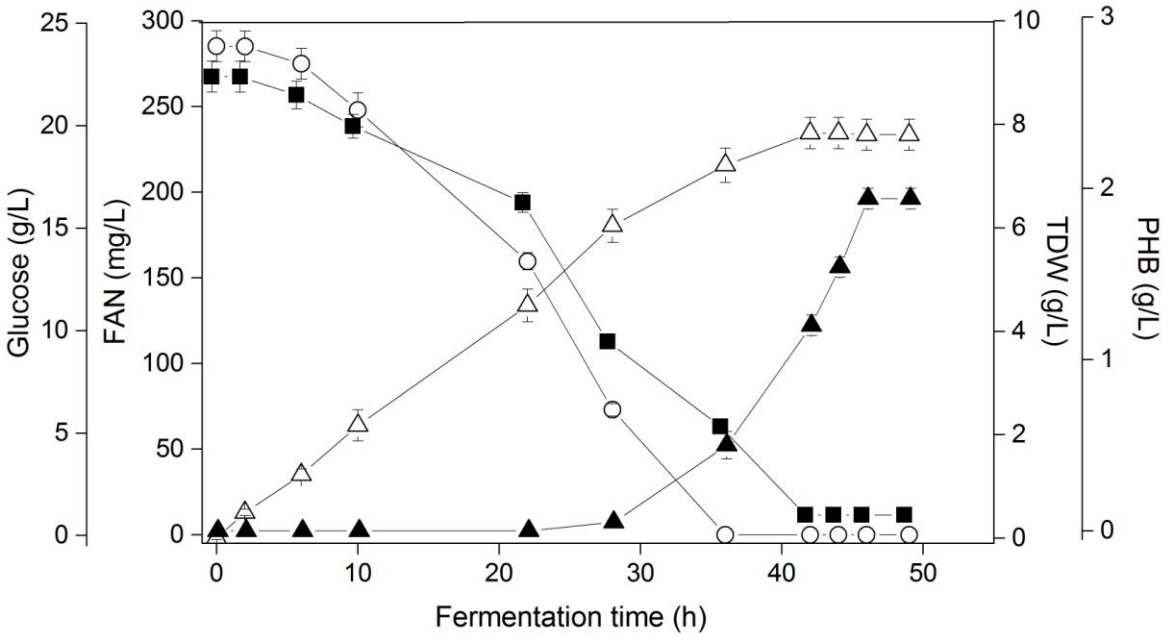


Figure 8.2: Consumption of FAN (○) and glucose (■) along with TDW (△) and PHB (▲) production, during shake flask fermentation using as fermentation feedstock flour-based and rapeseed meal hydrolysates of 275 mg/L and almost 20 g/L initial FAN and glucose concentrations, respectively. *C.necator* DSZM 545 was the strain used (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

These results indicate that the low PHB concentration achieved could be attributed to the low glucose concentration that was available for PHB accumulation after the depletion of FAN. Complete FAN consumption occurred at around 35 h, whereas glucose depletion occurred at around 45 h. This means that further addition of glucose was required in order to extend the production of PHB, which is triggered after 30 h due to depletion of either FAN or minerals (e.g. phosphorus).

Koutinas *et al.* (2007b) and Xu *et al.* (2010) reported that PHB accumulation took place after the exhaustion of FAN when the bacterial strain *C. necator* NCIMB 11599 was cultivated in wheat-based media. Garcia *et al.* (2013) reported higher PHB production (7

g/L), TDW (15.1 g/L) and PHA content (46.3 %), than those attained in this set of experiments, when the bacterial strain *C. necator* DSMZ 545 was cultivated in shake flasks using sunflower meal hydrolysates and crude glycerol as the sole fermentation feedstocks.

8.3.2 Novel wine lees nutrient supplements

Fractionated wine lees of 50 g/L and 25 g/L (on dry basis) initial concentration, derived after the extraction of ethanol, tartrate salts and antioxidants were hydrolysed using crude enzymes (produced by SSF of *Aspergillus oryzae* at 40 °C) for the production of nutrient supplements (Chapter 7). Nine experiments were carried out studying the effect of three initial FAN concentrations (100 g/L, 200 g/L and 300 g/L) and three initial glucose concentrations (10 g/L, 15 g/L, 20 g/L) on microbial growth and PHB accumulation. Table 8.3 presents the highest TDW, PHB concentration and RCM as well as PHB yield, productivity and content achieved using flour-based and wine lees hydrolysates as feedstock for PHB production during shake flasks fermentations of *C. necator* DSM 545.

Table 8.3: Production of TDW, PHB and RCM as well as PHB content, yield and productivity achieved in shake flasks fermentations of *C. necator* DSZM 545 using flour-based and wine lees hydrolysates as fermentation feedstock in varying initial FAN and glucose concentration (all values represent the mean of three replicates)

Glucose (g/L)	FAN (mg/L)	TIME (h)	TDW (g/L)	PHB (g/L)	RCM (g/L)	PHB yield (g/g)	PHB productivity (g/L/h)	PHB content (%)
10	100	30	2.35	0.7	1.65	0.07	0.023	29.78
15	100	36	4.02	1.1	2.92	0.07	0.03	27.36
20	100	46	6.15	2.1	4.05	0.1	0.045	34.14
10	200	52	3.91	0.8	3.11	0.08	0.015	20.46
15	200	73	4.17	1.02	3.15	0.07	0.013	24.46
20	200	80	5.89	1.54	4.35	0.08	0.019	26.14
10	300	57	4	0.7	3.3	0.07	0.012	17.5
15	300	85	4.91	1.14	3.77	0.08	0.013	23.21
20	300	83	5.04	1.26	3.78	0.03	0.015	25

Among the experiments carried out, as it can be seen in Table 8.3, the highest total dry weight (6.15 g/L), biopolymer production (2.1 g/L), PHB productivity (0.045 g/L/h), intracellular PHB yield on glucose (0.1 g/g) and PHB content (34.14 %) were achieved when initial FAN and glucose concentrations were 100 mg/L and 20 g/L, respectively. Based on the results presented in Table 8.3, it can be deducted that wine lees and flour-based by-products could be used as the sole feedstock for PHB production. The initial FAN and glucose concentration affect microbial growth and PHB production. Marudkla *et al.* (2012) reached higher maximum PHB concentrations (9.4 g/L) in shake flasks fermentations containing 10 g/L glucose, with the same strain. To the best of our knowledge, mixtures of hydrolysates from wine lees and flour-based by-products have not been used before for the production of PHB.

8.3.3 Evaluation of glucose to FAN concentration

Trying to enhance PHB production, optimization of initial glucose to FAN ratio was attempted. The initial glucose concentration was set at 20 g/L, while the initial FAN concentration was 40 mg/L, 60 mg/L, 80 mg/L and 100 mg/L (Figure 8.3). It can be easily observed that the higher PHB concentration was achieved at an initial FAN concentration of 80 mg/L. At initial FAN concentrations of 40 to 80 mg/L, the final PHB concentration is gradually increased, but at an initial FAN concentration of 100 mg/L the PHB concentration is decreased. PHA accumulation starts earlier implying that PHB production is triggered by the exchaustion of an essential nutrient (FAN or IP).

Maximum PHB (2.3 g/L) and TDW (7 g/L) production as well as PHB content (32.85 %), productivity (0.05 g/L/h) and PHB yield on glucose (0.11 g/g) were achieved when the initial FAN concentration was 80 mg/L after 42 h of fermentation (Figure 8.4). Observing the results presented in Figure 8.4, it can be deducted that PHB accumulation is triggered not due to FAN depletion but as a sress response to the exchaustion of another essential nutrient. It was assumed that inorganic phosphorus was the essential nutrient that served as the limiting factor of growth. The initial concentration of inorganic phosphorus was 25 mg/L and 7 mg/L in the experiment that initial FAN concentration was 80 mg/L and 40 mg/L, respectively. It should be pointed out that under the conditions tested higher initial inorganic phosphorus concentration could not be achieved.

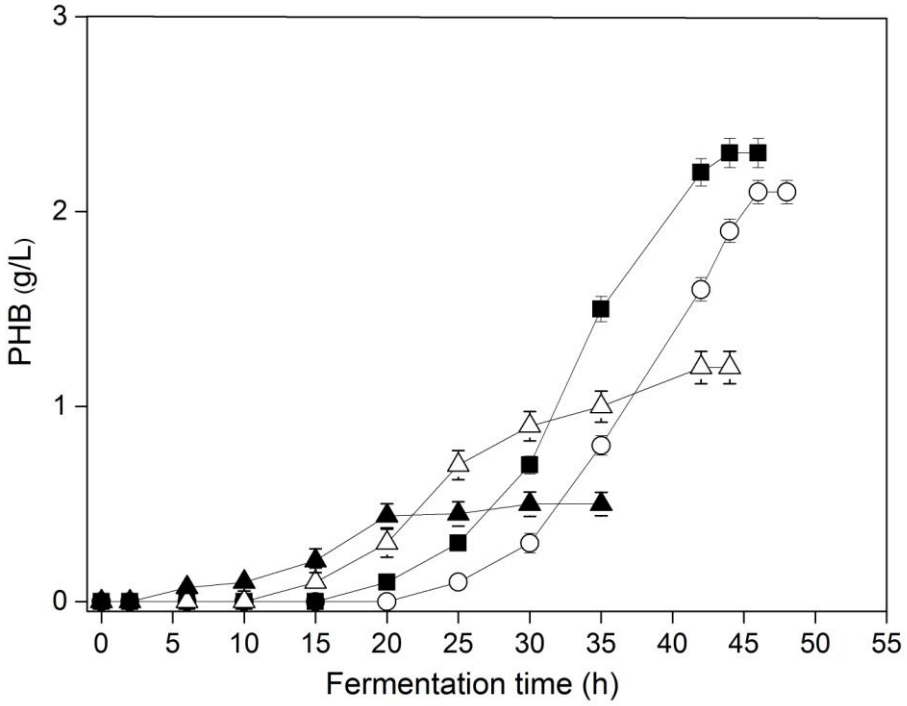


Figure 8.3: PHB production during shake flask fermentations of *C. necator* DSMZ 545, using flour-based and wine lees hydrolysates as fermentation feedstock. The initial FAN concentration was 40 mg/L (▲), 60 mg/L (△), 80 mg/L (■) and 100 mg/L (○), whereas 20 g/L of intial glucose concentration was used in all fermentations (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation).

The bacterial strain *C. necator* belong to the class of bacteria that requires limitation of an essential nutrient, such as nitrogen, oxygen or phosphorus, so as to produce PHB (Lee *et al.*, 1996b; Shi *et al.*, 2007). Depletion of other minerals, besides phosphorus, can also trigger PHB accumulation in the case that there is surplus of carbon, nitrogen and phosphorus sources still present in the fermentation broth (Avnimelech, 2009). Honqi *et al.* (1998) reported that PHB accumulation by *Alcaligenes eutrophus* can be maximised via phosphorus limitation in synthetic glucose based medium.

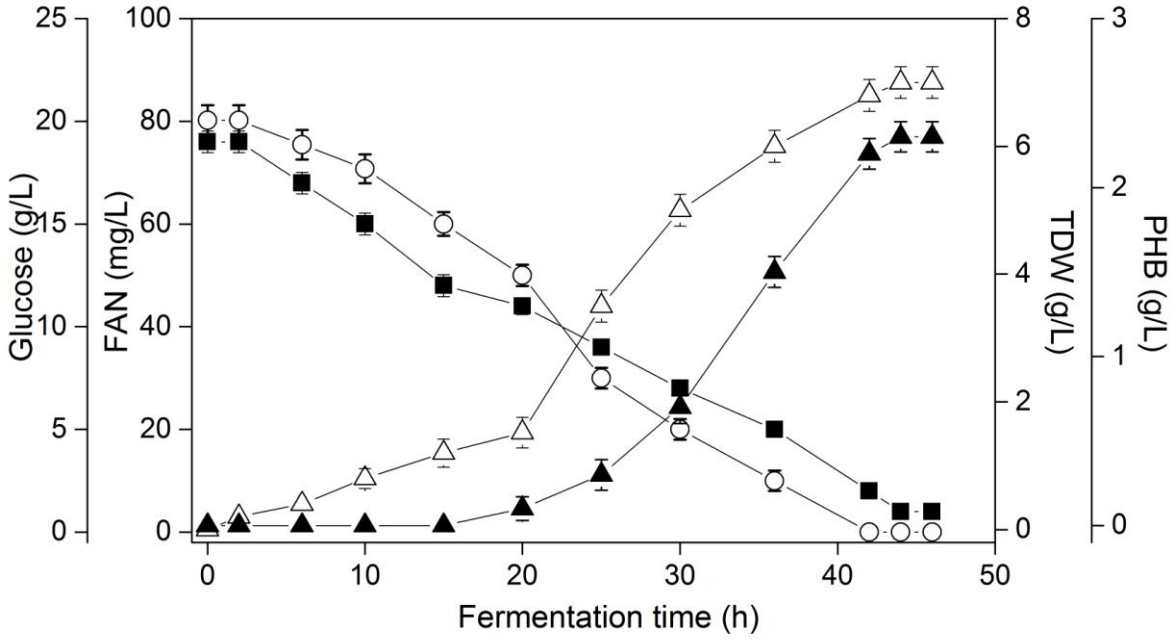


Figure 8.4: Consumption of FAN (○) and glucose (■) along with production of TDW (△) and PHB (▲) during shake flask fermentations of *C. necator* DSMZ 545 using as fermentation media flour based derived media mixed with wine lees hydrolysates of 80 mg/L and 20 g/L initial FAN and glucose concentration (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

8.3.4 PHB production using wine lees hydrolysates, rapeseed meal and flour based by-products by *C.necator* DSM 545: Comparative analysis

Flour based hydrolysates are glucose rich streams that apart from providing the fermentation medium with vital carbon sources for microbial growth and PHB accumulation also enrich the fermentation medium with vitamins, mineral salts and FAN. Nitrogen sources, such as rapessed meal hydrolysates and wine lees hydrolysates, provide to the microorganism nitrogen in assimilable form, FAN, and other nutrients such as trace elements. To the best of our knowledge the aforementioned feedstocks have never been evaluated as co-feedstocks for the production of PHB by *C. necator* DSZM 545. Figure 8.5

presents the maximum PHB production achieved in shake flasks fermentations by *C. necator* DSM 545 with flour-based hydrolysates alone or after supplementation with rapeseed meal hydrolysates or wine lees hydrolysates.

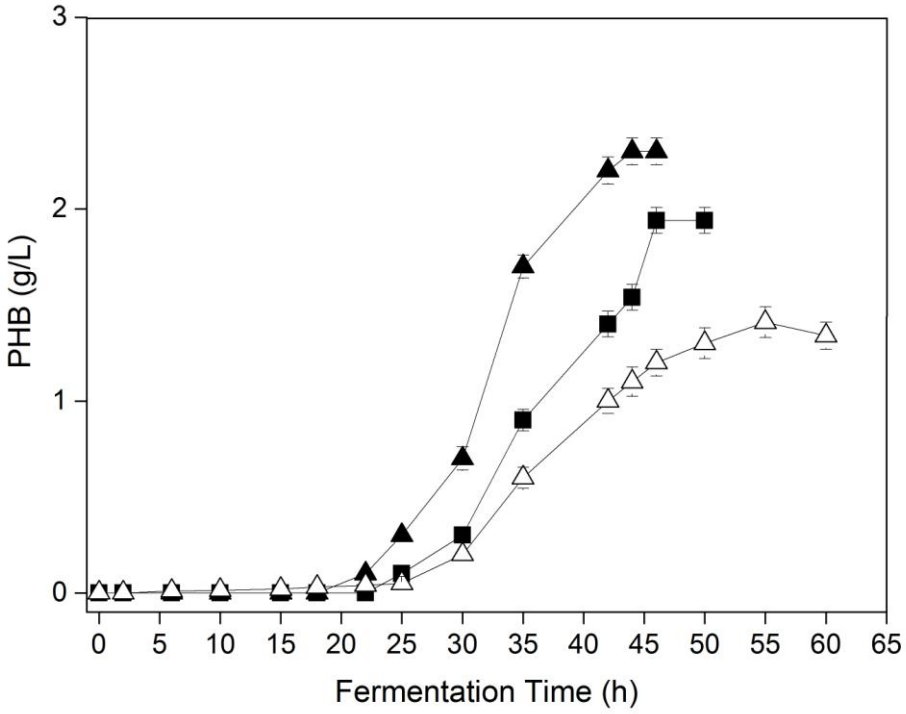


Figure 8.5: Maximum PHB production using (△) flour-based hydrolysates as sole nutrient source (with optimum initial glucose and FAN concentration of 25 g/L and 120 mg/L, respectively), (■) flour based hydrolysates supplemented with rapeseed meal hydrolysates (with optimum initial glucose and FAN concentration of 20 g/L and 275 mg/L, respectively), and (▲) flour based hydrolysates supplemented with wine lees hydrolysates (with optimum initial glucose and FAN concentration of 20 g/L and 80 mg/L, repectively). All fermentations were carried out in batch mode using *Cupriavidus necator* DSM 545 (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Different nutrient supplements lead to different PHB accumulation efficiency. Nutrient supplements are decisive not only for PHB production but also microbial growth. PHB production was enhanced when flour based hydrolysates were mixed with

proteinaceous hydrolysates. Wine lees hydrolysates were better nutrient source than rapeseed meal derived media for PHB production, using *C. necator* DSZM 545.

8.4 Flour-based hydrolysates as fermentation media for PHB production using *C. necator* DSMZ 7237

In preliminary experiments carried out with flour-based hydrolysates as the sole nutrient source at varying initial glucose concentrations (10 g/L, 20 g/L, 25 g/L and 30 g/L), it was confirmed that PHB production by *C. necator* DSMZ 7237 was feasible. However, both TDW and biopolymer production was lower than those presented in Table 8.1 when *C. necator* DSMZ 545 was used. More specifically, maximum PHB, TDW and PHA content of 0.55 g/L, 4.13 g/L and 13.31 % (w/w), respectively, were achieved when the initial glucose concentration was 25 g/L. The low PHB production achieved by *C. necator* DSMZ 7237 when cultivated on flour-rich hydrolysates indicates that nutrient supplementation is necessary.

8.4.1 Rapeseed meal derived media as nitrogen-rich supplement for *C. necator* DSMZ 7237 bioconversions

Trying to enhance PHB accumulation in flour-rich hydrolysates by *C. necator* DSMZ 7237, supplementation with rapeseed meal hydrolysates was evaluated. The effect of varying initial FAN concentration (150 mg/L, 212 mg/L, 396 mg/L, 501 mg/L and 598 mg/L) on PHB production was evaluated (Figure 8.6). The maximum microbial mass (10 g/L) and PHB production (1.87 g/L) were achieved at initial FAN concentration of 501 mg/L. Increasing the initial FAN concentration from 150 to 501 mg/L both TDW and PHB concentration increased, whereas PHB content dropped (from 24.33 % to 16.88 %). Further increment in initial FAN concentration above 500 mg/L resulted in both lower microbial growth and PHA synthesis. The same trend, regarding PHB intracellular synthesis, has been mentioned in previous publications (Koutinas *et al.*, 2007a,b).

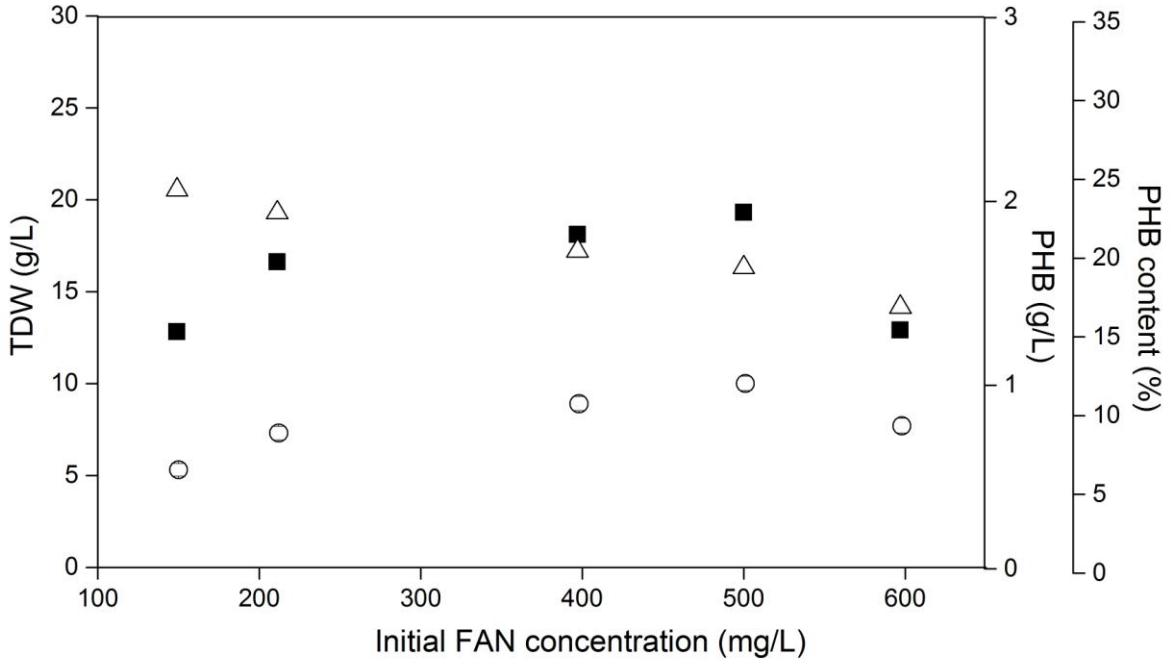


Figure 8.6: Maximum TDW (△) and PHB (■) concentration along with maximum intracellular PHB content (○) achieved during shake flask fermentations of *C. necator* DSZM 7237 carried out in a fermentation feedstock comprised of flour-based and rapeseed meal hydrolysates, at varying initial FAN concentrations (150 mg/L, 212 mg/L, 396 mg/L, 501 mg/L, 598 mg/L). The initial glucose concentration (25 g/L) was constant in all fermentations (the experimental data presented are the mean values of three replicates).

Figure 8.7 presents FAN and glucose consumption as well as total dry weight and PHB production during fermentation carried out by *C. necator* DSMZ 7237 at initial FAN and glucose concentrations of 501 mg/L and 25 g/L, respectively. Figure 8.7 shows that PHB synthesis is triggered after FAN depletion from the fermentation broth. PHB accumulation begins after 27 h when FAN depletion is observed. At this point, microbial growth enters the stationary growth phase.

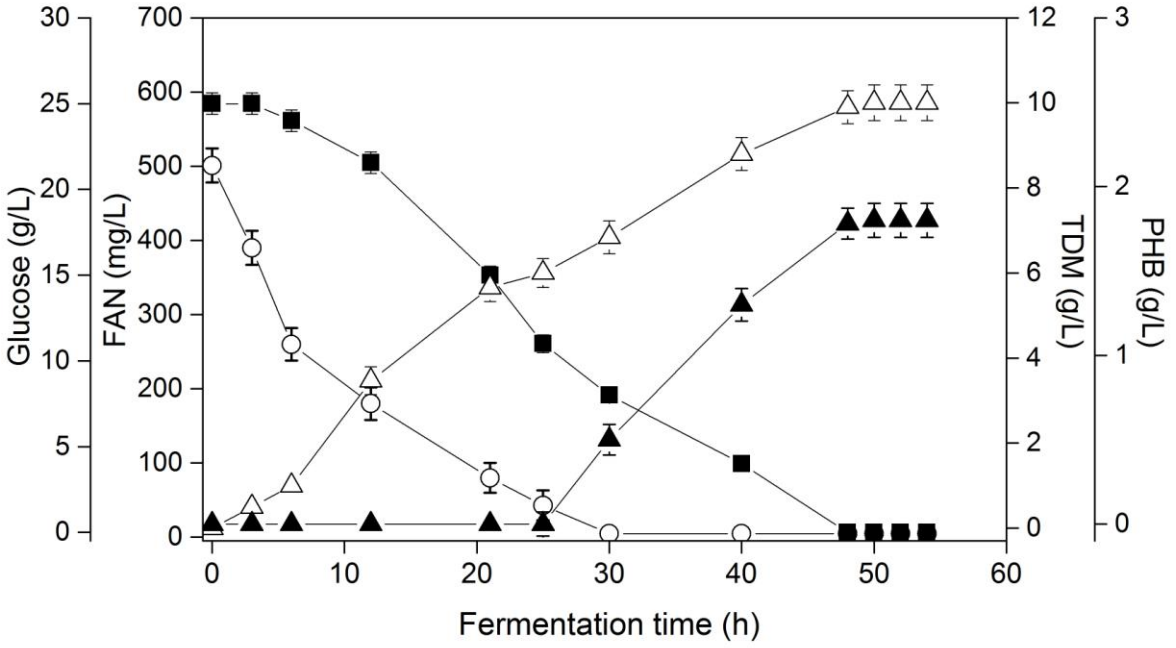


Figure 8.7: Consumption of FAN (○) and glucose (■) along with production of TDW (△) and PHB (▲) during shake flask fermentations carried out in flour based hydrolysates mixed with rapeseed meal hydrolysates with initial FAN and glucose concentration of 501 mg/L and 25 g/L, using *C. necator* DSMZ 7237 (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Comparing TDW and PHB production achieved during shake flasks fermentations using the same carbon (glucose rich feedstock) and nitrogen-rich source (rapeseed meal hydrolysates), the bacterial strain *C.necator* DSM 545 (Figure 8.2) leads to higher TDW and PHB concentrations than *C. necator* DSM 7237 (Figure 8.7). Haas *et al.* (2015) have recently reported that the PHA production and microbial cell growth achieved by three *Cupriavidus necator* strains varied even though all these strains were cultivated on the same substrate.

8.5 PHB production by *Cupriavidus necator* DSZM 7237 using wine lees hydrolysates

The production of PHB by *C. necator* DSZM 7237 was also evaluated when wine

lees hydrolysates were used as nutrient supplements. Shake flask fermentations were carried out by *C. necator* 7237 cultivated on mixtures of flour-based and wine lees hydrolysates of different initial glucose (30 g/L, 23 g/L and 15 g/L) and FAN (433.23 g/L, 295.42 g/L, 156.78 g/L) concentrations. The maximum total dry weight (7.85 g/L) and PHB production (2 g/L) were achieved when the initial glucose and FAN concentrations were 25 g/L and 295.42 mg/L, respectively (Table 8.4). The maximum PHB content was 25.47 % (Table 8.4). Lower concentrations of TDW (3 g/L) and PHB (0.7 g/L) were attained at initial glucose and FAN concentrations of 30 g/L and 433.33 g/L, respectively. These results demonstrate that wine lees hydrolysates can be used as nutrient supplements for *C. necator* DSM 7237 bioconversions. To best of our knowledge, wine lees hydrolysates have not been reported as nutrient supplement for the production of PHB.

Table 8.4: TDW, PHB concentration, PHB content, yield and productivity achieved by *C. necator* DSZM 7237 in shake flask cultures carried out with flour-based hydrolysates alone or supplemented with wine lees derived hydrolysates and rapeseed meal hydrolysates

Glucose (g/L)	**FAN (mg/L)**	**TIME (h)**	**TDW (g/L)**	**PHB (g/L)**	**PHB yield (g/g)**	**PHB productivity (g /L/h)**	**PHB content (%)**
A) Flour based hydrolysates							
25	120	56	4.13	0.55	0.022	0.009	13.31
B) Wine lees hydrolysates							
25	295	50	7.85	2	0.086	0.04	25.47
C) Rapeseed meal hydrolysates							
25	501	54	10	1.87	0.074	0.03	18.7

Table 8.4 shows that the PHB production and bacterial growth achieved with different renewable resources varies depending on the nutrient composition. In the different cultures presented in Table 8.4, the initial glucose concentration was the same (25 g/L), but the initial FAN concentration varied significantly (120 mg/L, 295 mg/L and 501 mg/L for cases A, B and C as presented in table 8.4). By mixing two different crude hydrolysates, it is not easy to maintain the same concentration of all essential nutrients for bacterial growth and PHB production. For this reason, the initial FAN concentration varied, although the same intial glucose concentration was used. However, all

combinations of crude hydrolysates led to bacterial growth and PHB production. Further optimization of each nutrient source is necessary in order to optimise PHB production. The utilization of crude nitrogen-rich supplements could lead to enhanced PHB production (Bormann *et al.*, 1998; Lee *et al.*, 1995). Lee *et al.* (1995) and Lee and Chang (1994) reported that the addition of small amounts of complex nitrogen sources in fermentation media increased significantly the final PHB concentration and yield by recombinant *E. coli* in both shake flask and fed-batch bioreactor cultures. This finding was based on the fact that provision of amino acids increases the availability of acetyl-CoA and/or NADPH in microbial metabolism resulting in more PHB production. Kimura *et al.* (2003) used various amino acids as sole carbon sources for the production of PHB by a wild-type *C. necator* strain. Mahishi *et al.* (2003) also reported increased PHB accumulation by recombinant *E. coli* when amino acids were used as the sole nutrient source.

8.6 Conclusions

In this chapter, the potential of side streams derived from the confectionary industry, biodiesel production plants and wineries have been evaluated as fermentation feedstocks for PHB production. All nutrient supplements, namely flour-based hydrolysates, wine lees hydrolysates and rapeseed meal hydrolysates, could be used for the production of biodegredable plastics. This work can form the basis for restructuring existing industrial processes into novel biorefineries incorporating the production of biodegradable polymers for packaging applications.

Wine lees derived hydrolysates is a novel source of nutrients for the production of PHB. This may be attributed to the fact that wine lees hydrolysates enrich the fermentation medium with amino acids and oligopeptides derived from the hydrolysis of proteins. Amino acids such as aspartic acid, glutamic acid, serine, glycine, a-alanine, tyrosine, and leucine are present in wine lees (Alcaide-Hidalgo *et al.*, 2008). Wine lees derived hydrolysates also contain inorganic phosphorus that can be produced from yeast cells. Khanna and Srivastava (2005) reported that different nitrogen sources lead to varying microbial cell growth and PHA production by *C. necator*. This observation is consistent with the results presented in this study. Since wine lees is a promising source of nutrient, research in the following chapter focused on the utilization of this waste stream for the

production of PHB.

CHAPTER 9

POLY-(3-HYDROXYBUTYRATE) PRODUCTION USING WINE LEES DERIVED HYDROLYSATES AND CRUDE GLYCEROL

9.1 Introduction

In this chapter, wine lees hydrolysates and crude glycerol were evaluated as the sole renewable resources for the production of PHB. The bacterial strains *Cupriavidus necator* DSMZ 7237 was used for the production of PHB employing the aforementioned crude renewable resources.

The crude glycerol was evaluated as carbon source. The crude glycerol used in this study was a highly concentrated by-product stream with a purity of 91% (w/w) derived from transesterification of sunflower oil with methanol in the presence of KOH for biodiesel production. The wine lees hydrolysates were produced as described in Chapter 7. The final solid fraction (Figure 7.11) separated from wine lees is rich in yeast cells and could be easily transported to a biodiesel production plant due to its solid nature. The hydrolysis of wine lees could take place by the biodiesel producer in order to produce the nutrient supplement to be combined with crude glycerol.

The successful utilisation of crude glycerol and wine lees hydrolysates for the production of PHB would set an example of potential synergies between different industrial sectors leading to the production of bio-based chemicals and polymers in the bio-economy era. In this manner, sustainable and novel value-chains could be developed where novel integrated biorefineries could be created through the restructuring of conventional industrial sectors (e.g. wineries, biodiesel producers from oilseeds).

9.2 Batch bioconversions of *C. necator* DSMZ 7237 using crude glycerol and wine lees hydrolysates as fermentation feedstock

Crude glycerol produced by biodiesel plants is considered a by-product stream and its final applications depend on its purity. Despite the fact that pure glycerol is a significant industrial feedstock with applications found in many industrial fields (such as food,

pharmaceutical, cosmetics and tobacco industries), crude glycerol from biodiesel plants has a relative low market value due to the presence of impurities (such as methanol, potassium salts and fatty acids) (see Chapter 2). Crude glycerol could be used as carbon source in fermentation processes. However, the nitrogen content in crude glycerol is negligible and therefore supplementation with appropriate nutrient supplements is necessary. For this reason, in this study, crude glycerol from biodiesel production processes has been supplemented with wine lees hydrolysates in order to formulate a nutrient complete medium for PHB production. This concept was initially evaluated in shake flask fermentations using *C. necator* DSM 7237 cultivated on mixtures of crude glycerol and wine lees hydrolysates of varying initial FAN concentrations (ranging from 100 to 500 mg/L) and the same initial glycerol concentration (25 g/L). Table 9.1 presents the initial FAN concentrations along with total dry weight, intracellular PHB concentration, residual cell mass (RCM) and PHB content attained in different shake flask cultures.

Table 9.1: Production of TDW, PHB and RCM during shake flask fermentations with *C. necator* DSM 7237 using crude glycerol and wine lees hydrolysates as feedstock (all values represent the mean of three replicates)

Initial Glycerol (g /L)	Initial FAN (mg/L)	Time (h)	TDW (g/L)	PHB (g/L)	RCM (g/L)	PHB content (%)
25	100	47	7	4.7	2.3	67.14
25	200	53	7.6	5.1	2.5	67.1
25	300	52	11.1	5.8	5.3	52.25
25	400	48	11.9	5.7	6.2	47.89
25	500	55	10.5	5	5.5	47.61

The results presented in Table 9.1 demonstrate that the maximum PHB concentration (5.8 g/L) was achieved at an initial FAN concentration of 300 mg/L, while it is slightly lower (5.7 g/L) at an initial FAN concentration of 400 mg/L. The highest PHB content (67.1%, w/w) was achieved at initial FAN concentrations of 100 mg/L and 200 mg/L. The shake flask cultures carried out in the highest FAN concentrations (400 mg/L and 500 mg/L) resulted in the lowest PHB content. A similar finding has been reported by

Koutinas *et al.* (2007a,b) during fermentation carried out with a *C. necator* strain cultivated on wheat hydrolysates. Figure 9.1 presents the consumption of glycerol, FAN and IP along with the production of TDW and PHB, when the initial FAN concentration was 400 mg/L and the initial glycerol concentration was 25 g/L.

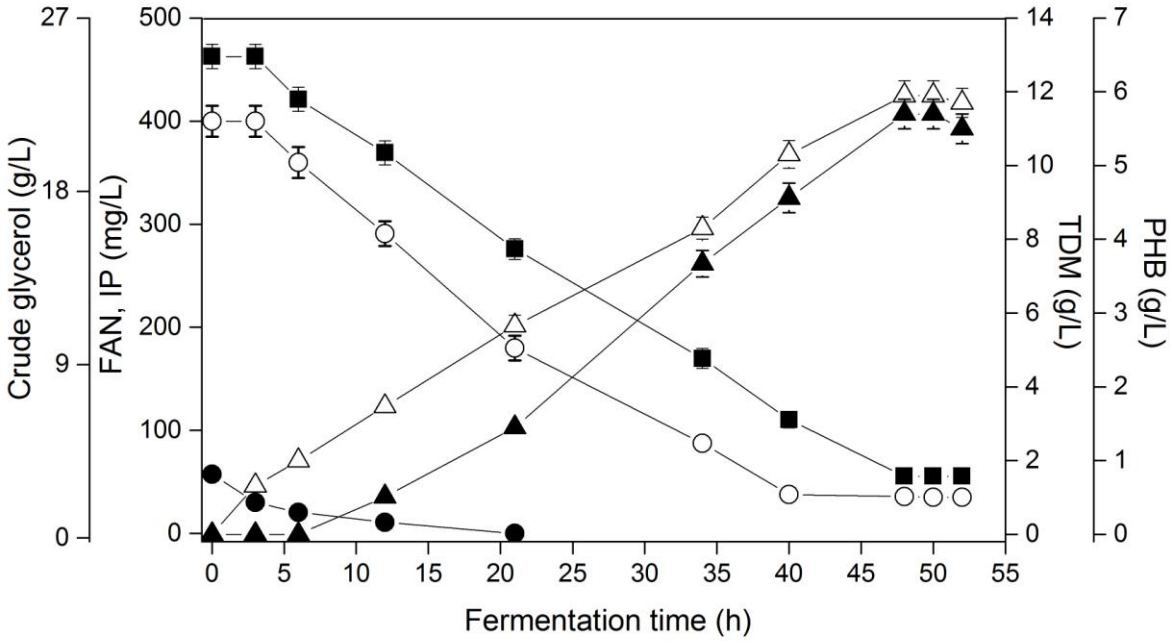


Figure 9.1: Consumption of FAN (○), IP (●) and crude glycerol (■) as well as production of TDW (△) and PHB (▲) during shake flask fermentations with *C. necator* DSMZ 7237 carried out in wine lees hydrolysates and crude glycerol with initial FAN and crude glycerol concentrations of 400 mg/L and 25 g/L (the experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

Figure 9.1 shows that PHB accumulation begins when the IP is almost depleted from the fermentation broth, indicating that IP limitation triggers the intracellular production of PHB. The maximum PHB production takes place until 48 h when the maximum concentration of 5.7 g/L is obtained along with a total dry weight of 11.9 g/L. During the PHB accumulation phase (12-48 h), the residual cell mass remained almost constant (6-6.2 g/L), implying that the microbial growth of *Cupriavidus necator* DSM 7237 has entered the stationary phase. In preliminary fermentations using crude glycerol

(25 g/L) as fermentation feedstock supplemented with mineral salts, it was observed that 0.7 g/L of PHB concentration and 2.4 g/L of TDW was produced, implying that the addition of wine lees hydrolysate was vital in order to enhance bacterial growth and PHB synthesis. The results presented in Table 9.1 and Figure 9.1 demonstrate that wine lees hydrolysates is a suitable nutrient supplement when used togrther with crude glycerol.

The bacterial strain *Cupriavidus necator* is able to produce PHB under non-growth-associated conditions. Most literature-cited studies apply nitrogen limitation (Cavalheiro *et al.*, 2012; Kim *et al.*, 1994; Pradella *et al.*, 2012), while some studies apply phosphate limitation in order to stimulate PHB production (Grousseau *et al.*, 2013; Shang *et al.*, 2007). When wine hydrolysates are used as nutrient supplements, it seems that phosphorus limitation triggers PHB production.

The maximum PHB concentration and content achieved in shake flask cultures are among the highest reported in the literature using crude glycerol as carbon source. Koller *et al.* (2005a) produced the same maximum PHB concentration (5.9 g/L) combining different nitrogen sources (meat and bone hydrolysates) in shake flasks fermentations. Tamiris *et al.* (2014) reported the production of 2.82 g/L PHB after 72 h of fermentation using crude glycerol as carbon souce. Mothes *et al.* (2007) and Calvaheiro *et al.* (2009) produced 50 % PHB content. It should be stressed that there are few publications reporting the production of PHB from mixtures of crude glycerol and crude hydrolysates derived from renewable resources (Koller *et al.*, 2005a, 2005b; Obruca *et al.*, 2011).

9.3 Fed-batch fermentations using crude glycerol and wine lees hydrolysates

Fed batch bioreactor fermentations were carried out aiming to optimize the addition of wine lees hydrolysate at the beginning of fermentation, using crude glycerol as carbon source. Five fed-batch fermentations were carried out at varying initial FAN concentration ranging from 300 mg/L to 1100 mg/L. The initial glycerol concentration was 25 g/L in all fermentations and optimization was focused on the initial FAN content. Wine lees hydrolysates with different FAN concentrations were produced, as described in Chapter 7 (under optimized conditions) via enzymatic hydrolysis of 100 and 50 g/L initial solid concentration (derived from wine lees by the process presented in Figure 7.18). The initial protease activities used during hydrolysis were 12 and 24 U/mL. Table 9.2 presents the

maximum TDW, PHB, RCM, PHB productivity and content achieved, at initial FAN concentrations varying from 300 to 1100 mg/L.

Table 9.2: Effect of different FAN concentrations on *C. necator* DSM 7237 fed-batch bioreactor fermentations using crude glycerol (25 g/L) and wine lees hydrolysates (data presented are the mean values of three replicates)

FAN (mg/L)	Time (h)	TDW (g/L)	RCM (g/L)	PHB (g/L)	PHB content (%)	Productivity (g/L/h)
300	41	5.3	3.4	1.9	35.8	0.05
500	47	10.6	6.5	4.1	38.7	0.09
700	46	25.8	15.1	10.7	41.5	0.23
950	75	16.8	10.8	6.0	35.6	0.08
1100	80	9.2	6.4	2.8	30.3	0.03

The results presented in Table 9.2 indicate that different initial FAN concentrations led to varying maximum PHB concentration, content and productivity. The maximum PHB accumulation and content were 10.7 g/L and 41.5% (w/w) with a productivity of 0.23 g/L/h, when the initial FAN concentration was 700 mg/L. Increasing the initial FAN concentration from 300 to 700 mg/L resulted in gradually increasing final TDW along with PHB production. However, further increase of initial FAN concentration above 700 mg/L led to lower PHB production. The maximum yield coefficient for PHB with respect to crude glycerol consumption was 0.3 g PHB per g glycerol. Thus the optimum FAN concentration in fed-batch fermentations was 700 mg/L. Figure 9.2 presents the consumption of crude glycerol, inorganic phosphorus and FAN as well as the production of total dry weight and PHB when the initial FAN concentration was 700 mg/L.

From Figure 9.2 it could be deducted that the fermentation can be divided into two distinct phases. The first phase, in which microbial proliferation took place, lasted approximately for 15 h, until IP was depleted from the fermentation broth. The second phase started after the completion of IP consumption and was associated with PHB accumulation. These results demonstrate that PHB accumulation occurred due to IP depletion from the medium. Taking into consideration all fermentation results, it could be

stressed that besides the carbon source, which is decisive for both microbial growth and PHB biosynthesis, sufficient and balanced supply of nitrogen and phosphorus at the beginning of each fermentation is of high significance for *Cupriadus necator* DSM 7237 growth and PHB synthesis. It has been reported that an insufficient or even excessive supply of nitrogen and phosphorus can be regarded as the main regulator factor for biomass production and redirection of carbon flux from biomass to PHA accumulation (Chakraborty *et al.*, 2009; Zinn *et al.*, 2001). It should be stressed that no supplementation of minerals was carried out in this fermentation.

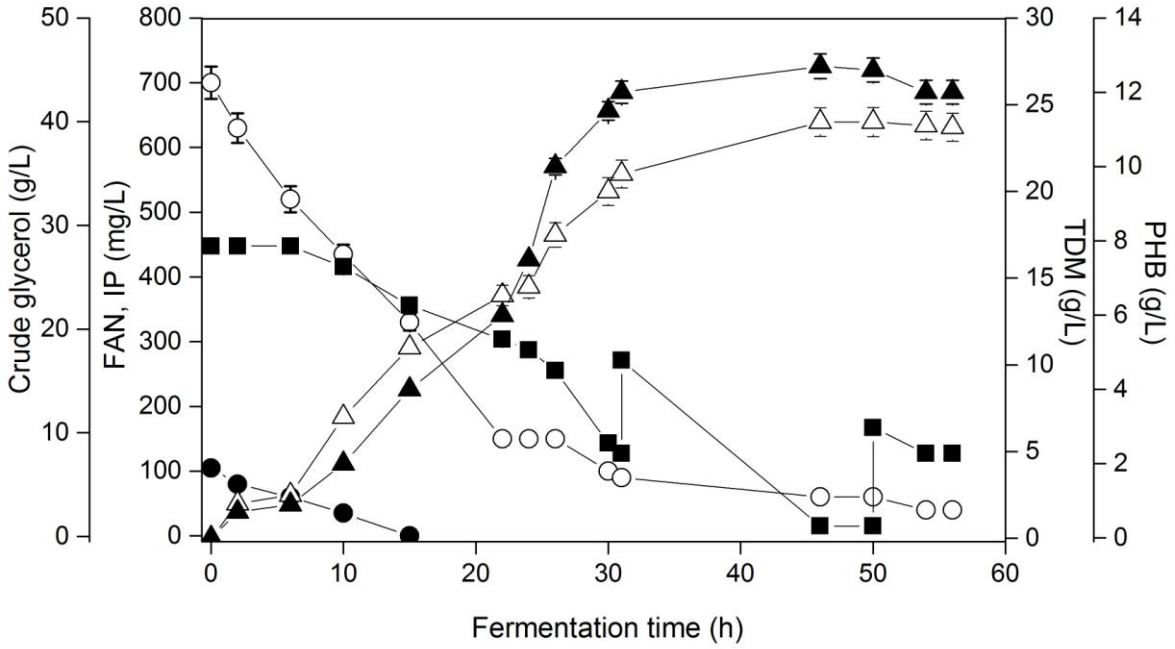


Figure 9.2: Consumption of FAN (○), IP (●) and crude glycerol (■) as well as production of TDW (△) and PHB (▲) during bioreactor fed-batch bioreactor cultures carried out by *C. necator* DSMZ 7237 in wine lees hydrolysates with initial FAN and crude glycerol concentrations of 700 mg/L and 27 g/L, without any mineral supplementation in the medium (The experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

9.4 PHB production achieved by supplementation with mineral salts

The PHB concentration achieved in the set of bioreactor fermentations presented in Table 9.2 was only 10.7 g/L with a total dry weight of 25.8 g/L. Although this fermentation was carried out using entirely wine lees derived hydrolysates and crude glycerol, it was considered that the content of minerals may have not been optimal for PHB production. In order to enhance the production of PHB, subsequent fermentations were supplemented with trace elements at the beginning of each culture. The initial glycerol concentration was 25 g/L in all fermentations. The fermentation conditions used were the same as in the fermentations carried out without supplementation of minerals (Table 9.2). The initial FAN concentrations used varied from 300 to 1100 mg/L.

Table 9.3 presents the total dry weight, the final PHB concentration, the residual cell mass, the PHB content and productivity achieved at the fermentation time that maximum PHB concentration was obtained. These results demonstrate that the addition of trace elements resulted to a significant increase of PHB accumulation. More specifically, addition of trace elements led to a maximum PHB concentration of 30.1 g/L, an intracellular PHB content of 71.3% (w/w), and a PHB productivity of 0.56 g/L/h and a PHB yield on glycerol of 0.36 g PHB per g glycerol consumed.

Table 9.3: Effect of trace elements addition in different fed-batch bioreactor fermentations using wine lees hydrolysates and crude glycerol (~25 g/L) as feedstock for *C. necator* DSM 7237 bioconversions (data presented are the mean values of three replicates)

FAN (mg/L)	Time (h)	TDW (g/L)	RCM (g/L)	PHB (g/L)	PHB content (%)	Productivity (g/L/h)
300	60	21	10.7	10.3	49	0.17
500	62	24.8	11.8	13	52.4	0.21
700	54	42.2	12.1	30.1	71.3	0.56
950	70	26.3	9.8	16.5	62.7	0.24
1100	80	21	13	8	38.05	0.1

Based on the fermentation results presented in Figure 9.3, is obvious that the fermentation can be divided into two distinct phases. The first phase of microbial

proliferation lasts up to approximately 9 h. During the bacterial growth phase, there is no PHB accumulation. The second phase is initiated when the IP is depleted from the fermentation broth. After 9 h, PHB accumulation begins and lasts until approximately 55 h. At this stage, the maximum total dry weight and PHB concentration are achieved. When IP is depleted from the fermentation broth, the FAN concentration is still around 400 mg/L. The FAN is completely consumed from the fermentation medium at approximately 30 h fermentation.

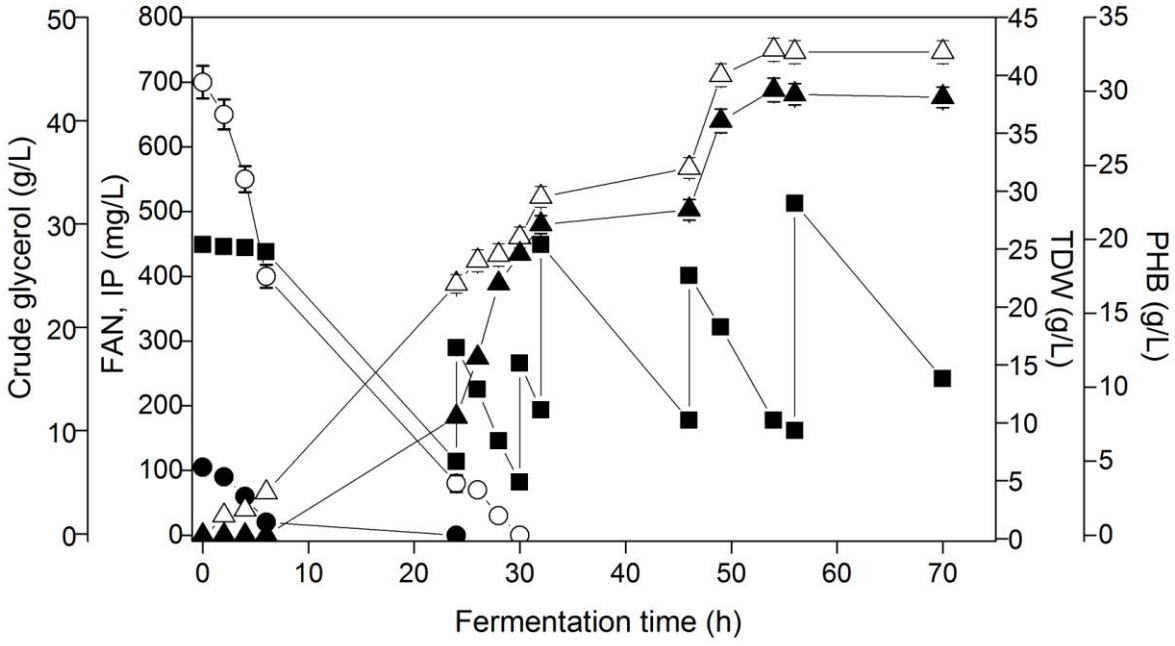


Figure 9.3: Consumption of FAN (○), IP (●) and crude glycerol (■) as well as production of TDW (△) and PHB (▲) during bioreactor fed-batch fermentations carried out with initial FAN and crude glycerol concentrations of 700 mg/L and 27 g/L, respectively. This fermentation was supplemented with minerals. (The experimental data presented are the mean values of three replicates and error bars represent their respective standard deviation)

The intracellular PHB content (71.3%, w/w), productivity (0.56 g/L/h) and yield for conversion of crude glycerol to PHB (0.36 g/g) achieved are among the highest reported in the literature when crude glycerol and crude nutrient supplements have been used as the sole fermentation feedstocks for PHB production. The highest intracellular content of

PHAs (75.4% co-polymer) produced by biodiesel industry by-products was recently reported by Hermann-Krauss *et al.* (2013). The highest productivity using crude glycerol (1.1 g/L/h) as carbon source (but with commercial nutrients as nitrogen source) was reported by Cavalheiro *et al.* (2009).

9.5 Evaluation of initial carbon to FAN ratio

One of the most important factors that decide the level of PHB production during fermentation is the ratio of carbon to FAN at the beginning of the fermentation. Since wine lees constitute a suitable crude nutrient supplement for the production of PHB by *C. necator*, subsequent experimental work focused on the evaluation of the carbon to FAN ratio. In order to calculate this ratio, the carbon contained in glycerol has been taken into consideration. All fermentations were supplemented with mineral salts as described in section 9.4. Three fermentations were carried out in the bioreactor using the same conditions as the fermentations carried out in section 9.4, besides the initial carbon to FAN ratio that varied between experiments (8.6 g/g, 12.3 g/g, and 14 g/g). The initial FAN concentration was 700 mg/L. The initial crude glycerol concentration in the three fermentations was 15.3 g/L, 22 g/L and 25 g/L, respectively, in order to achieve the targeted carbon to FAN ratio. All fermentations were carried out under fed-batch mode and each fermentation was terminated when the PHB production stopped.

Table 9.4 presents the TDW, the maximum PHB concentration, the residual cell mass, PHB content, productivity and yield achieved in the three fermentations carried out with the aforementioned initial carbon to FAN ratios. The results presented in Table 9.4 show that the optimum ratio of carbon to FAN was 8.6 g/g that led to the highest TDW and intracellular PHB content of 47.8 g/L and 77.2 % (w/w), respectively achieved after 65 h fermentation. These results demonstrate that the carbon to FAN ratio is crucial in order to optimise PHB production via fermentation. It should be mentioned that due to the complexity of the medium, only crude glycerol has been taken into consideration in the calculation of the carbon source. The quantity of wine lees hydrolysate added at the beginning of fermentation was the same in all fermentations and therefore the effect of wine lees hydrolysate on bacterial growth and PHB production was the same in all fermentations. The other two carbon to FAN ratios used resulted in lower PHB

concentration at the end of the fermentation. When the lowest carbon to FAN ratio of 8.6 g/g was used, the lowest PHB concentration (9.4 g/L) was achieved. The initial carbon to FAN ratio influenced also the productivity achieved in each fermentation. The highest productivity of 0.56 g/L/h was achieved at 12.3 and 14 g/g of carbon to FAN ratios. A similar RCM was achieved in all fermentations as was expected because the same concentration of nutrients was used at the beginning of fermentation.

Table 9.4: Evaluation of initial C/FAN ratio in different fed-batch bioreactor fermentations using the strain *C. necator* DSM 7237 cultivated on wine lees hydrolysates with initial FAN concentration of 700 mg/L and various initial crude glycerol concentrations (data presented are the mean values of three replicates)

Carbon to FAN ratio (g/g)	Time (h)	TDW (g/L)	RCM (g/L)	PHB (g/L)	PHB content (%)	Productivity (g/L/h)
8.6	35	20.8	11.4	9.4	45.2	0.27
12.3	65	47.8	10.9	36.9	77.2	0.56
14	54	42.2	12.1	30.1	71.3	0.56

It seems that finding the optimum initial carbon to FAN ratio is an important parameter that influences significantly PHB production. It is well known that the metabolic pathway leading to PHB synthesis begins with the condensation of two acetyl-CoA molecules for the production of acetoacetyl-CoA. Utilization of acetyl-CoA for growth via the TCA cycle occurs only when there is no nutrient limitation. If there is not sufficient microbial growth, even when there is a limitation of an essential nutrient, PHB might be produced but in lower quantities (Wei *et al.*, 2011).

The novel biorefinery developed in Chapter 7 could lead to the production of several value added products such as antioxidants, tartrate salts and ethanol. The remaining solid fraction could be hydrolysed into a generic fermentation feedstock. This complex nutrient source containing amino acids, peptides and phosphorus among other nutrients was evaluated together with crude glycerol as carbon source for PHB production. Table 9.5 presents the productivity, glycerol conversion yield and content achieved in literature-cited

publications using crude glycerol for the production of PHB via fermentation. Table 9.5 shows that the intracellular PHB content (77.2%, w/w), the productivity (0.56 g/L/h) and the PHB concentration achieved in this study are among the highest achieved in the literature when crude glycerol and renewable resources are used as the sole fermentation feedstocks.

Table 9.5: Fermentation efficiency reported in literature-cited publications and in this study using crude glycerol as carbon source

Strain	PHB (g/L)	PHB content (%, w/w)	PHB productivity (g/L/h)	Reference
Ralstonia eutropha DSM 11348	-	47	0.2	Bormann *et al.*, 1998
R. eutropha DSM 11348	-	65	0.37	Bormann *et al.*, 1998
C. necator DSM 545	38.1	50	1.1	Cavalheiro *et al.*, 2009
Haloferax mediterranei DSM 1411	-	75.4	0.12	Hermann-Krauss *et al.*, 2013
C. necator DSM 7237	36.9	77.2	0.56	This study

9.6 Conclusions and future goals

Thinking that fermentation media formulation accounts for 50% of the production cost of PHAs, the use of cheap feedstocks is a field of high interest. Hence, biorefining of food supply chain waste could lead to the development of sustainable processes with diversified market potentials. Wine lees, derivered from wine making of a well known grape variety, Merlot, was evaluated in this chapter as nutrient supplement for the production of PHB.

CHAPTER 10

TECHNO-ECONOMIC EVALUATION OF A NOVEL WINE LEES REFINERY FOR THE PRODUCTION OF VALUE ADDED PRODUCTS

10.1 Introduction

Wine lees constitute approximately 2-6% of the initial quantity of grapes used in winemaking and therefore 0.42-1.26 t of wine lees are generated annually in Europe (Bai *et al.*, 2008). Wine lees are currently used for commercial production of calcium tartrate and ethanol (Braga *et al.*, 2002). The EC 479/2008 revokes the EC regulation 1493/99 that necessitated the utilization of wine lees in distilleries. Holistic valorization of wine lees as well as all by-product streams produced by wineries should be achieved in order to develop novel products and sustainable processes in line with the requirements of bio-economy development.

Various reports have focused on the extraction of value added products, including antioxidant rich-extracts, seed oil, fibres, tartaric acid, squalene and ethanol from winery by-products (Versari *et al.*, 2001; Naziri *et al.*, 2012, Perez-Seradilla *et al.*, 2011; Bordiga *et al.*, 2015). Wine lees, as well as the carbohydrate fractions from grape stalks and grape marc, have been employed as fermentation media for the production of platform chemicals such as ethanol, lactic acid and xylitol (Bustos *et al.*, 2004; Perez- Bibbins *et al.*, 2004). Therefore, winery by-product streams could be ideally employed for the separation of several value-added products via the development of biorefinery concepts. Some reports have focused on the development of novel integrated biorefineries focusing on the exploitation of the full potential of winery by-product streams (Martinez *et al.*, 2016). Chapter 7 demonstrated the production of a nutrient-rich fermentation supplement from the yeast cells that are contained in wine lees after the separation of antioxidants, ethanol and tartrate. The nutrient-rich hydrolysate was used for the production of poly(3-hydroxybutyrate) in Chapter 9 using the bacterial strain *Cupriavidus necator* and crude glycerol as carbon source. Martinez *et al.* (2016) focused on the development of a cascade refining process of red grape pomace leading to the sequential production of polyphenols by supercritical CO_2 extraction, poly(hydroxyalkanoates) from volatile fatty acids as

carbon source for the cultivation of *Cupriavidus necator* and biogas via anaerobic digestion of the remaining solid stream.

The successful implementation of biorefining concepts strongly depends on sustainability issues and screening of process alternatives should be carried out based on cost-competitiveness, environmental benefits and socio-economic aspects. Process design and techno-economic evaluation of biorefining processes focusing on the production of numerous end-products from winery wastes following cascade principles should be carried out in order to assess the feasibility of process scale-up. The main aim of this chapter was:

1) To carry out process design and preliminary techno-economic evaluation of the wine lees refining process presented in Chapter 7 leading to the production of four end-products, namely ethanol, an antioxidant-rich extract, calcium tartrate and a solid fraction rich in yeast cells. The later can be used either as animal feed or for the production of fermentation nutrient supplements similar to yeast extract (Chapter 7).

2) To evaluate the effect of the unitary market price of the antioxidants-rich fraction on the overall profitability of the whole process of wine lees refining for the production of various value-added products.

10.2 Description of the process design strategy

Process design and preliminary techno-economic evaluation of the proposed biorefinery concept was carried out for processing a capacity of 500 kg/h of wine lees, which is the approximate quantity of wine lees produced by a large-scale winery in Greece (Vlyssides *et al.*, 2005). The wine lees refining process was assumed to operate on a seasonal basis at 120 days (approximately 4 months) per year due to the seasonal nature of the wine making process. Thus, the biorefinery uses 1440 t/y of wine lees. The life time of the plant was set at 30 years and the interest rate at 10%. The material and energy balances were validated using the commercial process simulator UniSim (Honeywell).

10.3 Composition and fractionation of wine lees

The fractionation process evaluated in this study has been developed in lab-scale

using wine lees derived from the wine making process of Merlot grape variety and were provided by the winery Ampelou Techni-Theodoros Stavrakis (Tyrnavos, Greece). The wine lees, employed as initial feedstock in Unisim database library contained 69.4% (w/w) water, 5.7% (w/w) ethanol and 31.4% (w/w) solids on a dry basis (db), according to the producer company.

The results used in the techno-economic evaluation have been obtained in lab-scale experiments using the following experimental protocols for the recovery of each component. The schematic diagram presented in Figure 7.18 illustrates the refinery concept and the material balances. Centrifugation is initially applied to the original wine lees for the production of liquid and solid fractions. Ethanol is recovered from the liquid fraction via distillation. The ethanol stream is subsequently used for the extraction of a phenolic-rich extract from the solid fraction at an ethanol to water ratio of 70:30 (v/v). The phenolic-rich extract constitutes 0.8% (w/w, db) of the initial wine lees. The use of ethanol than other solvent system was employed because it is available in the original wine lees and, therefore, the cost of the whole process can be reduced. It should be stressed that the extraction protocol of the polyphenol-rich extract has not been optimized and further work is needed in order to maximize the extraction of phenolics from wine lees. The solids that remain after the extraction of phenolics are processed for the recovery of calcium tartrate using the methodology proposed by Salgado *et al.* (2010). The recovered calcium tartrate constitutes 6.5% (w/w) of the initial wine lees. The remaining solids after the separation of the phenolic-rich extract and the calcium tartrate accounts for the 24.1% of the initial wine lees. These solids contain around 35-45% of yeast cells produced during the wine making process.

10.4 Process description and simulation

The process flow diagram (PFD) for the valorization of wine lees is shown in Figure 10.1. The process was divided into three subsections, namely the recovery of ethanol, the extraction of the phenolic-rich extract and the extraction of calcium tartrate.

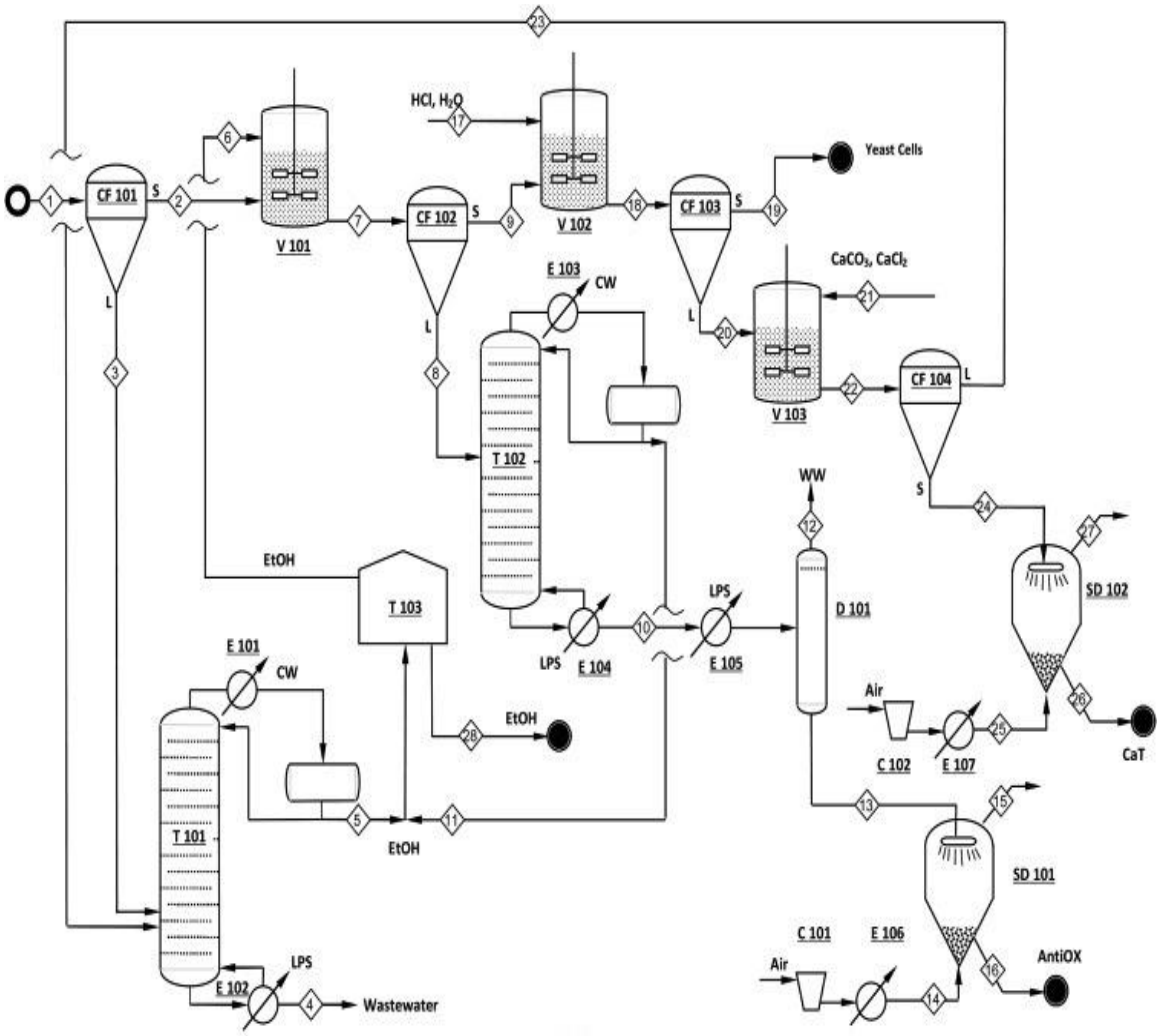


Figure 10.1: Process Flow Diagram for the biorefinery process based on wine lees fractionation for the production of ethanol, antioxidant-rich extract, calcium tartrate and solids enriched in yeast cells

10.4.1 Ethanol separation via distillation

The stream of wine lees (stream 1) is fed into a solid/liquid separation unit (CF-101) where the solids are separated from the liquid. The resulting streams (solids: stream 2 and liquid: stream 3) are determined by material balances considering that the solid fraction has a content of 50% solids and 50% moisture. The liquid stream is fed to the distillation column T-101 where ethanol and water at azeotropic composition, 95% (w/w) in ethanol, is obtained as a top product stream (stream 5). The product stream of the bottom (stream 4) contains mainly water and is fed to the wastewater treatment facility.

10.4.2 Extraction of antioxidants

Stream 2 contains all solids present in the wine lees and it is mixed with the ethanol:water mixture at azeotropic composition from tank T-103, so as to achieve an ethanol to water ratio of 70:30. The combined stream is then fed to the holding and mixing tank V-101. The aim here is to use ethanol as solvent so as to extract the antioxidants present in the solids. A solid/liquid separation unit (CF-102) is then used to recover most of the liquid containing the extracted antioxidants (stream 8) that is fed to a second distillation column (T-102) that operates under vacuum and produces an ethanol-water mixture at azeotropic composition (stream 11) and a bottoms product (stream 10) that contains the phenolic-rich extract. A single stage evaporator (D101) is then used to remove most of the water and the concentrated antioxidant-rich stream (stream 13) is fed to the spray dryer (SD-101) where hot air is used to remove the remaining water and produce practically a moisture-free phenolic-rich fraction (stream 16).

10.4.3 Extraction of calcium tartrate

Stream 9 contains all solid components after the removal of antioxidants and is mixed with a hydrochloric acid aqueous solution (stream 17) so as the total solids are approximately 15% (w/w). The initial solid content of stream 9 was 50% (w/w). Streams 9 and 17 are fed to the holding and mixing tank V-102. In the presence of HCl, the tartrate salt (calcium tartarate), that is practically insoluble in water, is transformed to the water soluble tartaric acid according to reaction (R1).

$$CaC_4H_4O_6 + 2HCl \rightarrow C_4H_6O_6 + CaCl_2 \qquad (R1)$$

Tartaric acid is recovered in stream 20 using the solid/liquid separation unit CF-103. Stream 20 is mixed with $CaCO_3$ and $CaCl_2$ to transform the water soluble tartaric acid to practically water insoluble calcium tartrate according to reaction (R2). The process used for the extraction of calcium tartrate was based on the methodology employed by Rivas *et al.* (2006).

$$C_4H_6O_6 + CaCO_3 \rightarrow CaC_4H_4O_6 + CO_2 + H_2O \qquad (R2)$$

The tartrate salt is separated from the liquid in the solid/liquid separation unit CF-104 and the solid stream (stream 24), containing 50% solids, is fed to a spray dryer (SD-102) where moisture free solids are obtained (stream 26) using compressed hot air. The liquid stream (stream 23) that contains mostly water and ethanol is recycled back to the distillation unit (T-101) for recovering the ethanol. The solid stream (stream 19) from the solid/liquid separation unit CF-103 contains the yeast cells which are used either as animal feed or for the production of a nutrient-rich fermentation supplement as presented in Chapter 7.

10.5 Process design in Unisim

Figure 10.2 presents the PFD of wine lees refining (Figure 10.1) developed in the commercially available software UniSim Design (Honeywell). Tartaric acid and calcium tartrate were introduced to the database of UniSim. Tartaric acid ($C_4H_6O_6$, MW:150.10) has a density in aqueous solutions of 1.506 kg/m^3. The heat capacity is also calculated from the same reference (Sijpkes *et al.*, 1989). Experimental and calculated values are compared in Figure 10.3 as an indication of the accuracy of the process simulation. The heat of formation of tartaric acid given is 1,295.7 kJ/mol (Kochergina *et al.*, 2006) and corresponds to the hypothetical state of undissociated tartaric acid at finite dilution. The value used for the heat of formation is validated with the calculated in UniSim Design heat of combustion (calculated: -1,135 kJ/mol) which compares well with the experimental value (−1,120.3 ± 0.9 kJ/mol) also given in the same reference. Data for calcium carbonate and calcium chloride are taken from Dean (1999). The heat of formation of calcium carbonate and calcium chloride are 1,220 kJ/mol and 877.1 kJ/mol, respectively. The NRTL/PR thermodynamic model is used to simulate the process under study.

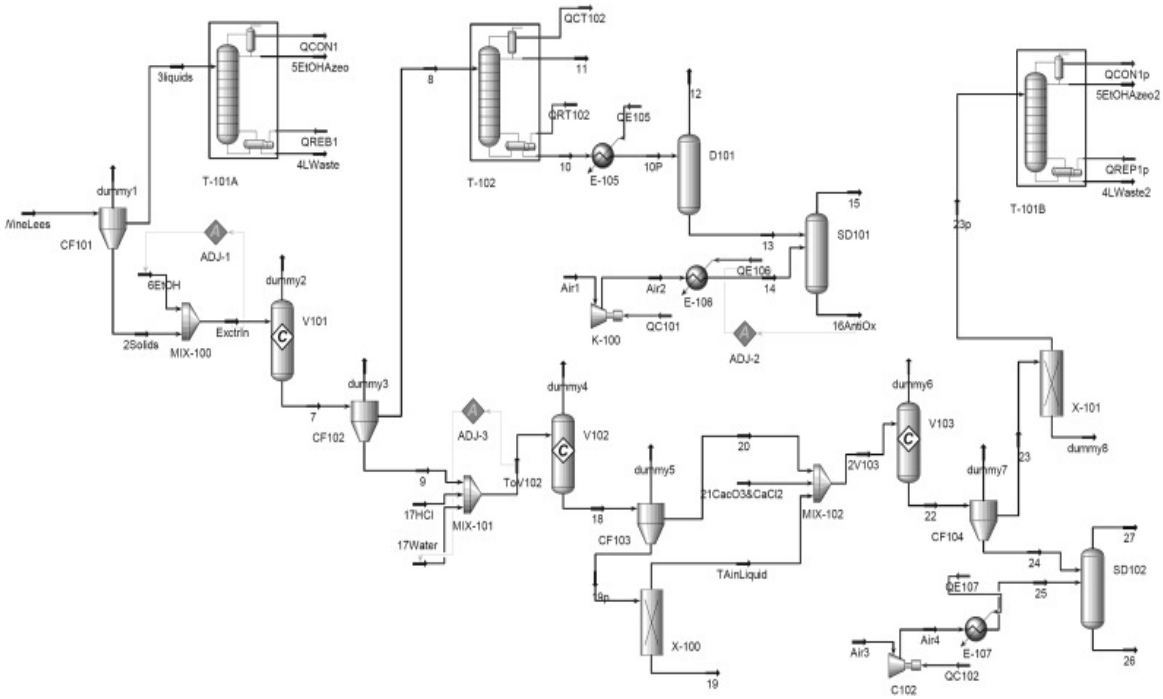


Figure 10.2: Simulation in UniSim Design of the process shown in Figure 10.1

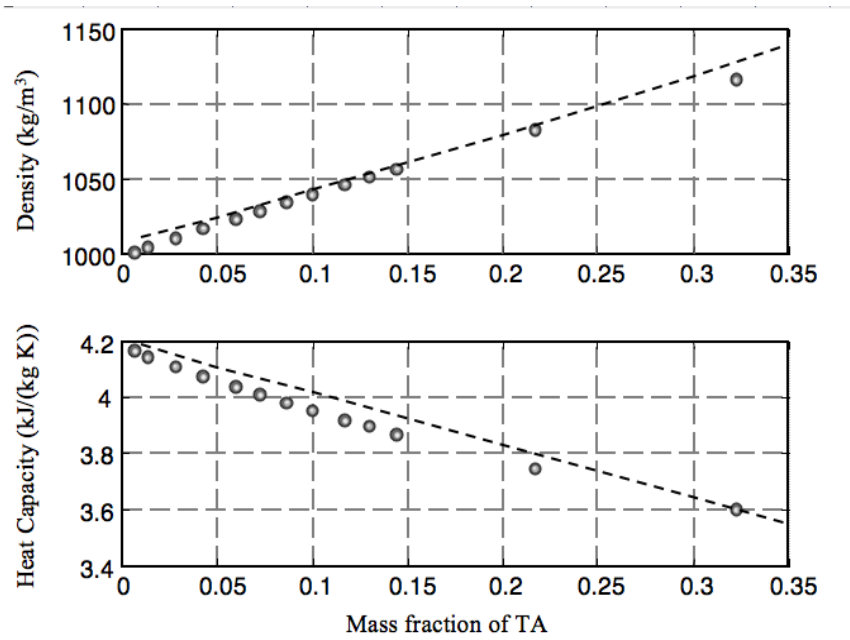


Figure 10.3: Comparison of experimental and calculated values for aqueous solutions of tartaric acid (lines: UniSim Design, circles: experimental).

The material balances of representative streams in the process flow sheet presented in Figure 10.1 are presented in Table 10.1.

Table 10.1: Material balances of representative streams presented in Figure 10.1

STREAM	**1**	**2**	**3**	**5**	**6**	**7**	**9**	**10**	**11**	**12**	**13**	**18**	**24**
T (^{o}C)	25	25	25	77.8	25	27	27	100	77.8	100	100	42	32
P (bar)	1	1	1	1	1	1	1	1	1	1	1	1	1
Components mass flowrate (kg/h)													
H_2O	314.5	143.9	170.5	0.93	46.63	190.6	29.0	121.4	40.2	109.4	11.9	302.1	4.63
EtOH	28.5	13.05	15.45	15.4	797.9	811	123.4	0.07	687.5	0.06		123.4	1.86
Solids	120.5	120.5	0.00			120.5	120.5					120.5	
AntOx	4.0	4.00	0.00			4.0	0.6	3.4	0.0		3.39	0.61	0.01
CaT	32.5	32.50	0.00			32.5	32.5					0.00	29.2
HCl												7.92	0.1
TA												25.93	
$CaCl_2$												19.17	15.7
TOTAL	**500.0**	**314.0**	**186.0**	**16.3**	**844.6**	**1158**	**306.0**	**124.8**	**727.7**	**109.5**	**15.3**	**599.6**	**51.8**

10.6 Estimation of the fixed capital investment

Based on the process flow diagram presented in Figure 10.1 and the material and energy balances estimated and validated in UniSim, the equipment type was selected and the characteristics of each equipment were determined based on standard engineering procedures and rules of thumb for the design and sizing of process equipment (Peters and Timmerhaus, 2003; Ulrich *et al.*, 1984; Turton *et al.*, 2009; Humbird *et al.*, 2011; Green and Perry, 2008). The equipment purchase cost (C_P) was used for the estimation of the fixed capital investment (FCI) using the equation FCI ≈ $5 \times C_P$. This equation was used because it has been proposed that the FCI can be estimated using the equipment purchase cost and a multiplier in the range of 3-6 (Peters and Timmerhaus, 2003; Turton *et al.*, 2009). When the sizing of equipment has been completed then data from the textbooks of Peters and Timmerhaus (2003) and Green and Perry (2008) were used in combination with equation 10.1.

$$C_p = \frac{CEPCI_t}{CEPCI_{t_0}} C_{p,0} \left(\frac{X}{X_0} \right)^n \qquad \text{Equation 10.1}$$

where $C_{p,0}$ is the purchase cost of a particular type of equipment at year t_0 with characteristic size X_0, C_p is the purchase cost of the same type of equipment in 2013 with the determined characteristic size X as was estimated in this study, n is the exponent that is characteristic to the particular type of equipment and $CEPCI_t$ is the chemical engineering plant cost index at year t published monthly in the Chemical Engineering magazine.

10.7 Estimation of the cost of manufacture

The cost of manufacture without depreciation is calculated based on Equation 10.2 (Turton *et al.*, 2009) that includes the FCI, the cost of utilities (C_{UT}), the cost of raw materials (C_{RM}), the cost of operating labor (C_{OL}) and the cost of wastewater treatment (C_{WT}).

$$COM_{woD} = 1.23 (C_{RM} + C_{UT} + C_{WT}) + 2.73C_{OL} + 0.18FCI \qquad \text{Equation 10.2}$$

The utility requirements (electricity, low pressure stream and cooling water) are determined based on the material and energy balances and their cost (C_{UT}) is then calculated. To determine the operating labor cost (C_{OL}), the workers necessary for each equipment unit per shift are initially estimated and then the overall number of workers necessary for the operation of the plant is determined. The cost of raw materials (C_{RM}) is calculated based on the unit price of each feedstock used and the annual consumed amount. The cost of the waste treatment (C_{WT}) for wastes produced from unit operations was determined by considering that the cost per t of non-toxic wastes was 50 $ (Turton *et al.*, 2009).

10.8 Estimation of the minimum selling price

To assess the feasibility and viability of the proposed process for wine lees valorization the potential revenues must be calculated and the cash flows must be assessed. This is necessary in order to estimate several economic indices such as the net present value and the payback period. Discounted cash flow (DCF) analysis was carried out in order to determine the conditions under which the proposed process for wine lees valorization is viable. The DCF focus on the calculation of the selling price, in $/kg, of a product for which the net present value (NPV) is zero. This selling price is defined as the minimum selling price (MSP) of the product.

The DCF analysis was carried out according to the assumptions presented in the 2011 NREL bioethanol production report (Humbird *et al.*, 2011). According to this report, the assumptions followed include a 10% discount rate (or internal rate of return), 30 years of plant lifetime, 100% of equity financing, 7 years depreciation based on the Modified Accelerated Cost Recovery System (MACRS), 35% of corporate tax rate, 3 years duration of plant construction, working capital equal to 5% of FCI and zero salvage value for equipment and land. During the construction period, it is considered that the distribution of plant construction costs is 8%, 60% and 32% for the 1st, 2nd and 3rd year of the plant construction period, respectively. In order to determine the MSP at different plant capacities, the raw material cost, the utilities cost, the waste treatment cost and revenues are scaled linearly with the amount of wine lees processed. The FCI is determined analytically and it was observed that it scales approximately according to equation 10.3.

$$FCI = FCI_0 \left(\frac{WL}{WL_0} \right)^{0.534}$$ Equation 10.3

where FCI_0 is the fixed capital investment for processing 500 kg/h of wine lees (this is the starting wine lees capacity processed, WL_0) and WL is the amount of wine lees processed in kg/h.

The cost of operating labor is scaled according to the following empirical equation 10.4 (Green and Perry, 2008)

$$C_{OL} = C_{OL,0} \left(\frac{WL}{WL_0} \right)^{3/4}$$ Equation 10.4

where $C_{OL,0}$ is the labor cost for processing 500 kg/h of wine lees.

10.9 Estimation of purchase equipment cost and FCI

Table 10.2 presents the purchase cost of each unit operation and the FCI of the process presented in Figure 10.1. The calculations were based on the processing of 500 kg/h of wine lees. The unit operations that contribute the highest cost to the total purchase equipment cost are the four centrifugation units that cost $87,000 each. In addition, the distillation columns T-101 and T-102 with 22 and 20 sieve plates, respectively, contribute a total purchase cost of $119,100. The total purchased equipment cost was estimated to be M$ 0.776 and this led to a fixed capital investment of M$ 3.879.

10.10 Estimation of the utilities cost

Table 10.3 presents the utilities required per unit operation as well as the total utilities cost for processing 500 kg/h of wine lees.

Table 10.2: Estimation of equipment cost and fixed capital investment

Unit		Characteristics	Data source	Purchase Cost (k$ 2013)
CF101	Disc Centrifuge	D=9 in, motor size of 20hp (14.9kW), it can be used at flow rates up to 9m^3/h	Green and Perry, 2008	87.0
T101	Distillation Column	H=14.9, D=0.35, 22 sieve plates	Peters and Timmerhaus, 2003	49
E101	Heat exchanger	Douple pipe, A=3.38 m^2	Peters and Timmerhaus, 2003	3.0
E102	Heat exchanger	Douple pipe, A=3.95 m^2	Peters and Timmerhaus, 2003	3.1
V101	Mixing tank	Glass lined, V=2.6 m^3, working volume 50%	Peters and Timmerhaus, 2003	86.2
CF102	Disc Centrifuge	D=2 in, 20hp motor	Green and Perry, 2008	87.0
T102	Distillation column	H=13.5m, D=0.85m, 20 sieve plates	Peters and Timmerhaus, 2003	69.4
E103	Heat Exchanger	Shell and Tube, A=19.2 m^2	Peters and Timmerhaus, 2003	3.5
E104	Heat Exchanger	Reboiler, A=19.3 m^2	Peters and Timmerhaus, 2003	3.5
E105	Heat Exchanger	Double pipe, A=1.15 m^2	Peters and Timmerhaus, 2003	2.8
D101	Drum	H=0.7 m, D=0.23m	Peters and Timmerhaus, 2003	4.3
C101	Blower	0.1 m^3 air/s, 3 psi max discharge	Peters and Timmerhaus, 2003	6.5
E106	Heat exchanger	Air Preheat, CS, A=1.62m^2	Peters and Timmerhaus, 2003	3.0
SD101	Spray Dryer	Max evap. Rate 0.03 Kg H_2O/s	Peters and Timmerhaus, 2003	27.3
V102	Mixing Tank	Glass lined, V=1.2 m^3, working volume 50%	Peters and Timmerhaus, 2003	54.5
CF103	Disc Centrifuge	D=9 in, 20hp motor	Green and Perry, 2008	87.0
V103	Mixing Tank	Glass lined, V=0.9 m^3, working volume 50%	Peters and Timmerhaus, 2003	46.0
CF104	Disc Centrifuge	D=9 in, 20hp motor	Green and Perry, 2008	87.0
C102	Blower	0.3 m^3 dry air/s, 3 psi max discharge	Peters and Timmerhaus, 2003	13.2
E107	Heat Exchanger	Air preheat, CS, A=0.54m^2	Peters and Timmerhaus, 2003	2.0
SD102	Spray Dryer	Max evap rate 0.03 Kg H_2O/s	Peters and Timmerhaus, 2003	27.3
T103	EtOH Storage Tank	CS Shop Fabricated tanks 20m^3	Peters and Timmerhaus, 2003	22.5
TOTAL PURCHASE EQUIPMENT COST (M$)				0.776
FIXED CAPITAL INVESTMENTS (M$)				**3.879**

Table 10.3: Consumption and cost calculation of utilities requirements

Unit operation	Electricity (kWh/y)	Low pressure steam (t/y)	Cooling Water (t/y)
CF101	42,910		
V101	3,745		
CF102	42,910		
C101	5850		
V102	1,730		
CF103	42,910		
V103	1,270		
CF104	42,910		
C102	2,880		
E102		1180	
E104		5,752	
E105		343	
E106		57	
E107		19	
E101			48,400
E103			274,830
TOTAL	187,115	7,351	323,230
Unit Price	0.06 $/kWh	12 $/t	0.015 $/t
Total Cost ($/y)	11,230	88,212	4850
Utilities Cost (M$/y)			**0.105**

Specific unit operations, such as the mixing tanks and centrifugation units, require electricity, while other unit operations, such as heat exchangers, require low pressure steam (heaters) or cooling water (coolers). The unit prices of the electricity, low pressure steam and cooling water were set to 0.06 $/kWh, 12 $/t and 0.015 $/t according to Turton *et al.* (2009). Approximately 84.6% of the utilities cost is due to steam requirements and specifically to the E-104 boiler of the distillation column T-102 (5752 t/y), where water

and antioxidants are separated from the ethanol water mixture at azeotropic composition. The utilities cost was estimated at 0.105 million $ per year.

10.11 Estimation of labor cost

The labor cost was calculated according to the number of workers needed per shift multiplied by 4.5 and the annual average salary of workers which is equal to 35,000 $ (Humbird *et al.*, 2011). In this process, due to the seasonal operation of the plant, salaries are accounted only for 120 days (approximately 4 months) per year resulting in a C_{OL} equal to 0.093 million$ per year.

10.12 Estimation of the cost of raw materials

The unit price of raw materials, the annual cost of raw materials and their annual consumption are presented in Table 10.4. The total cost of raw materials is lower (0.064 million $ per year) than the C_{OL} and C_{UT} mainly due to the low processing capacity of the plant and the low purchase price of wine lees (0.01 $/kg).

Table 10.4: Raw material consumption and cost calculation summary

Raw Material	Consumption (kg/h)	Consumption (t/y)	Unit Price[a] ($/kg)	Cost (M$/y)
Wine Lees	500	1,440	0.01	0.015
HCl 37 %	55.45	159.70	0.22	0.035
$CaCO_3$	15.79	45.48	0.15	0.007
$CaCl_2$	15.79	45.48	0.15	0.007
TOTAL RAW MATERIALS COST (M$/y)				**0.064**

[a] Pérez-Bibbins *et al.*, 2015

10.13 Estimation of the wastewater treatment cost

Another cost contributing to the total manufacturing cost is the cost of wastewater

treatment (C_{WT}). Liquid wastes are mainly produced from the bottom of the distillation column T-101 (stream 4 in Figure 10.1), but also from the evaporator D-101 (stream 12 in Figure 10.1). The above waste streams account for 169.7 kg/h from stream 4 and 110 kg/h from stream 12 and lead to an annual cost for waste treatment of 0.04 million $ per year.

10.14 Estimation of the cost of manufacture

The cost of manufacture is calculated by Equation 10.2 as M$ 1.21 in the case that 500 kg/h of wine lees are processed in the proposed biorefinery concept. Table 10.5 present the total capacity of end-products fractionated from wine lees and the associated revenues. It is important to note that for the three commodity products (namely ethanol, calcium tartrate and yeast cells) the selling prices do not vary significantly and the values used in this study are representative for these end-products. Calcium tartrate is mainly used in the food industry. Ethanol from wine lees is used as potable ethanol or alternatively could be used in several applications, such as platform chemical. However, the market price of the antioxidants may vary significantly depending on their purity, the origin and the final application.

Table 10.5: Annual capacity of end-products and associated revenues

Material	Production (kg/h)	Recovery (%)	Production (t/y)	Unit Price ($/kg)	Revenues (M$/y)
EtOH	14.11	49.51	40.63	0.6[a]	0.025
Antioxidants	3.39	84.75	9.76	x	0.00976x
Calcium tartrate	29.25	90.00	84.24	5.0[a]	0.421
Yeast cells for animal feed	120.50	100.00	347.04	1.0[a]	0.347
TOTAL REVENUES					**0.793+0.00976x**

[a]Pérez-Bibbins *et al.*, 2015

Tao *et al.* (2014) optimized the extraction of total phenolics from wine lees (58.76 mg of gallic acid equivalents per g of dried wine lees) via ultrasound-assisted extraction using aqueous ethanol solution of 43.9% ethanol. Wu *et al.* (2009) reported the extraction of 24.1% of total polyphenols using an ethanol:water mixture with 95% ethanol

content and a soxhlet extractor. In the studies of Tao *et al.* (2014) and Wu *et al.* (2009), the composition of phenolics in the extract was not identified. Perez-Serradilla and Luque de Castro (2011) carried out microwave-assisted extraction of phenolics from the dried solid phase of wine lees that was separated from the liquid phase via centrifugation. An acidified ethanol:water mixture of 75% (v/v) ethanol was used as extraction solvent. The dried phenolic-rich extract contained 364 mg of gallic acid equivalents per g of wine lees extract powder with an antioxidant activity of 3930 μmol of Trolox equivalents per g of wine lees extract powder. The main phenolics in the dried extract were p-coumaroyl derivatives, quercetin, quercetin-3-β-glucoside, myricetin, p-coumaric acid and caffeic acid (Perez-Serradilla and Luque de Castro, 2011). Delgado de la Torre *et al.* (2015) reported that the extracts obtained from wine lees by ethanol-water mixtures and microwave-assisted extraction were rich in numerous compounds such as primary amino acids, anthocyans, flavanols, flavonols, flavones and non-flavonoid phenolic compounds, among others. Varying efficiencies on the extraction of phenolic compounds from wine lees can be achieved by different technologies such as solid-liquid extraction, grinding, soxhlet extraction, microwave-assisted extraction, ultrasound-assisted extraction, high pressure extraction, pulsed electric fields extraction and supercritical fluid extraction (Tao *et al.*, 2014; Wu *et al.*, 2009; Barba *et al.*, 2016).

In this study, an ethanol:water mixture with 70% ethanol has been used as the solvent system for the extraction of polyphenols from the solid fraction of wine lees. The proposed process exploits the ethanol content in wine lees, which can be exploited for the extraction of antioxidants from the same raw material. In this way, the simultaneous production of a value-added product (potable ethanol) and extraction of antioxidants using recycling of ethanol can be achieved. It should be stressed that the extraction of polyphenols from the solid fraction of wine lees has not been optimized. The total polyphenol content determined by the Folin-Ciocalteu assay in this study was 26.1 mg of gallic acid equivalents per g of dry wine lees, which is lower that the respective values reported by Tao *et al.* (2014) and Perez-Serradilla and Luque de Castro (2011), namely 58.76 mg of gallic acid equivalents per g of dried wine lees and 364 mg of gallic acid equivalents per g of wine lees extract powder, respectively. However, the optimization of polyphenol extraction from wine lees involves the comparison of different extraction technologies, such as ultrasound or microwave assisted extraction among others (Barba *et al.*, 2016). In this study, the profitability margin of the proposed wine lees refining process

has been presented using a simple solid-liquid extraction system. The development of a polyphenol-rich extract with higher polyphenol content and extraction yield together with cost estimation of the optimized system and the identification of market outlets should be the focus of future studies. Polyphenol-rich extracts from winery wastes and by-products of varying purities could be used as additives for food and cosmetic applications (Barba *et al.*, 2016).

10.15 Calculation of the MSP of antioxidants for different wine lees processing capacities

Due to the fact that it is difficult to estimate a market price of the antioxidant-rich fraction, the profitability of the plant processing the wine lees will depend on the revenue achieved from the antioxidant-rich fraction. The approach followed in this study was based on the calculation of the MSP of the antioxidants-rich fraction (that result in zero NPV at the end of the lifetime of the industrial facility) as a function of the amount of the processed wine lees. For each capacity of processed wine lees, the NPV was assessed using the COM, FCI and C_{OL} values estimated using Equations 10.2, 10.3 and 10.4, respectively. The results of the DCF analysis are presented in Figure 10.4.

The MSP of the antioxidants-rich fraction is 122 $/kg when 500 kg/h of wine lees are processed but decreases significantly when the amount of wine lees processed is increased ten times (i.e. when 5000 kg/h of wine lees are processed) where the MSP becomes 11.06 $/kg. In order to assess the potential implementation of the proposed process, the wine production capacities in Southern European countries could be taken into consideration (FAOSTAT, 2015). The utilization of grapes for wine making in Greece, Italy, France and Spain in 2012 was 0.68, 4.65, 5.28 and 4.97 Mt, respectively. Taking into consideration that an average of 4% of wine lees is produced as related to the grape capacity used in wine making, then the annual wine lees production in Greece, Italy, France and Spain could be estimated as 27,000 t, 186,000 t, 211,000 t and 199,000 t, respectively. The techno-economic evaluation was carried out considering that the plant operates for 2880 h per year processing 14,400 t wine lees per year. According to the sensitivity analysis carried out in this study (Figure 10.4), the proposed wine lees refining concept could be implemented in major wine producing countries, such as Italy, Spain and

France.

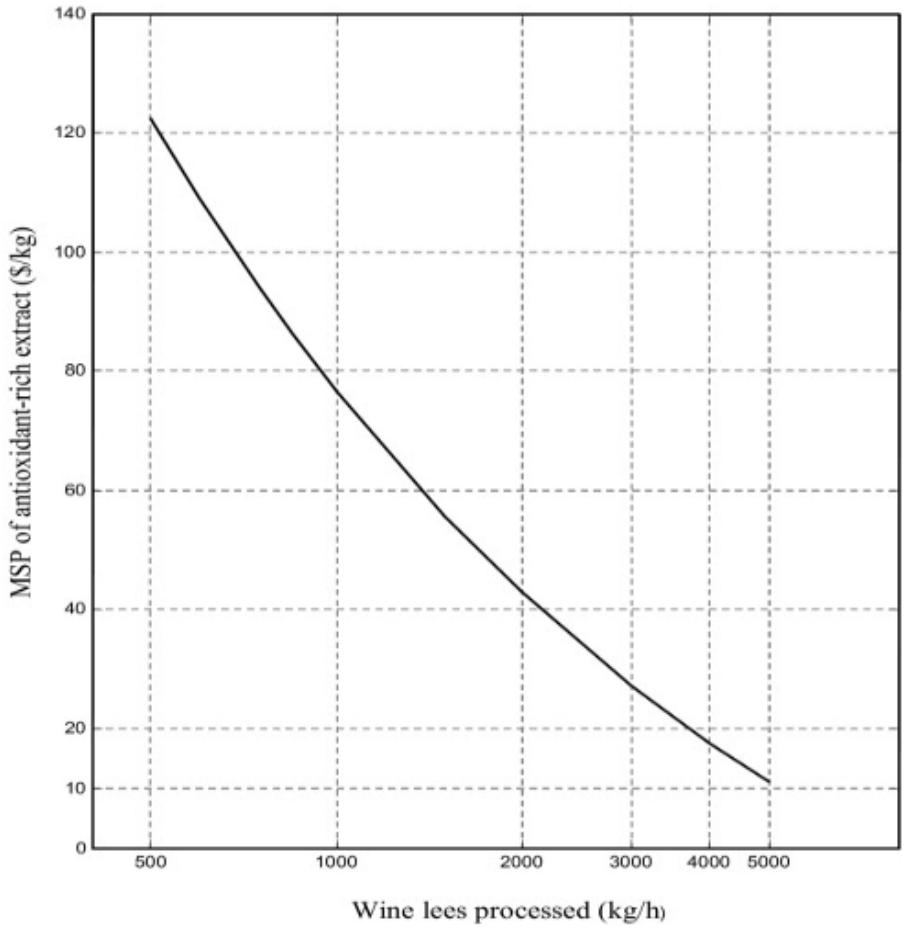


Figure 10.4: Minimum selling price of antioxidants-rich fraction as a function of the amount of the wine lees processed

The yeast cells produced in the proposed biorefinery concept could be used as animal feed, the extraction of value-added components in yeast cells or the production of a nutrient-rich fermentation supplement equivalent to yeast extract. Value-added components, such as β-glucan and mannoprotein compexes (Borchani *et al.*, 2014; de Silva Araújo *et al.*, 2014), could be also extracted from yeast cell walls increasing the final products derived from the proposed biorefinery concept leading to further diversification of market outlets. Lin *et al.* (2014) has proposed the utilization of similar wine lees derived hydrolysates for the production of microbial oil using oleaginous yeasts. The utilization of such complex sources of nutrients from industrial waste and by-product streams would be essential in order to enhance the fermentation efficiency of fastidious microorganisms, such as the production of succinic acid by *Actinobacillus succinogenes* and lactic acid by

Lactobacillus strains. Pérez-Bibbins *et al.* (2015) presented the potential exploitation of lees from wine, beer and cider production processes for sustainable generation of yeast extract via autolysis or cell disruption. Such yeast extracts could be also produced via acid hydrolysis of yeast cells.

The MSP of antioxidant-rich extracts estimated in this study was in the range of 11.06-122 $/kg, which is within the range of the market price (10-100 $/kg or higher depending on the purity and the active compounds contained in the extract) of antioxidant-rich extracts isolated from grapes (www.alibaba.com, accessed 1/2016).The market prices of assai extract powder vary in the range of 9.5-25 $/kg (Vieira *et al.*, 2013). The extraction of antioxidant-rich extracts from various renewable raw materials has been estimated in literature-cited publications. The COM of crude extracts recovered from jussara pulp were estimated in the range of 87.32-167.48 $/kg (Vieira *et al.*, 2013). Farias-Campomanes *et al.* (2013) reported that the extraction of phenolics using grape bagasse from Pisco residues by supercritical CO_2 will result in a COM of 133.16 $/kg considering a plant capacity of 0.5 m^3 with an expected phenolic content of 23 g/kg of extract. Furthermore, the process used for the extraction of antioxidant-rich fractions is crucial in order to reduce the COM as reported by Santos *et al.* (2012) for the extraction of three fractions rich in bioactive compounds (i.e. crude fraction, anthocyanin-rich fraction and fraction rich in phenolic compounds) via pressurized liquid extraction or low pressure solvent extraction using jabuticaba skins. The pressurized liquid extraction process resulted in 40-fold reduced COM for producing fractions of similar recovery yields as was achieved by the low pressure solvent extraction method. The cost of manufacture of different antioxidant-rich extracts reported in the literature, the market prices of antioxidant-rich extracts and the MSP of antioxidant-rich extracts achieved in this study show that the development of biorefinery concepts for the extraction of antioxidant-rich extracts as well as other value-added products could lead to the implementation of cost-competitive processes.

It should be stressed that if the extraction yield and the purity of the final antioxidant-rich extract is further increased then higher market prices could be achieved, thus improving the profitability of the whole process. However, the cost of manufacture of extracts recovered by different extraction methods should be estimated in order to evaluate the cost-competitiveness of the advanced process.

10.16 Conclusions

The economic potential for the development of a biorefinery based on wine lees valorization leading to the production of antioxidant-rich extract, calcium tartrate, ethanol and yeast cells, has been presented in this chapter. The sensitivity analysis showed the plant capacities required in order to develop cost-competitive processes depending on the MSP of antioxidant-rich extracts. The holistic utilization of all winery by-products, grape stalks, grape pomace and wine lees, could lead to the development of integrated biorefineries for the production of many products with diversified market outlet.

CHAPTER 11

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Food wastes and by-products from biodiesel production processes could be used for the development of integrated biorefineries. Biodegradable polymers, such as PHB, will be one of the main products of integrated biorefineries. However, the high cost of manufacture is one of the main problems preventing the industrial production of PHB. The only way to create sustainable technologies for the production of PHB is through the development of integrated biorefineries leading to the production of various value-added products. Based on the concepts mentioned above, the present PhD thesis has focused on the development of a biorefinery concept using wine lees for the production of four different products, namely ethanol, antioxidant-rich extract, calcium tartrate and nutrient-rich hydrolysates as fermentation supplements.

The study was initiated with the development of the biorefinery concept focusing on the extraction of ethanol, antioxidants and calcium tartrate followed by the optimization of hydrolysis of the remaining solids in order to evaluate the production of nutrient-rich hydrolysate (Chapter 7). The crude enzyme consortia used for the hydrolysis of wine lees derived solids were produced via solid state fermentation carried out with the fungal strain *Aspergillus oryzae*. Different parameters affecting enzymatic hydrolysis were evaluated, including pH value, temperature, initial wine lees derived solid concentration and initial enzymatic activity. The maximum free amino nitrogen concentration achieved was 1400 mg/L corresponding to 28 g/L of commercial yeast extract.

Subsequent experiments (Chapter 8) focused on the evaluation of various crude renewable resources as fermentation feedstocks for the production of PHB by two *Cupriavidus necator* strains. Crude hydrolysates were produced from flour-based waste streams, wine lees and rapeseed meal. Crude glycerol was also used as carbon source. The production of PHB was optimized in fed-batch bioreactor cultures carried out by *C. necator* DSMZ 7237 using crude glycerol and wine lees derived hydrolysates (Chapter 8). Biosynthesis of a total dry weight of 47.8 g/L, a PHB concentration of 36.9 g/L and a productivity of 0.56 g/L/h was accomplished when a carbon to free amino nitrogen ratio of 12.3 g/g was used. This fermentation was also supplemented with some mineral salts.

The biorefinery concept developed in Chapter 7 was evaluated by process design and a preliminary techno-economic evaluation. The profitability potential of the whole biorefinery concept was evaluated by estimating the minimum selling price of the antioxidant-rich extract for different plant capacities. A sensitivity analysis was carried out, based on discounted cash flow analysis, so as to estimate the minimum selling price of the antioxidant-rich extract that should be achieved at different plant capacities in order to develop a profitable wine lees refining process. The minimum selling prices (122 – 11.06 $/kg) of the antioxidant-rich extract was identified for plant capacities with the range of 500 – 5000 kg/h considering 120 days of annual plant operation.

The results accomplished within the present study on the development of the biorefinery based on wine lees valorisation demonstrate the necessity of iimplementing the principles of biomass refining. However, future research should ffocus on the improvement of the holistic approach. The main topics that should be addressed include:

1) valorization of the other waste streams generated by wineries (e.g. stalks and marc) for the production of value-added products and sugar-rich hydrolysates from the cellulose and hemicellulose fractions. In this way, the profitability potential of the whole process will be improved further targeting the holistic valorization of all winery waste streams

2) utilization of the sugar-rich hydrolysates as carbon sources combined with wine lees derived nutrient supplements for the production of chemicals and polymers via fermentation. Besides biopolymers, microbial fermentation could focus on the production of various products, such as platform chemicals (e.g. succinic acid), microbial lipids, bio-colourants and bacterial cellulose.

3) utilize specific precursors as secondary carbon sources for the production of poly(hydroxyalkanoates) as co-polymers with improved properties

4) Life cycle assessment of the whole process.

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